France Mailly

MUTATIONS IN THE HUMAN LIPOPROTEIN LIPASE GENE AND THEIR RELATIONSHIP TO HYPERLIPIDAEMIA

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Centre for the Genetics of Cardiovascular Disorders Department of Medicine The Rayne Institute University College London
ABSTRACT

The enzyme lipoprotein lipase (LPL) is a key participant in the catabolism of triglyceride-rich plasma lipoproteins. The absence of LPL activity leads to type I hyperlipoproteinaemia and it has been suggested that partial deficiency may be involved in the development of combined hyperlipidaemia. This thesis describes the detection and identification of mutations in the human lipoprotein lipase (LPL) gene and investigates their potential biological significance and impact on plasma lipid levels.

Single-stranded conformation polymorphism (SSCP), in combination with sequencing, was used to screen a group of twenty individuals with type I hyperlipoproteinaemia for mutations in exons 2 to 9 of the LPL gene. Thirteen separate mutations were identified, six were in exon 5 and four in exon 6. Twelve of these were single nucleotide substitutions, of which ten resulted in an amino acid change and there was one small deletion, leading to premature termination. Eight of these mutations were novel to this study. The previously reported G188E substitution was the only mutation present in more than one proband. The A158T and S193R substitution were shown by in vitro mutagenesis and expression in mammalian cells to completely abolish enzyme activity, while the N291S decreased it by 50%.

The approach described above was also applied to screen a subset of 35 hyperlipidaemic individuals with combined hyperlipidaemia and/or low lipase activity. One SSCP variant was identified in exon 2 in three individuals resulting in the substitution of Asn for Asp at amino acid 9. Both in vivo and in vitro studies showed that D9N decreased both LPL activity and mass. A DNA pooling strategy was developed which allowed rapid
screening of over 2200 individuals from six studies for this substitution (1000 patients/hyperlipidaemics and 1200 controls). The carrier frequency of the Asn9 variant was almost twice as high in the patient group compared to controls and the study of a subset of carriers with regards to plasma lipid levels suggested that the Asn9 variant was associated with lower LPL activity and a tendency to elevated triglycerides. The commonest LPL mutation found in type I subjects (G188E) was rare (<1%) or absent in the various study subsets. In contrast, the N291S substitution was relatively common (2.8% in the general population control sample) and had a weak impact on triglyceride levels. Thus, biologically important variants of the LPL gene are present in the general population and are likely to contribute to the risk of developing hyperlipidaemia and heart disease.
ACKNOWLEDGEMENTS

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Abbreviations used in this thesis

A  adenine
aa  amino acid
Apo  apolipoprotein
ASO  allele-specific oligonucleotide
BMI  body mass index
C  cytosine
CAD  coronary artery disease
CE  cholesterol ester
CETP  cholesteryl ester transfer protein
CHL  combined hyperlipidaemia
CHD  coronary heart disease
Chol  cholesterol
dATP  deoxy adenosine triphosphate
dCTP  deoxy cytosine triphosphate
DMSO  dimethyl sulfoxide
DTT  dithiothreitol
<table>
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<th>Full Form</th>
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<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>FCHL</td>
<td>familial combined hyperlipidaemia</td>
</tr>
<tr>
<td>FDH</td>
<td>familial dyslipidaemic hypertension</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acids</td>
</tr>
<tr>
<td>FH</td>
<td>familial hypercholesterolaemia</td>
</tr>
<tr>
<td>G</td>
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<td>guanadinium hydrochloride</td>
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<td>hyperlipidaemia</td>
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<td>HSPG</td>
<td>heparan sulphate proteoglycans</td>
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<td>IAA</td>
<td>isoamylalcohol</td>
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<td>intermediate density lipoprotein</td>
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<td>low density lipoprotein</td>
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<td>lipoprotein</td>
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<td>lipoprotein lipase</td>
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<td>LRP</td>
<td>LDL receptor related protein</td>
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<td>microsomal triglyceride transfer protein</td>
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<td>monoclonal antibody</td>
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<tr>
<td>MI</td>
<td>myocardial infarction</td>
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<tr>
<td>MOPS</td>
<td>3-(N-morpholino)propanesulfonic acid</td>
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<tr>
<td>NIDDM</td>
<td>non-insulin dependent diabetes mellitus</td>
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<tr>
<td>nt</td>
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<tr>
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<td>post heparin lipoprotein lipase activity</td>
</tr>
<tr>
<td>RAP</td>
<td>receptor-associated protein</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<td>SSCP</td>
<td>single-strand conformation polymorphism</td>
</tr>
<tr>
<td>T</td>
<td>thymine</td>
</tr>
<tr>
<td>Tg</td>
<td>triglyceride(s)</td>
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**VLDL** very low density lipoprotein  
**WT** wild-type

### One and three letter codes for amino acids:

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<td>T</td>
<td>Thr</td>
</tr>
<tr>
<td>Y</td>
<td>Tyr</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Three-letter code</th>
<th>Amino Acid</th>
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</thead>
<tbody>
<tr>
<td>R</td>
<td>Arg</td>
</tr>
<tr>
<td>D</td>
<td>Asp</td>
</tr>
<tr>
<td>Q</td>
<td>Glu</td>
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<tr>
<td>G</td>
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<td>I</td>
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<td>K</td>
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<td>F</td>
<td>Phe</td>
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<td>S</td>
<td>Ser</td>
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<tr>
<td>W</td>
<td>Try</td>
</tr>
<tr>
<td>V</td>
<td>Val</td>
</tr>
</tbody>
</table>

Throughout this thesis, the following convention has been arbitrarily chosen:

- amino acid substitutions are designated using the one-letter code for amino acids (i.e. G188E substitution, D9N mutation etc.), whereas specific mutant proteins are designated using the three-letter code (i.e. Asn9 variant, Asn9 carriers, etc.).
1 INTRODUCTION

1.1 General overview of lipoprotein structure and metabolism.

Most mammalian cells are dependent on a continuous, external supply of cholesterol, phospholipids and fatty acids for maintenance and growth, as their lipid requirements are only partially met by local synthesis (Myant, 1990; Durrington, 1989). Specialised cells such as hepatocytes, enterocytes and adipocytes, which possess high storage and/or synthetic capacity, play a major role in maintaining homeostasis of other tissues. This is achieved in part by a two-way delivery system between the specialised tissues and the periphery via the plasma circulation, where lipids are transported by emulsion-like particles called lipoproteins. These are large complexes of lipids and specialised proteins (apolipoproteins) displaying extensive heterogeneity with respect to size and lipid/apolipoprotein content, with a general structure consisting of a phospholipid/cholesterol monolayer surrounding a hydrophobic core of triglycerides and cholesterol ester (reviewed by Dolphin, 1986). Apolipoproteins help maintain the structural integrity of the particles or act as ligands for specific cell surface receptors whose expression may be modulated by local cellular needs.

Lipoproteins originate either from the intestine (dietary fat) or the liver (endogenously synthesised lipids) and their metabolism can be divided into two distinct, yet connected branches, reflecting the different metabolic fate of lipoproteins according to their site of synthesis. Both intestinal and hepatic lipoproteins are routed to the various tissues and organs, via the capillary circulation, through a complex series of lipolysis steps, spontaneous and active exchanges between lipoproteins, and interactions between protein ligands and cell-surface receptors (reviewed by Havel and Kane, 1989). These pathways and the main participants will be reviewed briefly in the next two sections and are illustrated in Figure 1.1. The relative proportion of lipid moieties together with the protein content determine the size and buoyancy of a lipoprotein. This provided the basis for the separation of lipoproteins into five major classes by preparative
ultracentrifugation, a method originally devised by Havel and colleagues many years ago (Havel et al., 1955) and still in use today. The characteristics of each class, by increasing order of density, are presented in Table 1.1.

1.1.1 Lipids of dietary origin.

Extensive processing of dietary lipid is required for efficient absorption by the gut to take place (reviewed by Durrington et al., 1989). Food entering the digestive tract is broken down into an emulsion of coarse lipid droplets through the action of proteases and gastric lipase. The latter hydrolyses accessible triglycerides and phospholipids to fatty acids as well as mono- and diglycerides. As the bolus progresses through to the intestinal lumen, it is emulsified by biliary salts, thereby greatly enhancing the hydrolysis of acyl glycerol molecules by pancreatic lipase (PL) together with its co-factor peptide colipase. Bile acids, free cholesterol, monoglycerides, fatty acids and phospholipids then combine to form mixed micelles which enable close contact between the absorption surface (the microvilli) and the products of fat hydrolysis. Free fatty acids (FFA) and cholesterol molecules can then readily diffuse into enterocytes. In the cytoplasm of these cells, medium-chain fatty acids (C8 to C12) form complexes with albumin and enter the blood circulation directly via the portal system. These complexes provide a significant proportion of the fuel required by the heart and skeletal muscles. Simultaneously, triglycerides are re-synthesized from long-chain FFA and glycerol. These are packaged with cholesterol (mostly in the free form), phospholipids and apolipoproteins (apos) A-I, B48 and C-II into a triglyceride-rich (Tg-rich) particle called chylomicron (Havel and Kane, 1989)(Fig.1.1). The nascent lipoprotein is first secreted into the lymph, where it acquires additional A and E apoproteins (apo AIV and apo E), then it is transported to the plasma compartment. Large chylomicrons, with triglycerides constituting 80-95% of their mass, have a very short half-life in normal subjects (less than 10 minutes) (Olivecrona et al., 1989) as they are rapidly metabolised mainly through the action of
Figure 1.1 Outline of lipoprotein metabolism. Details in text. Adapted from figures courtesy of R. Peacock and P. Talmud.

SR: surface remnants; Cs: apolipoproteins CII and CIII.
Table 1.1 Composition and physical characteristics of plasma lipoproteins. Based on Thompson (1989) and Durrington (1989).

<table>
<thead>
<tr>
<th>Lipoprotein class (origin)</th>
<th>Density (g/ml)</th>
<th>Particle diameter (nm)</th>
<th>% mass</th>
<th>protein</th>
<th>Tg</th>
<th>PL</th>
<th>Chol/CE</th>
</tr>
</thead>
<tbody>
<tr>
<td>chylomicrons (intestine)</td>
<td>&lt; 0.95</td>
<td>100 - 1000</td>
<td>1 - 2%</td>
<td>80 - 90%</td>
<td>4%</td>
<td></td>
<td>2% / 3%</td>
</tr>
<tr>
<td>VLDL (liver)</td>
<td>0.95 - 1.006</td>
<td>30 - 80</td>
<td>8%</td>
<td>55%</td>
<td>15%</td>
<td></td>
<td>7% / 12%</td>
</tr>
<tr>
<td>IDL (intestine/liver)</td>
<td>1.006 - 1.019</td>
<td>25 - 30</td>
<td>19%</td>
<td>26%</td>
<td>20%</td>
<td></td>
<td>9% / 29%</td>
</tr>
<tr>
<td>LDL (liver)</td>
<td>1.019 - 1.063</td>
<td>18 - 25</td>
<td>22%</td>
<td>7%</td>
<td>22%</td>
<td></td>
<td>8% / 42%</td>
</tr>
<tr>
<td>HDL (liver)</td>
<td>HDL2</td>
<td>1.063 - 1.125</td>
<td>9 - 12</td>
<td>40%</td>
<td>5%</td>
<td>33%</td>
<td>5% / 17%</td>
</tr>
<tr>
<td></td>
<td>HDL3</td>
<td>1.125 - 1.210</td>
<td>5 - 9</td>
<td>55%</td>
<td>3%</td>
<td>25%</td>
<td>4% / 13%</td>
</tr>
</tbody>
</table>
several lipoprotein lipase (LPL) molecules. This enzyme is bound to the luminal surface of the capillary endothelium where it captures circulating lipoproteins and hydrolyses their core triglycerides. The reaction is activated by the presence in chylomicrons of an essential co-factor of LPL, apo CII, and FFA released by LPL are readily taken up by surrounding tissues (Wang et al., 1992). As more core triglyceride molecules are hydrolysed, chylomicrons shed excess surface components, and become smaller and relatively enriched in cholesterol (remnants). Chylomicron remnants acquire increasing amounts of apolipoprotein CIII (apo CIII), and lose phospholipids as well as apo CII, AI and AIV through exchange with HDL (reviewed by Nestel, 1987) prior to clearance via hepatic receptors (Sherrill et al., 1980). This hepatic uptake appears to be mediated by apo E since it is specifically inhibited by antibodies raised against apo E in vitro (Hui et al., 1984) or by the presence of a defective-binding species of apo E (Schneider et al., 1981) (see section 1.1.4). Moreover, apo B48 does not possess a receptor-binding domain (Marcel et al., 1987) and is thus unable to interact with hepatic or peripheral cell receptors (Hui et al., 1984). Uptake can occur via the low-density-lipoprotein (LDL) receptor (Goldstein et al., 1985) as well as via a distinct, apo E-specific receptor present on dog and human liver membranes (Mahley et al., 1981). The physiological significance of the apo E-specific receptor is supported by the observation that remnant lipoproteins do not accumulate in the plasma of subjects with familial hypercholesterolaemia (FH), (Mahley and Innerarity, 1983). There is now increasing evidence that the low-density receptor related protein (LRP), a liver surface receptor with the ability to bind apo E, may represent the elusive apo E-specific remant receptor and may participate in the removal of remnant particles (Beisiegel et al., 1989; Nykjaer et al., 1993). In addition, the presence of LPL on the particle may stimulate the binding and uptake (both receptor-dependent and independent) of lipoproteins by hepatocytes through interaction with heparan sulphate proteoglycans (HSPG) (Mulder et al., 1992; Chappell et al., 1994).
1.1.2 Lipoproteins of endogenous origin and their catabolism.

Very low density lipoproteins (VLDL) are synthesised by the liver and function as the major endogenous triglyceride carriers in plasma, although they are denser and contain proportionately more cholesterol, phospholipids and protein than their dietary lipid counterpart, the chylomicrons. VLDL secretion by hepatocytes is driven mainly by the availability of FFA for triglyceride synthesis (Dixon et al., 1991; Boren et al., 1993). The major protein constituent of VLDL, apo B100, is produced constitutively and degraded intracellularly unless it is packaged co-translationally with triglycerides, phospholipids and apolipoproteins CII, CIII and E into a lipoprotein particle (Davis, RA et al., 1990; Boren et al., 1992). It has been demonstrated that the assembly process is controlled by the microsomal triglyceride transfer protein (MTP), which catalyses the transport of neutral lipids between membranes (Gordon et al., 1994; Leiper et al., 1994). Individuals who are deficient in MTP cannot synthesise and secrete apoB-containing lipoproteins (Wetterau et al., 1992).

Newly secreted VLDL are also catabolised through the action of LPL, providing FFA for tissues, albeit at a much slower rate than chylomicrons (Kane and Havel, 1989). The smaller size of VLDL compared to chylomicrons probably decreases the number of LPL molecules which can act upon a VLDL particle at any one time (Olivecrona et al., 1989). As triglyceride hydrolysis proceeds, the particle becomes progressively smaller and denser to become intermediate density lipoproteins (IDL), losing more surface components including apolipoproteins. IDL may be further lipolysed by hepatic lipase (HL) in the liver capillary beds and metabolised to the final product of the lipolytic cascade, low density lipoproteins (LDL), the major cholesterol carrier in human plasma. At that point, apo B100 remains the sole protein moiety and mediates the removal of LDL from circulation via the ubiquitous LDL receptor (Goldstein et al., 1985). The crucial role of the LDL-receptor in the maintenance of cholesterol homeostasis has been elucidated by the pioneering work of Goldstein and Brown on familial
hypercholesterolaemia (FH), an autosomal dominant disorder where a decreased number of cell surface receptors leads to raised plasma LDL levels and premature heart disease.

Based on lipoprotein turnover studies, the precursor-product relationship between VLDL and LDL is well established (reviewed by Eisenberg, 1990) but the actual proportion of VLDL giving rise to LDL is still a matter for some debate. It is believed that large proportion of VLDL particles are rapidly lipolysed then removed from plasma by virtue of the interaction between apo E molecules and hepatic receptors (Gianturco and Bradley, 1991). Also, it has been suggested that a fraction of plasma LDL represents direct hepatic secretion into the LDL compartment (Janus et al., 1980a). A recent report from Zambon et al. (1993) indicates that this fraction, if it does exist, is not quantitatively important. These authors have identified a proband with complete LPL deficiency (section 1.2.1.1) who is also a FH heterozygote. In this individual, plasma VLDL are low due to reduced synthesis (owing probably to low supply of fatty acids), but LDL also fail to accumulate, suggesting that there is no major pool of independently secreted LDL.

High density lipoproteins (HDL) are the densest particles of the group, depleted in cholesterol and triglycerides and highly enriched in proteins and cholesterol esters. Nascent HDL have an unusual discoid appearance until they acquire esterified cholesterol (Barter, 1993). Unlike their lighter counterparts which provide lipids for peripheral tissues, these lipoproteins collect excess cholesterol from cells and actively transport it from tissues back to the liver for excretion (reverse cholesterol transport)(Barter, 1993). It is not yet clear whether HDL bind specifically to a liver receptor (Havel and Kane, 1989) or whether they simply transfer their esterified cholesterol load to VLDL via the action of the cholesteryl ester transfer protein (CETP). HDL and VLDL/chylomicron remnants are known to interact in vivo where excess surface components generated by lipolysis (phospholipids, unesterified cholesterol) are exchanged for apo Cs and esterified cholesterol (Goldberg et al., 1990a; Barter, 1993). The well-known inverse relationship
between HDL cholesterol and triglycerides (Davis et al., 1980) is determined in part by the action of HL and LPL (Kuusi et al., 1989; Breslow et al., 1993). Elevated HDL levels are associated with lower incidence of heart disease (Castelli, 1986) and Barter (1993) has suggested that a high HDL concentration is in fact a marker for efficient VLDL metabolism.

1.1.3 Heterogeneity within lipoprotein classes.

Through the development and use of immunoassays, it has been established that lipoproteins (Lp) within each density class represent a mixture of species differing in the nature of the apolipoproteins they carry (Alaupovic, 1982). Both simple particles, containing a single apolipoprotein species (LpB, LpAI), and complex particles, carrying two or more different apolipoproteins (LpE:B, LpCIII:B, LpCIII:E) have been identified, and variation in apolipoprotein content has been associated with altered function or metabolic fate. For instance, LpAI particles promote cholesterol efflux from cells while LpAI:AII particles are unable to do so (Fruchart and Ailhaud, 1992). Both particles are present within the HDL range but the HDL2 class is enriched in LpAI and the latter may represent the anti-atherogenic lipoprotein fraction (Parra et al., 1992). Similarly, LpE:B, LpCIII:B and LpCIII:E:B particles which are found throughout the VLDL/IDL density range display markedly different affinity for the LDL receptor (Agnani et al., 1991). LpE:B particles, in contrast to LpCIII:B, can interact with the LDL receptor (Agnani et al., 1991) and may be rapidly cleared from plasma in normal individuals, while LpCIII:B may be converted to LpB (LDL)(Genest et al., 1991).

1.1.4 Impact of the apo E polymorphism on lipid levels.

Family studies and biochemical analyses have shown that the apo E locus is polymorphic, with three alleles (E2, E3 and E4) giving rise to three plasma isoforms as a result of Arg/Cys substitutions at position 112 and 158 of the polypeptide chain (Zannis and Breslow, 1981; Weisgraber et al., 1981). Apo E2 has cysteine residues in both
positions, apo E3 has cysteine in position 112 but an arginine residue at position 158, while both positions are occupied by arginine in apo E4. In most Caucasian populations, E3, the commonest allele, shows a frequency close to 0.77 while for the two rarer alleles the frequency of E4 is higher than that of E2 (0.15 vs. 0.08) (Davignon et al., 1988).

Experiments carried out in the early 1980s had established that apo E2 displays markedly reduced affinity for hepatic lipoprotein receptors (Schneider et al., 1981; Rall et al., 1982) In spite of this, most E2E2 homozygotes are normolipidaemic and have lower serum cholesterol levels than individuals with other apo E phenotypes (Utermann et al., 1979a,b; reviewed by Dallongeville et al., 1992). This is thought to be due to the up-regulation of the LDL-receptor resulting from the reduced binding and hepatic uptake of VLDL and IDL particles (Boerwinkle and Utermann, 1988). Nevertheless, these individuals have an increased susceptibility to develop hypertriglyceridaemia (HTG) and/or hypercholesterolaemia in the face of an environmental challenge (Davignon et al., 1988, see section 1.3.3). Interestingly, normolipidaemic carriers of the apo E4 isoform often tend to have higher LDL-cholesterol levels, even though both apo E3 and apo E4 display similar affinity for the LDL-receptor (Davignon et al., 1988). This is believed to result from the replacement of the cysteine residue (apo E3) by arginine (apo E4) at position 112 which prevents the formation of disulphide bridge between apo E4 and apo AII as well as Apo E homodimers. This accelerates the transfer of apo E4 from HDL to VLDL (Weisgraber and Shinto, 1991). This greater enrichment in apo E molecules in turn stimulates the hepatic removal of lipoproteins dependent on apo E for their uptake, and produces a compensating down-regulatory effect on LDL-receptors, which in turn leads to slower clearance of lipoproteins and thus hyperlipidaemia.

1.1.5 Relationship between lipid levels and the development of atherosclerosis.

The development of atherosclerotic lesions, which may eventually lead to coronary heart disease (CHD) and myocardial infarction, is a complex, multi-event
process and has been recently reviewed by Ross (1993). The early lesion in coronary arteries is thought to involve either the infiltration of the intimal layer by monocytes/macrophages through damaged endothelium, or injury to endothelial cells, both of which cause growth factor secretion and smooth muscle cell proliferation (intimal thickening). The macrophages actively take up lipoproteins and become lipid-laden foam cells, the debris of which are not cleared upon cell death, which may lead in turn to fatty streak formation. Macrophages may also secrete growth factors such as platelet-derived growth factor, fibroblast growth factor and epidermal growth factor. This further promotes smooth muscle cell proliferation and the production of connective/fibrous material, leading to more severe narrowing of the arterial lumen, a characteristic of the more advanced lesion, the fibrous plaque. A myocardial infarction (MI) may result from the complete occlusion of the vessel, or through thrombosis following the rupture of an unstable plaque and the ensuing clotting process.

The elaborate system for lipid transport and redistribution is designed to preserve plasma lipid homeostasis and prevent lipid deposition in inappropriate locations, despite being subjected to a variety of challenges. When the system’s catabolic capacity is exceeded, hyperlipidaemia in the fasting state may be detected. Elevated plasma cholesterol levels have long been recognised as a risk factor for CHD. Longitudinal cohort studies and primary intervention trials carried out in the U.S over the past decades such as the Framingham and Lipid Research Clinics studies established and confirmed the clear relationship between plasma cholesterol levels, specifically LDL cholesterol (LDL-chol) and CHD (Gordon et al., 1977b; Lipid Research Clinics Program, 1984). Low levels of HDL cholesterol were also believed to favour the appearance of CHD as this would decrease anti-atherogenic reverse cholesterol transport (Gordon et al., 1977a). These data agreed well with the findings of pathologists in experimental (animal) and human atherosclerosis since cholesterol and cholesterol ester deposits often featured prominently in the lesions associated with the disease (reviewed by Davies, 1991).
Plasma triglyceride levels were also found to be good predictors of CHD in univariate analysis (Castelli, 1986). However, an independent association between triglycerides and CHD could not be demonstrated beyond doubt, as the relationship usually disappeared when factors such as HDL levels and obesity were taken into account (reviewed by Austin et al., 1991). This may be due in part to the complex interrelationships between HDL and VLDL, the main triglycerides carrier in fasting plasma. It is well documented that elevation of triglyceride levels is often associated with low HDL levels (Gordon et al., 1977a; Davis et al., 1980). Consequently, the status of triglycerides in the hierarchy of risk factors for CHD still remains to be clarified. Additionally, the impact of specific risk factors may differ between men and women. A recent prospective study in Swedish women found that Tg levels were strong predictors of MI events in this cohort, while LDL cholesterol had little predictive value (Bengtsson et al., 1993).

1.2 Lipid disorders associated with elevated triglycerides.

Serum triglyceride levels are determined by the balance between the catabolism and the synthesis of TG-rich lipoproteins. In this section, metabolic disorders which lead to hypertriglyceridaemia through increases in chylomicrons and/or VLDL levels will be reviewed. The involvement of lipoprotein lipase, apo CII, CIII and E, as well as other potential candidates in the development of hypertriglyceridaemia will be examined. Finally, the possible role of triglyceride-rich lipoproteins in the pathogenesis of CHD and atherosclerosis will be discussed.

1.2.1 Elevation of chylomicron triglycerides.

1.2.1.1 Familial hyperchylomicronaemia syndrome (LPL and apo CII deficiencies).

This rare, autosomal recessive disorder (estimated frequency 1:1 000 000) was first recognized as a distinct clinical entity by Burger and Grutz (1932) but almost thirty more years elapsed before Havel and Gordon related the defective clearance of
chylomicrons to low post-heparin (PH) lipolytic activity (1960). The disorder is characterised primarily by severe fasting hypertriglyceridaemia and the massive accumulation of chylomicrons in fasting plasma, with abnormally low levels of LDL and HDL-cholesterol (Brunzell, 1989). Serum triglyceride levels are typically above 20 mmol/l (normal <1.5mmol/l, Thompson, 1989), and elevation up to and above 100 mmol/l may occur as affected individuals reach adulthood. The hyperchylomicronaemia is often accompanied by an increase in VLDL triglycerides as affected individuals reach adulthood. The lipoprotein phenotype is designated as type I (chylomicrons only) or type V (chylomicrons and VLDL) hyperlipoproteinaemia (HLP) according to the Fredrickson classification (Fredrickson and Lees, 1966). The nature of the lipaemia (chylomicron accumulation) can be readily ascertained by visual inspection of plasma stored overnight: a thick, creamy layer appears, floating above clear or turbid (indicative of increased VLDL) plasma.

The syndrome is usually diagnosed in early childhood. In a series of 43 patients reviewed by Lees et al. (1973), more than 80% of subjects had been diagnosed by the end of the first decade of life. The clinical features of the disorder have recently been reviewed (Brunzell, 1989; Santamarina-Fojo and Brewer, 1991). In addition to lipaemia, affected individuals often present with recurrent episodes of abdominal pain or pancreatitis attacks which are triggered by dietary fat intakes. The precise mechanism by which the elevation in triglycerides precipitates the attacks is poorly understood. This may be partly due to difficulties in diagnosing/documenting pancreatitis accurately as the presence of inhibitors and high lipid levels interfere with the standard serum amylase assay (Fallat et al., 1973). Other clinical features include hepatosplenomegaly due to lipid accumulation, eruptive xanthomata on the buttocks, arms and legs resulting from phagocytosis of chylomicrons by macrophages in the skin, and lipaemia retinalis (with TG > 35 mmol/l). These symptoms are reversible with a reduction of plasma TG to 10-20 mmol/l, a level which can usually be achieved with severe dietary fat restriction (15%
or less calories derived from fat). The major morbidity associated with the hyperchylomicronaemia syndrome is the episodic occurrence of pancreatitis (Brunzell, 1989). Individuals do not appear to be at increased risk of atherosclerosis, an unsurprising finding considering the low LDL levels and the absence of atherogenic remnant lipoproteins.

Although the disorder is very rare, an unusually high frequency has been reported among French-Canadians from the Lac St-Jean/Charlevoix region in the province of Quebec (Gagné et al., 1989). The estimated prevalence of homozygosity in that area is 1/5000, which represents a two-hundred fold increase relative to the frequency observed elsewhere. The phenomenon is likely to be due to a founder effect since this population is derived from a small number of settlers (approximately 8000 individuals) who lived in relative isolation after emigrating from France in the 17th and 18th centuries, and had a high birth rate (Charbonneau and Robert, 1987). Other rare genetic conditions, including familial hypercholesterolaemia and hereditary tyrosinaemia are also present at an increased frequency in this population (De Braekeleer et al., 1991).

### 1.2.1.2 Molecular defects.

The accumulation of Tg-rich lipoproteins in the plasma of patients with type I HLP is caused by the absence of LPL activity on the luminal surface of capillaries. In recent years, the elucidation of the LPL gene structure and its coding sequence (Deeb and Peng, 1989, Oka et al., 1990, Wion et al., 1987) have stimulated research into the molecular basis for this deficiency. Studies from several laboratories have demonstrated that defects in the LPL structural gene are responsible for the majority of cases of familial hyperchylomicronaemia. Missense mutations are most commonly found, followed by nonsense and splice-junction mutations and much rarer major gene deletions or insertions (Table 1.2). In all, over forty distinct mutations have been reported worldwide. These are not distributed evenly along the nine LPL coding exons (Deeb and
Table 1.2 Mutations identified in the lipoprotein lipase gene of type I hyperlipidaemic individuals. The nucleotide and amino acids substituted are indicated for missense mutations.

<table>
<thead>
<tr>
<th>Amino acid position</th>
<th>Nucleotide (exon)</th>
<th>Amino Acid</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
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<td>LPL 9</td>
<td>G-&gt;A (2)</td>
<td>Asp-&gt;Asn</td>
<td>Lohse et al., 1991</td>
</tr>
<tr>
<td>LPL 61</td>
<td>G-&gt;A (3)</td>
<td>Tyr-Stop</td>
<td>Gotoda et al., 1992</td>
</tr>
<tr>
<td>LPL 64</td>
<td>nr</td>
<td>Try-Nonsense</td>
<td>Sprecher et al., 1992</td>
</tr>
<tr>
<td>LPL 69</td>
<td>G-&gt;C</td>
<td>Val-Leu</td>
<td>Bruin et al., 1994b</td>
</tr>
<tr>
<td>LPL 64</td>
<td>C-&gt;A</td>
<td>Tyr-Stop</td>
<td>Wilson et al., 1993</td>
</tr>
<tr>
<td>LPL 73</td>
<td>G-C</td>
<td>Val-Leu</td>
<td>Wilson et al., 1993</td>
</tr>
<tr>
<td>LPL 75</td>
<td>A-&gt;C</td>
<td>Arg-Ser</td>
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</tr>
<tr>
<td>LPL 86</td>
<td>T-&gt;C</td>
<td>Trp-Arg</td>
<td>Ishimura-Oka et al., 1992a</td>
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<td>LPL 136</td>
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<td>Deeb et al., 1991</td>
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<td>LPL 139</td>
<td>G-&gt;A</td>
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<td>Bijvoet et al., 1994</td>
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<td>LPL 142</td>
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<td>Gly-Glu</td>
<td>Ameis et al., 1991</td>
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<tr>
<td>LPL 154</td>
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<td>Gly-Ser</td>
<td>Bruin et al., 1993</td>
</tr>
<tr>
<td>LPL 156</td>
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<td>Asp-Gly</td>
<td>Ma et al., 1992a; Faustinella et al., 1991a</td>
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<td>LPL 156</td>
<td>G-&gt;A</td>
<td>Asp-Asn</td>
<td>Ma et al., 1992a</td>
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<tr>
<td>LPL 157</td>
<td>C-&gt;G</td>
<td>Pro-Arg</td>
<td>Bruin et al., 1992</td>
</tr>
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<td>LPL 172</td>
<td>C-&gt;G</td>
<td>Ser-Cys</td>
<td>Ma et al., 1993a</td>
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<td>LPL 176</td>
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<td>Ala-Thr</td>
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<td>Ile-Ser</td>
<td>Reina et al., 1992</td>
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<tr>
<td>LPL 216</td>
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<th>Amino acid position</th>
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<td>LPL 225</td>
<td>T→C (5)</td>
<td>Ile-Thr</td>
<td>Henderson et al., 1993</td>
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<td>Cys-Stop</td>
<td>Takagi et al., 1994</td>
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<td>Arg-His</td>
<td>Dichek et al., 1991</td>
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<td>LPL 243</td>
<td>C→T</td>
<td>Arg-Cys</td>
<td>Ma et al., 1994a</td>
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<td>Ser-Thr</td>
<td>Hata et al., 1990a</td>
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<td>Asp-Asn</td>
<td>Ma et al., 1992b; Ishimura-Oka et al., 1992b</td>
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<td>Ser-Cys</td>
<td>Bijvoet et al., 1993</td>
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<td>Ala-Thr</td>
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<td>G→A</td>
<td>Glu-Lys</td>
<td>Previato et al., 1994</td>
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<td>LPL 447</td>
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<td>Ser-Stop</td>
<td>Hata et al., 1990b</td>
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Intron 1 donor splice site
- G→C
  - v. low mRNA
  - Chimienti et al., 1992

Intron 2 donor splice site
- C→A
  - no mRNA
  - Hata et al., 1990a

Intron 2 acceptor splice site
- G→A
  - v. low mRNA
  - Gotoda et al., 1991b

2kb insertion
- Dupl exon 6
- Langlois et al., 1989; Devlin et al., 1990

6kb deletion
- Exons 3-5
- Langlois et al., 1989

5bp insertion
- GGGCT at 559 (3)
  - Premature stop
  - Henderson et al., 1990

4bp deletion
- 614-617 (4)
  - Premature stop
  - Ma et al., 1993b

1bp deletion
- 916 (5)
  - Premature stop
  - Takagi et al, 1992

1bp deletion
- 925 (5)
  - Premature stop
  - Deeb et al., 1991

Dupl = duplication
Del = deletion
ins = insertion
Peng, 1989, Oka et al., 1990) but rather appear to concentrate in exons 4, 5 and 6 which encode the central, catalytic domain of the enzyme (Santamarina-Fojo, 1992). This will be further discussed in section 1.4.

A much rarer cause of the chylomicronaemia syndrome, responsible for an estimated 5% of cases, is a functional deficiency in the essential co-factor for LPL activity, apoCII (reviewed by Santamarina-Fojo, 1992). Its absence was shown to result in a type I phenotype similar to LPL deficiency by Breckenbridge and colleagues (1978) although the clinical manifestations associated with apoCII deficiency generally appear to be less severe than in LPL deficient subjects, possibly due to residual LPL activity. In particular, individuals tend to be diagnosed later in life and tolerance to dietary fat appears higher (Brunzell, 1989). Studies in apo CII deficient probands and their relatives have shown that it is an autosomal recessive disorder which can be caused both by missense mutations and premature termination codons in the gene coding for apoCII (Santamarina-Fojo, 1992).

Unusual cases of inherited LPL deficiency have also been reported. Brunzell et al. (1980) and Lithell et al. (1978) provided early evidence of subjects with type I HLP where the LPL deficiency appeared to be tissue-specific. A later study demonstrated defective transport and processing of the enzyme from its site of synthesis in a proband with very variable Tg levels. LPL activity was present at low levels within adipose tissue but not in PH plasma (Fager et al. 1990). Finally, an interesting proband identified by Brunzell and collaborators (1983a) was shown to have a circulating plasma inhibitor to LPL resulting in an apparently autosomal dominant mode of transmission. The inhibition was likely to be caused by autoantibodies against LPL, as was demonstrated in a Japanese patient (Kihara et al., 1989).
1.2.2 Elevation of VLDL triglycerides.

1.2.2.1 Familial hypertriglyceridaemia (familial HTG).

This common disorder with an estimated population frequency of 1% (Goldstein et al., 1973a) is associated with a type IV lipoprotein phenotype (Fredrickson and Lees, 1966), which is characterised by an elevation of VLDL triglycerides. Familial HTG is often classified as a monogenic disorder transmitted as an autosomal dominant trait (Goldstein et al., 1973b, Brunzell et al., 1983b). Typically, subjects present in adulthood with a moderate increase in triglycerides (2.5-6.5 mmol/l), low levels of HDL cholesterol but normal levels of total cholesterol, LDL-cholesterol and apoB (reviewed by Kane and Havel, 1989). Conditions such as obesity and diabetes tend to worsen the lipid profile. The absence of raised apoB levels differentiates familial HTG from hyperapobetalipoproteinaemia, a syndrome where both plasma VLDL triglycerides and apoB can be elevated (see section 1.2.2.3). Affected individuals are generally detected through a routine lipid measurement in the course of another investigation or through affected relatives. Dermatological abnormalities are not usually present.

The nature of the underlying molecular defect in familial HTG is still unknown. While a role for defective VLDL clearance has not been entirely ruled out, post-heparin LPL activity is often within the normal range (reviewed by Taskinen, 1987). Studies which have reported low levels of muscle or adipose tissue LPL in type IV subjects often did not take into account confounding factors such as obesity or diabetes (Taskinen, 1987). Additionally, it is likely that only a subset of individuals presenting with a type IV phenotype have a familial form of HTG as opposed to secondary hyperlipidaemia (see section 1.2.2.4). Helio et al. (1994) have reported the exclusion of the LPL, CIII and apo B genes in the etiology of familial HTG, based on co-segregation studies. This suggests that a deficit in LPL activity due to mutations within the LPL gene is unlikely to play a major role in this disorder. Two recent articles have nevertheless provided evidence for LPL involvement in a small proportion of HTG cases. Takagi et al. (1994)
have described a nonsense LPL gene mutation detected in a type IV subject with low LPL activity. LPL molecular defects were also found in 17% of subjects in a group of French Canadians with type IV HLP where secondary causes of HTG had been excluded (Minnich et al., 1995). All of the individuals identified through the screening procedure were either heterozygotes or compound heterozygotes for LPL mutations previously shown to impair or abolish LPL activity. The relatively high prevalence detected in the latter study may reflect in part the high frequency of LPL mutations in the French Canadian population (Gagné et al., 1989) and may not be applicable to other populations.

Kinetic studies have demonstrated that the type IV lipoprotein phenotype appears to be due mainly to hepatic overproduction of large, TG-enriched VLDL particles (Janus et al., 1980b). Cianflone et al. (1992) have shown that in HepG2 cells, a widely used model for hepatocyte function, incubation with carbohydrates but not FFA can mimic this phenotype. This suggests that a possible role for defective glucose/insulin metabolism in this hyperlipidaemia.

Although familial HTG was reported many years ago to be present in 5% of survivors of a myocardial infarction (MI) (Goldstein et al., 1973b), the proportion of clinical endpoints (MI) that are actually attributable to the hypertriglyceridaemia per se is unclear. As mentioned before, HTG is frequently accompanied and exacerbated by potentially atherogenic conditions such as obesity and diabetes (Chait and Brunzell, 1983) whose contribution was not evaluated in the original study. Moreover, the evidence obtained from epidemiological studies examining the role of triglycerides as an independent risk factor for CHD is contradictory (see section 1.2.3).

1.2.2.2 Familial dysbetalipoproteinaemia (type III HLP) and the role of apo E.

This rare disorder (frequency 1:10 000) is characterised by an elevation of both cholesterol and triglyceride levels due to defective clearance of remnant lipoproteins (type III HLP) (reviewed by Havel, 1982). When plasma from type III patients is
subjected to electrophoresis, the cholesterol-enriched chylomicron and VLDL remnants exhibit a unique mobility shift from the pre-beta (VLDL) to the beta (LDL) position. Familial dysbetalipoproteinaemia is often diagnosed in early adulthood due to the appearance of distinctive palmar and tuberoeruptive xanthomas as a result of lipid deposition. Premature atherosclerosis and CHD usually develop by the age of 50 in males and a decade later in females (Davignon et al., 1988).

Several years ago, Utermann and colleagues made several key observations leading to the understanding of the pathogenesis of type III HLP (Utermann et al., 1977, 1979b). They noted that several isoforms of apo E, a component of VLDL, were present in human plasma, and that subjects with type III HLP were almost invariably homozygous for the rarest form (apo E2). Apo E2-containing remnant lipoproteins were cleared more slowly from plasma (Gregg et al., 1981) because of the markedly reduced affinity of apo E2 for its receptors (Schneider et al., 1981; Rall et al., 1982, 1983), partly explaining the lipoprotein accumulation seen in type III HLP.

In most populations studied, the frequency of E2/2 homozygotes is approximately 1% (Davignon et al., 1988). While all these individuals have chylomicron and VLDL remnants of abnormal composition in their plasma, reflecting reduced apo E-mediated clearance (Fainaru et al., 1982; Brenninkmeijer et al., 1987; Demant et al., 1991), only 1-2% of the subjects will develop the full-blown type III HLP phenotype (Mahley and Angelin, 1984). Therefore, additional genetic or environmental factors are required for full expression of the disorder ('two-hit' hypothesis). Type III HLP has been observed in individuals who had inherited both an LDL-receptor gene defect (causative of FH on its own) and a single apo E2 allele (Hopkins et al., 1991; Emi et al., 1991). Possible interactions between LPL mutations, pregnancy and apo E have been reported (Ma et al., 1994c). Conditions such as obesity (high-fat diet), diabetes and hypothyroidism have also been identified as triggers for type III HLP, leading to an increase in lipoprotein synthesis which overwhelms the sluggish clearance pathway in E2/2 homozygotes.
1.2.2.3 Familial combined hyperlipidaemia and related syndromes.

Familial combined hyperlipidaemia (FCHL) was first described by Goldstein and his colleagues based on the study of MI survivors from the Seattle area and their relatives, whose cholesterol and triglyceride levels had been measured (Goldstein et al., 1973b). FCHL was common in this cohort accounting for 11% of premature CHD cases (before age 60). The disorder is classically defined by elevated plasma levels of either cholesterol or triglycerides or both, in the proband and in at least one relative, with a relatively late age of onset (3rd-4th decade) (Goldstein et al., 1973a,b; Nikkila and Aro, 1973). Multiple lipid phenotypes (Fredrickson and Lees classification - IIa, IIb and IV) are expected within a family and these often change over time (Grundy et al., 1987). A positive family history for CHD should also be found.

The common metabolic defect in FCHL appears to be overproduction of Tg-rich apoB-containing particles from the liver (Sniderman et al., 1980; Janus et al., 1980a & b; Grundy et al., 1987) which accounts both for HTG and raised apo B levels (Venkatesan et al. 1993). This may be accompanied by increased number of small dense LDL particles in the blood (LDL pattern B, Austin et al., 1990a; Hokanson et al., 1993). Castro-Cabezas et al. (1993) and Sniderman et al. (1992b) have additionally proposed that defective free fatty acid (FFA) metabolism and hyperinsulinaemia are involved in the etiology of FCHL.

Although FCHL was originally described as a monogenic disorder with an autosomal dominant mode of inheritance, it is now widely believed to be a genetically heterogeneous condition (reviewed in Kwiterovich, 1993b). Several conditions which display a degree of overlap with FCHL may share a common genetic defect. Hyperabobetalipoproteinaemia (hyperapo B) is defined by an increase in apo B relative to LDL cholesterol (Sniderman et al. 1982 and 1992b). It is characterised by a very
similar, if slightly milder, lipid profile compared to FCHL (LDL cholesterol may be normal but Tg may be elevated) as well as premature atherosclerosis (Sniderman et al., 1980) and may not be a distinct entity from FCHL. In familial dyslipidaemic hypertension (FDH), early hypertension is associated with elevated LDL-cholesterol, low HDL-cholesterol or raised Tg (Williams RR et al., 1992). Of 63 individuals with FDH studied by Hunt et al. (1989), almost a third could be classified as having FCHL. FDH probands are often obese and hyperinsulinaemic. Taken together, the features of hypertension, obesity, hyperinsulinaemia and hyperlipidaemia are reminiscent of a disorder described by Reaven (1988) called syndrome X where a clustering of these conditions is observed.

1.2.2.3.1 Candidate genes for FCHL.

A number of potential causative genes for FCHL have been investigated. Given the metabolic abnormalities in FCHL, the apo B gene was a prime candidate for study but it has been ruled out through family and sib-pair linkage analysis studies (Rauh et al., 1990; Coresh et al., 1992; Nishina et al., 1992). Complex segregation analysis in FCHL families suggests that a major gene locus determines apo B levels (Jarvik et al., 1993) while in other pedigrees a major gene has been proposed to determine Tg levels (Cullen et al., 1994). These data do not unfortunately provide any additional information as to the identity of the gene involved. Kwiterovich (1993b) has recently put forward a 'two-hit' hypothesis whereby two genes with Tg and apo B raising effects might be necessary for full expression of FCHL. This pattern has been observed in the animal model for FCHL, the St.Thomas Hospital hyperlipidaemic rabbit (Beaty et al., 1989).

The proposed locus for the atherogenic lipoprotein phenotype (Austin et al., 1990b), characterised by predominance of small dense LDL, elevated Tg and elevated apo B, is linked to the LDL-receptor locus (Nishina et al., 1992). However, earlier studies indicate that mutations at this locus are unlikely to cause FCHL (Goldstein et al.,
Preliminary evidence suggests that another gene in close proximity to the LDL-receptor, the insulin receptor gene, may in fact be involved (Nishina et al., 1992).

Cianflone and co-workers (1989, 1990a) have isolated a small basic plasma protein which they named acylation-stimulating protein (ASP) as it stimulates Tg synthesis from FFA in fibroblasts from normal individuals. Importantly, the presence of ASP did not activate Tg synthesis to the same extent in cells from subjects with hyperapobetalipoproteinaemia (Cianflone et al., 1990a). This defective stimulation has been replicated by another group who had independently isolated ASP and termed it BP I (basic protein I) (Kwiterovich et al., 1994). Based on their results, Sniderman et al. (1992a) proposed that a receptor for ASP exists which is defective in patients with hyperapobetalipoproteinaemia. These authors further suggested that this causes an increase influx of FFA to the liver, thereby increasing VLDL apo B secretion, the major metabolic abnormality observed in FCHL (Sniderman et al., 1992b).

Co-segregation of variation at the AI-CIII-AIV gene cluster with FCHL has been reported in seven families (Wojciechowski et al., 1991). The peak LOD score of 6.86 at a recombination frequency of 0 suggested that the defect was in or very near this gene cluster. Of the three genes in the cluster, overproduction of apoCIII would appear to be the most likely cause of hypertriglyceridemia, as apoCIII is known to inhibit lipoprotein lipase (LPL) and hepatic lipase and to interfere with clearance of remnant lipoproteins (Brown and Baginsky, 1970) (see section 1.5.3.1). Other workers have not detected this linkage between FCHL and the apo AI-CIII-AIV gene cluster in other families (Wijsman et al., 1992; Xu et al., 1994) so that the involvement of this gene cluster remains to be confirmed.

One of the key factors determining the metabolism of triglyceride-rich lipoproteins is the activity of LPL. Patients who are homozygous for a mutation in the LPL gene causing LPL deficiency occur at a frequency of roughly one per million, and
have Type I hyperlipoproteinaemia with fasting chylomicronaemia (section 1.2.1.1); thus, carriers for such mutations may be as frequent as 1/500. The study of a large Type I kindred has shown that some relatives who are heterozygous for LPL deficiency have high plasma triglyceride concentrations and that this is most marked in individuals over 40 years (Wilson et al., 1990). In another report, Babirak et al. (1989) have noted the presence of hyperlipidaemia in obligate heterozygotes for mutations in the LPL gene, with multiple lipoprotein phenotypes reminiscent of FCHL. Recently, these workers have also demonstrated that a proportion (1/5 - 1/3) of FCHL patients have levels of post-heparin LPL activity and mass below the 10th percentile for the general population (Babirak et al., 1992). These data suggest that partial LPL deficiency, either genetic or acquired, may underlie the phenotype of FCHL in some patients. Based on observations made with HepG2 cells in culture, Williams et al. (1991) have proposed a mechanism linking partial LPL deficiency and FCHL. In the presence of LPL, a large proportion of newly secreted VLDL are rapidly re-absorbed by the cells. In vivo, this phenomenon might take place in the space of Disse (Williams et al., 1991) where active LPL has been detected. Thus, LPL deficiency would result in apparent apo B overproduction, as observed in FCHL. Additional evidence for this hypothesis is provided by the finding that LPL can act as a bridge between lipoproteins and the cell surface (see section 1.3.3.4).

In summary, several candidate genes have been proposed which may contribute to the FCHL phenotype in individuals and further studies are required to clarify their roles and the relationship between them.

1.2.2.4 Secondary causes of hypertriglyceridaemia.

Hypertriglyceridaemia also arises in humans secondary to another pathology and can be reversed by successful treatment of the latter. Clinically common disorders which lead to plasma accumulation of chylomicron and/or VLDL particles include poorly
controlled diabetes mellitus, obesity, hypothyroidism, alcohol abuse, and renal failure. (Durrington, 1989). The presumptive mechanism for the HTG induced by diabetes, obesity and alcohol abuse involves an increased availability of FFA for triglyceride synthesis in the liver (Castro-Cabezas et al., 1993; Durrington, 1989). In contrast, the hypertriglyceridaemic impact of renal disease and hypothyroidism appears to be mediated through inhibition of LPL activity (Durrington, 1989).

Infection and inflammation is also known to be associated with a massive and prolonged increase in plasma triglycerides (reviewed by Hardardottir et al., 1994). The administration of bacterial endotoxin to laboratory animals has been used to study the phenomenon and has been shown to stimulate cytokine production and VLDL synthesis while inhibiting LPL-mediated clearance. Since cytokines such as tumour necrosis factor (TNF) also stimulated VLDL production and appeared to inhibit LPL activity in rodents (Semb et al., 1987; Feingold et al., 1989), it was suggested that the Tg-raising effect of endotoxins was cytokine-mediated. While this is probably the case for increased hepatic lipoprotein synthesis, cytokines do not appear to impede the clearance of Tg-rich lipoproteins (Chajek-Shaul et al., 1989). Moreover, the increase in plasma triglycerides effected by the injection of TNF precedes the decrease in plasma LPL and thus the latter phenomenon is not responsible for the initial rise in triglyceride levels (Grunfeld et al., 1989).

1.2.3 Hypertriglyceridaemia and CHD risk.

As discussed in section 1.1.3, the elevation of plasma triglycerides has emerged as a strong predictor of CHD in univariate analysis but not usually in multivariate analysis. One possible confounding factor is that in contrast to cholesterol, plasma triglyceride levels vary considerably in response to dietary intake. Another important consideration which has become apparent is that total plasma triglyceride levels in the fasting state are unlikely to discriminate well between MI sufferers and healthy subjects,
as the different triglyceride-rich lipoprotein fractions do not all confer an increased risk of MI (Castelli, 1992). Individuals with type III HLP (remnant lipoproteins) or FCHL (small, dense LDL) are clearly at increased risk for developing premature CHD while subjects with type I HLP (chylomicrons) are not. In keeping with these observations, in vivo studies in the St. Thomas hyperlipidaemic rabbit strain have shown that remnant particles in the VLDL range as well as LDL can readily infiltrate the arterial wall in these animals and may thus be highly atherogenic (Nordestgaard et al., 1992). Moreover, apo E-mediated uptake of chylomicron and VLDL remnants has been demonstrated in macrophages in vitro (reviewed by Gianturco and Bradley, 1991), and Tg-rich liposomes analogous to chylomicron-like surface remnants have been detected within human atherosclerotic plaques (Chung et al., 1994). Native chylomicrons, on the other hand, do not interact with cellular receptors and are not readily taken up by cells, probably explaining the absence of atherosclerotic disease in type I subjects (Zilversmit, 1979). While the risk of CHD is probably not increased in subjects with familial HTG (Brunzell et al., 1983b), large abnormal VLDL particles (some of which may be chylomicron remnants) from some type IV individuals can nonetheless cause rapid lipid accumulation in macrophages and induce the foam cell phenotype in vitro (Gianturco et al., 1982). Intimal infiltration by such lipid laden cells may be minimal at the undamaged vascular endothelium but could increase if the properties of the endothelial layer were altered. Exposure to cigarette smoke has been shown to increase the permeability of the endothelial barrier to large molecules in dogs and could have a similar effect on human arteries (Allen et al., 1988).

In the Framingham study, many of the individuals who suffered MI did not have raised LDL-cholesterol and were not originally considered to be at high risk of CHD (Castelli, 1992). The combination of low HDL cholesterol and elevated triglycerides, a very common condition, accounted for a large proportion of MI cases in this study as well as in others (PROCAM and Helsinki Heart Study) and has been proposed as a
marker for the presence of remnant lipoproteins (Castelli, 1992). Thus, Tg elevation associated with Tg-rich remnant lipoproteins may well be predictive of atherosclerosis (Castelli, 1992). This is not altogether surprising since the absolute cholesterol content of such particles individually is far greater than that of LDL particles owing to their respective sizes (Gianturco and Bradley, 1991). This is often overlooked because of the low relative percentage of cholesterol in Tg-rich particles.

1.3 Features and functions of LPL.

1.3.1 Enzymatic properties and biochemical characteristics.

Much of the knowledge pertaining to the biochemistry of LPL has been obtained using enzyme purified from bovine milk, a protein closely related to human LPL (Hide et al., 1992). LPL is a glycosylated, basic enzyme with a relatively high pi (close to 9), which hydrolyses predominantly the sn-1 and sn-3 ester bonds of triglycerides at a pH optimum of 8.0 - 8.5 (Olivecrona and Bengtsson-Olivecrona, 1987). LPL activity is greatly enhanced by the presence of its co-factor apo CII, and deficiency of the latter leads to hyperchylomicronaemia and low LPL activity (section 1.2.1.2). In humans, the enzyme is released in circulation by an intravenous injection of heparin and binds with high affinity to heparin in vitro (Olivecrona et al., 1977). The presence of a serine residue within a serine protease consensus sequence (Gly-X-Ser-X-Gly) (Wion et al., 1987) together with the fact that LPL activity could be inhibited in vitro by protease inhibitors (Parkin et al., 1982; reviewed by Quinn, 1985) initially suggested that the mechanism for triglyceride hydrolysis involved a nucleophilic attack of the ester bond by this amino acid. By analogy with previously characterised serine proteases such as trypsin, it was hypothesised that the active serine residue of LPL and other lipases was part of a hydrogen-bonded charge relay network which included aspartic acid and histidine residues - the classic Ser-Asp-His catalytic triad (Kraut, 1977). This has now been confirmed by crystallographic data from the related PL enzyme and by molecular
Molecular sizing by sedimentation centrifugation revealed that 8% of LPL mass might be accounted for by complex N-linked carbohydrates (Iverius and Ostlund-Lindqvist, 1976). Glycosylation appears to be essential for efficient LPL secretion and extracellular enzymatic activity (Ben Zeev et al., 1992). Tunicamycin treatment of adipocytes to prevent N-glycosylation resulted in the production of an inactive LPL protein which remained sequestered in the endoplasmic reticulum of cells (Chajek-Shaul et al., 1985a; Ong and Kern, 1989). Based on their original centrifugation studies, Iverius and Östlund-Lindqvist (1976) reported that the bovine LPL dimer probably represents the smallest active molecular species. This suggestion was further supported by radiation inactivation and chemically-induced denaturation experiments which associated the 54 kd monomer species with the inactive fraction (Osborne et al., 1985; Olivecrona et al., 1985a) with rapid and irreversible loss of activity. More recently, Peterson and colleagues (1992) used monoclonal antibodies in ELISA assays to quantify the dissociation process and probe conformation changes brought about by denaturation. These authors concluded that the active LPL fraction was probably a homodimer, while monomeric proteins were inactive.

The main distinctive feature of LPL and related lipases (compared to other esterases) reside in their ability to hydrolyse a water-insoluble substrate in an aqueous environment. The process requires the adsorption of the substrate at a water-lipid interface which induces a substantial enhancement of the enzyme’s activity. This phenomenon is termed 'interfacial activation', a concept developed by Desnuelle and Sarda (Desnuelle et al., 1960). In fungal or mammalian triglyceride lipases, it appears to involve a conformation change, which is necessary to bring the substrate and the active site in close proximity, as these enzymes all have their active sites buried underneath a loop segment, termed the 'lid' (Brzozowski et al., 1991). Moreover, human PL and its essential co-factor colipase have recently been crystallised together in the
presence of bile salts (van Tilbeurgh et al., 1993), and colipase has been shown to
interact with the open lid structure to form the lipid-water interface. By analogy, apo CII
may also participate in the LPL conformation change thought to occur in vivo in the
presence of triglycerides (Derewenda and Sharp, 1993).

1.3.2 Synthesis, processing and sites of action.

Studies of rat, guinea pig and human tissues have demonstrated widespread
expression of LPL. Both mRNA and LPL activity were present at high levels in adipose
tissue, lactating mammary gland, adrenals and heart from all three species, whereas LPL
mRNA was absent from the adult liver. (Semenkovich et al., 1989a; Goldberg et al.,
1989; Wion et al., 1987; Olivecrona and Bengtsson-Olivecrona, 1987). In contrast, in
humans, moderate mRNA levels were seen in intestine and kidney tissue (Wion et al.,
1987) while such expression was absent from the corresponding rat tissues (Semenkovich
et al., 1989a). Levels in human adrenals were close to those observed for adipose tissue,
while they represented less than 10% of adipose tissue levels in rats. Low levels were
observed for one or the other species for lung, brain, ovaries and testes (Semenkovich
et al., 1989a; Goldberg et al., 1989). Macrophages can also synthesise and secrete LPL
(Chait et al., 1982) (section 1.3.4).

From the early work of Robinson and others who demonstrated the release of
LPL activity into the bloodstream within seconds of an intravenous heparin injection, it
was surmised that LPL was probably bound to the capillary endothelium surface through
ionic interactions (Olivecrona and Bengtsson-Olivecrona, 1987). The hypothesis was lent
additional support by the finding that purified labelled LPL could associate in a saturable
manner to high-affinity sites on cultured endothelial cells and that labelled LPL protein
was detectable in animal tissues minutes after being injected intravenously (Goldberg et
al., 1988). Moreover, immunocytochemistry studies in guinea pigs demonstrated the
presence of LPL at the endothelial surface of all blood vessels (Camps et al., 1990).

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Although the heparin-releasable activity only represented a fraction of the total activity which could be extracted from a tissue, it was likely to be the physiologically relevant fraction (Olivecrona and Bengtsson-Olivecrona, 1987). However, in situ hybridisation studies showed that LPL mRNA was absent from endothelial cells (Camps et al., 1990). Therefore, LPL synthesised by parenchymal cells within an organ or tissue must be secreted and then transported across the interstitial space and endothelial layer to the cell surface (Braun and Severson, 1992).

The intracellular processing of LPL has been extensively investigated, particularly in adipocytes (reviewed by Braun and Severson, 1992). As a secreted protein, LPL is synthesised on rough endoplasmic reticulum ribosomes and the nascent polypeptide co-translationally threaded through the membrane where the 27-amino acid signal peptide (Wion et al., 1987) is cleaved off. N-linked complex carbohydrates are transferred to LPL in the endoplasmic reticulum from the dolichol precursor and subsequently acted upon by glycosidases, yielding a high-mannose structure (Braun and Severson, 1992). Dimerisation is believed to occur at this stage. LPL is then transferred to the Golgi apparatus where mannose residues are removed and sialic acid, galactose and N-acetyl glucose residues added. Finally, the enzyme is transferred to secretory vesicles. Glycosylation is essential for LPL function as blocking of the initial, dolichol-mediated step with tunicamycin abolishes LPL activity in cultured cells (Chajek-Shaul et al., 1985; Simso et al., 1992a). Ben Zeev et al. (1992) have shown that glucose-trimming process is also necessary for enzyme activity but that transfer to the Golgi compartment is not. The importance of carbohydrate moieties for LPL function is further emphasised by the lethality of the homozygous combined lipase deficiency mutation in mice where a defect in the glycosylation process prevents the secretion of functional LPL and HL (Olivecrona et al., 1985b; Davis RC et al., 1990).

An elegant series of experiments by Saxena, Goldberg and colleagues has shed light on the complex pathway for the delivery of LPL from its site of synthesis to the
Figure 1.2. Sites of LPL synthesis, processing and secretion. Details in text. HSPG = heparan sulphate proteoglycans. SV = secretory vesicles. CV = cycling vesicles. CHO = carbohydrates. RER = rough endoplasmic reticulum.
endothelial surface. These workers showed that LPL binding to cultured endothelial cells via HSPG led to internalisation and recycling of LPL without degradation (Saxena et al., 1990b) and that transport from the basolateral to the apical surface also occurred (Saxena et al., 1991a). The HSPG binding sites were asymmetrically distributed on the cell surface, with a striking predominance on the apical surface, which is the physiologically relevant surface (Stins et al., 1992). A 220 kdal proteoglycan has been isolated and proposed to be the LPL endothelial cell surface receptor (Saxena et al., 1991b). The current model (shown in Figure 1.2) proposes that LPL secreted by parenchymal cells is released into the intercellular space. This portion of the LPL transport process is still poorly understood. In adipocytes, LPL is bound to the cell surface via higher affinity HSPG binding sites presumably distinct from the endothelial HSPG but can be released by FFA (Sasaki and Goldberg, 1992; Saxena et al., 1990a). LPL is then believed to bind to the 220 kdal receptor on the basal surface of endothelial cells, and is actively transported to the luminal side presumably through endocytosis and vesicle fusion with the luminal membrane (Braun and Severson, 1992). This process would occur in most tissues and organs with the notable exception of the liver, which produces its own related lipase - hepatic lipase (HL) - rather than LPL. In spite of this, small amounts of LPL activity can be released from (rat) liver capillary beds. This has led to the suggestion that LPL can become detached from HSPG, transported into circulation and captured by HSPG at another location and may represent a means of local regulation of LPL activity (see section 1.5.3.2). Additionally, the liver is believed to be responsible for the clearance of inactive LPL molecules (Wallinder et al., 1984), probably in association with lipoproteins (Vilella et al., 1993).

1.3.3 Role of LPL in the metabolism and remodelling of plasma lipoproteins.

1.3.3.1 In vitro studies of LPL activity.

The primary physiological substrates for LPL are insoluble, long-chain triacyl-
and diacylglycerol molecules present in lipoproteins (Wang et al., 1992) and the enzyme
does not appear to exhibit acyl chain length specificity in vivo. LPL can also hydrolyse
soluble substrates such as tributyrin (Olivecrona and Bengtsson-Olivecrona, 1987).
However, when substrates with different chain lengths are mixed and incubated with LPL
in vitro, a different picture emerges. LPL exhibits an increasing preference for Tg
molecules with shorter acyl chains with the eight carbon chain appearing to be the
optimal substrate (Wang et al., 1992). The presence of a cis double bond at position C9
improves the hydrolysis rate of Tg with longer fatty acid chains, possibly by decreasing
steric hindrance (Wang et al., 1992).

Human LPL can also release the phosphate moiety from phospholipids, although
the enzyme’s affinity towards this substrate is much lower than towards Tg (Deckelbaum
et al., 1992). Tsujita and Okuda (1994) have recently reported that rat LPL can
synthesize fatty acid ethyl esters at low rates. The physiological relevance of this activity
to lipoprotein metabolism in humans is not known.

1.3.3.2 Insights from genetic or artificially induced deficiency in LPL activity.

Havel and Gordon (1960) recognised that a deficiency in heparin-releasable,
plasma lipolytic activity in humans resulted in the massive accumulation of chylomicrons
in circulation and that it was accompanied by a decrease in other lipoprotein fractions
(type I lipoprotein phenotype)(Brunzell, 1989). Thus, LPL catalysed the rate-limiting
step in the metabolism of dietary lipids (Olivecrona and Bengtsson-Olivecrona, 1987) and
its absence was shown to be due to alterations in the gene coding for LPL (Langlois et
al., 1989; Monsalve et al., 1990; reviewed by Lalouel et al., 1992). More recently,
Ginzinger et al. (1993) have reported the molecular characterisation of LPL deficiency
in the cat, where a mutation in the LPL gene causes chylomicronaemia.

Although important information was obtained through the study of individuals
with complete LPL deficiency, the most extreme phenotype, other approaches are better
suited to evaluate the role of LPL in the metabolism of lipoproteins other than chylomicrons. Important insight was gained by inducing prolonged, reversible inhibition of LPL activity in monkeys with the injection of antibodies against LPL (Goldberg et al., 1988 and 1990a). In addition to a rapid rise in plasma Tg, abnormally low LDL and HDL levels became apparent in these animals. This provided additional evidence as to the crucial role played by LPL in the generation of LDL via the lipolytic cascade and the close relationship between Tg-rich lipoproteins and HDL. Tg clearance and restoration of the normolipidaemic state could be accomplished by injection of post-heparin plasma from healthy animals. Another strategy used to assess the function of LPL is to identify and characterise individuals with partial LPL deficiency. Such studies have been carried out in large families where the Gly188 to Glu mutation has been identified. Carriers for this mutation, which probably accounts for 25% LPL mutant alleles in Caucasians (Monsalve et al., 1990; Mailly et al., 1993), have half-normal activity in plasma (Babirak et al., 1989; Wilson et al., 1990; Miesenbock et al., 1993). While hyperlipidaemia is seen in some carriers (especially with increasing age or obesity), others have low LDL and HDL levels (Wilson et al., 1990). Even in carriers with normal levels, a dietary fat challenge may overwhelm the lipolytic system, leading to delayed clearance of chylomicrons and IDL and low HDL (Miesenbock et al., 1993). Overall, these studies show that LPL is directly involved or influences the metabolism of all lipoproteins classes.

1.3.3.3 Studies of LPL in transgenic mice.

The use of transgenic animals to investigate protein function has become widespread in recent years and several reports have been published in the last two years, describing the establishment of transgenic mouse lines expressing human LPL under a variety of promoters (Shimada et al., 1993; Liu et al., 1994). These studies have demonstrated that moderate overexpression of LPL (two-fold increase vs. non-transgenic
litter mates) induces changes in the lipoprotein profile of transgenic animals in both the fasting and fed states. Fasting triglyceride levels were lower in transgenic animals compared to wild-type animals (Shimada et al., 1993; Liu et al., 1994). Moreover, the increase in Tg normally seen following an overnight fast in mice is abolished by increased LPL activity in transgenic animals (Liu et al., 1994). These effects were shown to be due to decreases in the VLDL fraction and this was associated with an increase in LDL-chol in one study (Shimada et al., 1993). Finally, the impact of carbohydrate (increased Tg) and cholesterol-enriched (increased LDL-chol) diets on plasma lipid levels was only observed in control animals (Shimada et al., 1993). Thus, LPL overexpression appears to enhance the clearance of Tg-rich lipoproteins, a potentially beneficial alteration with regards to CHD risk (Liu et al., 1994).

1.3.3.4 Cell surface interactions and the clearance of remnant lipoproteins.

Much interest has been generated in recent years by the realisation that the presence of LPL can enhance the cell-surface binding and internalisation of lipoproteins (reviewed in Santamarina-Fojo and Dugi, 1994). Two distinct mechanisms have been invoked to explain this effect. Firstly, LPL may act as a bridge between HSPG and lipoproteins. Lipoproteins are then concentrated at the cell surface and can interact with receptors or be taken up directly via internalisation of HSPG (Obunike et al., 1994). The phenomenon occurs in a number of cell types including macrophages, fibroblasts and HepG2 cells and has been observed for chylomicrons (Eisenberg et al., 1992), VLDL and LDL (Mulder et al., 1992; Rumsey et al., 1992; Eisenberg et al., 1992). The second mechanism involves the direct interaction of LPL with the LRP, the putative chylomicron remnant receptor, (Beisiegel et al., 1991) which may be internalised with its ligand. Nykjaer et al. (1994) have observed binding of VLDL remnants to the LRP via LPL. No LPL activity is required for this binding and uptake to occur (Chappell et al., 1994). It has also been shown that LPL can enhance the uptake of lipoproteins by a process that
does not involve the LDL-receptor, is independent of HSPG turnover. This uptake is, however, inhibited by the 39 kdal receptor-associated protein (RAP) (Obunike et al., 1994), which is known to interfere with the binding of LPL to LRP (Chappell et al., 1992).

1.3.4 Expression of LPL in macrophages and smooth muscle cells: a potentially atherogenic role.

In view of the location of LPL on the vascular endothelium, Zilversmit (1973) proposed that this enzyme might play a role in the pathogenesis of atherosclerosis. In support of this proposal, immunofluorescence and in situ hybridisation studies have shown that LPL is synthesised and expressed in human and rabbit atherosclerotic lesions both in smooth muscle cells and macrophages (Yla-Herttuala et al., 1991). In culture, macrophages secrete LPL resulting in lipolysis of Tg-rich lipoproteins, increased uptake of FFA and increased endocytosis of remnant lipoproteins (Sofer et al., 1992), causing marked triglyceride accumulation in these cells. Blocking of LPL synthesis or incubation of lipoproteins with macrophages from LPL-deficient individuals prevents this accumulation (Sofer et al., 1992; Skarlatos et al., 1993). In smooth muscle cells isolated from bovine aorta, LPL promotes the uptake of beta-VLDL and it has been proposed that this could represent a mechanism for the intracellular accumulation of esterified cholesterol (Stein et al., 1993). Renier and colleagues (1993) have studied LPL expression in peritoneal macrophages isolated from atherosclerosis-susceptible and atherosclerosis-resistant mouse strains. They found that LPL mRNA, activity and mass were twice as high in macrophages from the susceptible strains. Thus, while the contribution of the macrophage LPL pool to overall lipoprotein metabolism may be modest, high LPL activity in this cell type may significantly increase the atherogenic risk as the lipid filled cells accumulate in the intima, contributing to the atherosclerotic lesion.
1.3.5 Implication of LPL activity in the coagulation cascade.

LPL activity has recently been implicated in the hypercoagulability associated with hypertriglyceridaemia (reviewed by Miller, 1993). Activation of factor VII via the intrinsic pathway is thought to require the presence of a negatively-charged surface, a role which might well be played by FFA assembled on the surface of endothelial cells. Increased factor VII activity is often observed in conditions where plasma Tg are elevated, such as type IV HLP and untreated diabetes mellitus (Miller, 1993). Moreover, both factor VII activity and antigen are correlated with Tg levels in healthy men and the association is strongest with Tg-rich lipoprotein concentrations (Mitropoulos et al., 1989). In contrast, individuals with complete LPL deficiency and massive HTG have normal factor VII levels, but these rise sharply when functional LPL is added to the plasma (Mitropoulos et al., 1992). These results suggest that FFA released by LPL from chylomicrons and VLDL may be physiologically important in the coagulation process.

1.4 Structure-function relationships in LPL.

In recent years, considerable advances have been made in the cloning and detailed characterisation of genes coding for pancreatic lipase (PL), HL and LPL from several species. Concurrent to this work, Winkler et al. (1990) and Brady et al. (1990) published three-dimension structural models of human PL and of Rhizobium Mucor lipase respectively, based on X-ray crystallographic data. These data have provided a framework to study both the common and specific properties of these lipases at the molecular level. In particular, our knowledge of the complex substrate-enzyme interactions which dictate in part the function of proteins operating at the lipid-water interface has progressed substantially. In combination with the analysis of sequence homology/divergence between lipases, the study of naturally-occurring and synthetic LPL mutants have generated a better understanding of LPL structure-function relationships, which will be reviewed in this section.
Fig. 1.3 Schematic structure of the human LPL gene. Stipled/filled boxes represent the coding portion of the gene, with the exon number above them, while introns are shown as clear boxes. Shaded areas indicate untranslated regions in exons 1 and 10. The approximate position of the alternative polyadenylation site in exon 10 is marked by a dotted line. Numbers at the right-hand side of each coding exon box denote the last amino acid encoded (or interrupted). Arrows show the position of polymorphisms located within the LPL gene.
1.4.1 Structure of the LPL gene.

The human LPL gene spans approximately 30 kb and is divided into ten exons, of which nine are protein coding and one codes for the entire 1.95 kb 3’ untranslated region (Deeb and Peng, 1989, Oka et al., 1990)(Figure 1.3). Exons 1-9 vary in length from 105 (exon 9) to 273 bp (exon 1) a size range comparable to that reported for other mammalian exons (Breathnach and Chambon, 1981). The first exon codes for the 5’ untranslated region, the 27-amino acid, hydrophobic signal peptide and the first two residues of the mature protein. RNase mapping and primer extension experiments were used by several investigators to identify the 5’ terminus of LPL mRNA (Wion et al., 1987; Kirchgessner et al., 1989a; Deeb and Peng, 1989; Oka et al., 1990). Four possible transcription initiation sites were detected with the most likely cap site being located 188 bp upstream from the first AUG codon (Kirchgessner et al., 1989a; Deeb and Peng, 1989). Exons 2-9 encode the remainder of the 448 amino acid protein. Exon 10 is unusually large at 1.95kb and is untranslated, with the exception of the first nucleotide of the exon representing the third base of the termination codon. Two AATAAAA polyadenylation sites, separated by 395 bp, are present in exon 10 and account for the 3.35 and 3.75 kb mRNA species detected in human tissues (Wion et al., 1987).

Introns 2-9 are similar in size (ca. 1-3kb) while intron 1 is 10kb long. Two inverted Alu sequences have been identified in intron 6 (Devlin et al., 1990) and intron 7 (Chuat et al., 1992). The latter is followed by (dA)_{20} and not flanked by direct repeats.

1.4.1.1 Common genetic variation at the LPL gene locus.

Several polymorphisms (rare allele frequency > 1%) located within or near the LPL gene have been reported so far in the literature and their position is shown on the schematic LPL gene map (Figure 1.3). They include:

- classical diallelic restriction fragment length polymorphisms (RFLPs) such as the PvuII
(intron 6), the *HindIII* (intron 8) and *BamHI* polymorphic sites (approximately 5 kb downstream of the gene) identified concurrently to the isolation of genomic clones (Fisher et al., 1987; Kirchgessner et al., 1989a; Oka et al., 1990);

- silent (*G*<sub>405</sub> to A - exon 3; *G*<sub>435</sub> to A - exon 4; *C*<sub>1164</sub> to A - exon 8) and potentially functional single-base substitutions (*A*<sub>1127</sub> to G, N291S; *C*<sub>1595</sub> to G, S447stop) within the LPL coding region identified by sequencing DNA amplified via the polymerase chain reaction (PCR)(Gagné et al., 1994; Lohse et al. 1991; Ma et al., 1993c; Hata et al., 1990b); these polymorphisms are detected by PCR followed by restriction digest or hybridisation with allele-specific oligonucleotide (ASO) probes;

- recently identified highly polymorphic markers containing repeated dinucleotide (CA, unspecified position in 5'end region) or tetranucleotide motifs (TTTA, intron 6), identified with repeat-specific probes and resolved by polyacrylamide gel electrophoresis (Ahn et al., 1992; Zuliani and Hobbs, 1992; Narcisi et al., 1993); these will be valuable in cosegregation studies but are unlikely to represent functional variants.

The stop codon 447 allele gives rise to a truncated LPL species where the C-terminal Ser-Gly dipeptide is deleted (Hata et al., 1990b). This mutation does not adversely affect LPL activity but it may alter lipid interfacial recognition (see section 1.4.5)(Kobayashi et al., 1992). Both the *PvuII* and *HindIII* RFLPs as well as the Ser447/stop polymorphism have been used in frequency and association studies to demonstrate that genetic variation in the LPL gene contributes to the determination of lipid levels (Chamberlain et al., 1991; Hata et al., 1990b; Peacock et al., 1992; Ahn et al., 1993; Mattu et al., 1994). An association has been observed between the *HindIII* RFLP and Tg levels where the H1 (cutting) allele of the *HindIII* polymorphism is associated with increased Tg, cholesterol and apoB levels and lower LPL activity in white Caucasians (Peacock et al., 1992). A recent study in Mexican American subjects has also shown that the *PvuII* polymorphism contributes to the determination of plasma
insulin levels (Ahn et al., 1993). LPL variants have also been examined in the context of atherosclerotic disease and the H1 allele of the HindIII polymorphism was found to be associated with the severity of atherosclerosis in a Welsh population (Mattu et al., 1994) and the progression of the disease in the Swedish YMI study (Peacock et al., 1992). As the HindIII RFLP itself does not change the coding sequence and is unlikely to be directly responsible for the association, it is probably in linkage disequilibrium with a functional mutation. Common LPL variants which alter the amino acid sequence such as the Ser447stop substitution (Hata et al., 1990b) do not appear to explain the associations detected between lipid traits and the LPL gene variants (Peacock et al., 1992; Peacock, 1994). This strongly suggests that there are as yet unknown functional genetic variants which are in linkage disequilibrium with the HindIII and PvuII polymorphisms and can directly influence lipoprotein metabolism.

1.4.2 Evolutionary relationships between the LPL gene and other members of the lipase gene family.

The chromosomal localisation of the human LPL and HL genes was first determined by Sparkes et al. (1987), by Southern blotting of genomic DNA from mouse-human somatic cell hybrids and by in situ hybridisation. Recent work by Mattei et al. (1993) has confirmed the original assignment of LPL to chromosome band 8p22.

The isolation and sequencing of cDNA clones from several species, including rat HL and LPL (Komaromy and Schotz, 1987; Brault et al., 1992), mouse LPL (Kirchgessner et al., 1987), guinea pig LPL (Enerback et al., 1987), bovine LPL (Senda et al., 1987), and human HL, PL, LPL (Datta et al., 1988; Lowe et al., 1989; Wion et al., 1987) as well as amino acid sequence data from porcine PL (De Caro et al., 1981) has allowed comparisons and alignments of derived amino acid sequences. These have shown extensive homology between these genes and led to the recognition of a lipase gene family comprising the vertebrate LPL, HL and PL genes (Komaromy and Schotz,
Kirchgessner and collaborators (1989a) have proposed a model whereby a primordial gene containing 14 introns gave rise to mammalian HL/LPL in one branch and PL in another through two duplications and separate intron loss events. The N-terminus and central portion of the LPL and PL proteins are more highly conserved than the C-terminal region. Human LPL and HL genes are more closely related to each other than to human PL, as assessed by 1) the lower proportion of amino acid differences (53% residue identity between HL and LPL vs. 35% identity of either with PL); and 2) the similarity in the organisation of exon/intron boundaries between LPL and HL (Kirchgessner et al., 1989a). Both HL and LPL have nine coding exons of comparable size (exons 1-9) interrupted by introns at coinciding codons and in the same phase (Cai et al., 1989; Oka et al., 1990). Amino acid sequence identity for mammalian LPL ranges from 90.1% between guinea pig and cow to 97.8% between human and mouse LPL while slightly lower residue identity exists between human and chicken LPL (76%) (Hide et al., 1992).

In addition to these vertebrate lipases, two Drosophila yolk proteins (YP I and II) that do not possess lipase activity but retain the ability to bind lipids, show similarities with LPL in a 105 residue stretch (aa 105-209) (Persson et al., 1989). The region includes 23 residues completely conserved between YPI, YPII, human LPL, human HL and porcine PL, and an additional 11 residues conserved in four of five species. This relatively high degree of conservation between such distantly related species points to the importance of this region.

1.4.3 Key features of human LPL amino acid sequence.

Mature human LPL contains 448 amino acids with a molecular mass of 50 398 daltons (Wion et al., 1987). Its main features include a consensus sequence shared by virtually all neutral lipases (G-X-S-X-G) as part of a putative active site (Derewenda and Sharp, 1993), two consensus sequences for N-glycosylation (N-X-S/T where X is any
amino acid except proline) at Asn43 and 359 respectively (Wion et al., 1987), ten conserved cysteine residues shown to exist in five disulfide linkages (Yang et al., 1989) and an arginine/lysine rich motif at residues 292-300, representing a putative heparin binding site (KVRAKRSSK) (Wion et al., 1987; Cardin and Weintraub, 1989; Enerback et al., 1987). Additionally, two other short, positively charged segments may interact with the LPL co-factor, apo CII (Lys147-148) or heparin (279-282) (Hata et al., 1993; Ma et al., 1994b) while the region from amino acids 125-142 may mediate interfacial lipid binding (Wion et al., 1987).

1.4.4 Secondary structure predictions and tridimensional structure of PL.

Much has been learned in recent years about the secondary and tertiary structure of lipases as a result of X-ray crystallography studies of human PL and of two fungal lipases from \textit{Rhizomucor miehei} (RmL) and \textit{Geotrichum candidum} (GcL) respectively (Winkler et al., 1990; Brady et al., 1990; Schrag et al., 1991). Determination of the crystal structures at 1.9 and 2.2 Å resolution has shown that in spite of a complete lack of sequence homology, all three enzymes share common structural features. These include a central β-pleated sheet with closely superimposable strands, including five parallel β strands, an active site comprising a Ser - Asp - His catalytic triad and a loop (lid) structure under which is buried the catalytic site (reviewed by Lawson et al., 1992).

The three-dimension model of human PL proposed by Winkler and colleagues (1990) indicated that PL consisted of two distinct folding units: the larger N-terminus comprises residues 1-336 in an α/β structure with a core parallel β-sheet whereas the smaller C-terminus (337-449), absent in fungal lipases, assumes a so-called β-sandwich conformation with two layers of four anti-parallel β chains (Derewenda and Sharp, 1993). Although a crystal structure has not yet been obtained for human LPL, the high sequence homology between the two enzymes, particularly in the central, aa108-320
region (Hide et al., 1992), strongly suggests that the folded 3D structure of LPL will closely resemble that of PL (Santamarina-Fojo, 1992; van Tilbeurgh et al., 1994). The conserved alignment of four pairs of disulfide-linked cysteine residues further strengthens this hypothesis (Wang et al., 1992). Derewenda and Cambillau (1991) have presented secondary structure predictions for LPL obtained with the Chou-Fasman algorithm (Chou and Fasman, 1978). The N-terminal portion of LPL (residues 1-308) includes nine parallel $\beta$ strands together with eight a helices whereas only $\beta$ strands are predicted to form in the smaller C-terminus region (309-448). This is in general agreement with the PL secondary structure although some differences are apparent, especially in the less conserved C-terminal region. More recently, van Tilbeurgh and colleagues (1994) have used a computer modelling approach to evaluate the effects of known amino acid substitutions in LPL on domain structure and intra-chain interactions by superimposing the LPL amino acid sequence onto the PL 3D coordinates, with adjustments for the effect of non-conservative amino acid substitutions.

1.4.5 Localisation of LPL functional domains.

The biochemical properties of human LPL have been extensively studied and indicate that the protein contains at least six functional domains: 1) a catalytic site including the Ser-Asp-His triad; 2) a heparin/heparan sulphate binding region; 3) a binding site for the essential LPL co-factor, apo CII; 4) a lipid binding domain; 5) a region interacting with LRP; and 6) an interfacial activation domain. Much progress has been achieved in the localisation of these domains in recent years and this will be reviewed in the next two sections. In addition, as yet unknown regions of LPL interact (non-covalently) to form the active dimer enzyme. Based on structural studies of LPL carried out with a series of monoclonal antibodies, Wong and collaborators (1994) have proposed a head-to-tail model of LPL with a two-fold axis of symmetry where the N-terminal domain of one monomer interacts with the C-terminal domain of the other.
Several of the mutations identified in subjects with type I HLP appear to inactivate LPL by virtue of the instability of the dimer rather than via a direct effect on the catalytic site (Hata et al., 1992; Previato et al., 1994). One of these mutations, the Gly188 to Glu substitution, results in the production of large amounts of monomeric protein (Hata et al., 1992). The introduction of the charged glutamic acid residue in a region in close proximity to the lid structure apparently impairs the dimerisation process, probably through a conformation change. Figure 1.4 presents the known LPL functional domains superimposed on the dimerised PL structure.

1.4.5.1 The use of naturally occurring and genetically engineered mutations.

This section describes the use of point mutations or small deletions towards the localisation of the functional domains of LPL. In the course of their study of PL, Winkler et al. (1990) identified Ser152 of porcine PL (Ser153 in human PL) as the possible active-site serine of the catalytic triad. The other two members of the triad in human PL, Asp177 and His264, are linked to Ser152 via hydrogen bonds (Winkler et al., 1990). The relevance of the triad residues to catalysis in PL was confirmed by site-directed mutagenesis (Lowe, 1992). The corresponding residues in human LPL are Ser132, Asp156 and His241 (Hide et al., 1992) and are thought to be buried in a hydrophobic pocket like the PL triad residues (Winkler et al., 1990). Substitution of Ser132 with glycine, alanine, threonine and aspartic acid by site-directed mutagenesis results in the production of catalytically inactive proteins (when tested against triolein) which maintain their affinity for heparin and lipids (Emmerich et al., 1992; Faustinella et al., 1991b). Faustinella et al. (1991b) also systematically mutated seven other serines which were conserved across species in LPL as well as in human HL or PL, and replaced most of them successively with alanine, threonine or glycine. While several mutants had decreased activity, at least one active mutant species could be identified for each replaced serine with the exception of Ser132, which was inactive for all
PROPOSED LOCATION OF FUNCTIONAL DOMAINS IN ACTIVE LPL DIMER

Fig 1. Details in text.
substitutions. The replacement of Asp 156 by glycine or asparagine also abolishes LPL activity when tested \textit{in vitro} (Emmerich et al., 1992). Moreover, these substitutions have been identified in LPL-deficient probands exhibiting type I HLP (Faustinella et al., 1991a; Ma et al., 1992a). Mutagenesis of the third residue of the triad, His241, again results in the inactivation of the protein (Emmerich et al., 1992). Additionally, Emmerich et al. (1992) showed that mutations of the catalytic residues abolished LPL esterase activity against tributyrin. Although no natural mutations have yet been reported at Ser 132 and His241, molecular defects have been identified in several neighbouring residues which cause LPL deficiency and HLP (positions 136, 139, 239, 243 and 244, see table 1.2). Each of the residues of the catalytic triad is encoded by a different exon (Ser132 in exon 4, Asp156 in exon 5 and His241 in exon 6). Of the large number of genetic defects described in the LPL gene of type I subjects, the majority are clustered in exons 3-6 (Lalouel et al., 1992). Some of these, such as Asp180 to Glu (Haubenwallner et al., 1993) are conservative amino acid substitutions. This is in keeping with the highly conserved nature of this region (Hide et al., 1992) and the tight packing of the residue side chains (van Tilbeurgh et al., 1994).

An important feature of LPL function is the ionic attachment on the endothelial cell surface via HSPG. As HSPG and heparin bear strong negative charges, it was expected that clusters of basic amino acids would be involved in the interaction with HSPG. Five clusters (three in the N-terminus and two in the C-terminus) have been investigated by site-directed mutagenesis with the replacement of lysine or arginine residues with neutral alanine (Hata et al., 1993; Ma et al., 1994b). Substitutions within segments spanning residues 279-282 or 291-304 were shown to alter the affinity of LPL towards a heparin-Sepharose column and these regions were concluded to be involved in the interaction with HSPG. The catalytic activity was usually maintained. van Tilbeurgh et al. (1994) reached a similar conclusion by calculating the electrostatic surface potential using their 3D computer-derived model of LPL except that Arg263 was
added to the first segment as it was adjacent to aa279-282 in the 3D model. No single mutation abolished the interaction between LPL and heparin, a finding consistent with the polyanionic nature of the binding site (Hata et al., 1993). The position and orientation of these regions on the 3D model (Fig. 1.4) - pointing away from the catalytic domain - is also in agreement with the known ability of LPL to simultaneously bind HSPG and substrate (Hata et al., 1993). Finally, LPL monomers have very weak affinity towards HSPG (Peterson et al., 1992) and it has been pointed out that charged clusters in both subunits probably operate together as a single heparin binding site (van Tilbeurgh et al., 1994).

Since the terminal negatively charged dipeptide of apo CII appears to be important for the interaction with LPL, positively charged pairs of residues were investigated (Bruin, 1994). Replacement of lysine 147 and 148 by alanine resulted in a moderate decrease of apo CII's ability to activate LPL. Thus, additional interactions involving other amino acids are probably necessary for the activation of LPL by apo CII.

Glycosylation of LPL is necessary for enzyme activity and two potential N-linked glycosylation sites, Asn43 and Asn359, have been identified (see section 1.3.2) (Ben Zeev et al., 1992). Ben Zeev et al. (1994) have shown that in COS cells transfected with the LPL cDNA, both sites are glycosylated (Ben Zeev et al., 1994). However, in vitro mutagenesis and in vivo studies demonstrate that glycosylation at Asn43 only is essential for enzyme activity and secretion (Kobayashi et al., 1994). The replacement of Asn359 by Ala prevents glycosylation but results in a fully active enzyme that is secreted normally (Semenkovich et al., 1990; Ben Zeev et al., 1994).

The two amphipathic helices which form the lid structure covering the catalytic site are located between cysteine residues 216 and 239, themselves linked through a disulfide linkage (Yang et al., 1989; Winkler et al., 1990). It has been suggested that the lid structure forms part of the interfacial activation site (in addition to residues 125-142) (van Tilbeurgh et al., 1993 and 1994). Based on X-ray studies with PL, it is predicted
that the interaction between a lipoprotein substrate and the LPL lid causes a conformation change in the latter, thereby exposing previously buried hydrophobic residues (van Tilbeurgh et al., 1993). The fact that the deletion of the lid abolishes LPL activity against triolein but not against tributyrin, a soluble substrate, further supports this claim (Dugi et al., 1992). In contrast to other regions of exons 4-6, this segment of exon 5 is poorly conserved between LPL and HL (Bensadoun, 1991) and this suggests that it could determine at least in part LPL substrate specificity (Santamarina-Fojo and Dugi, 1994). Replacement of the LPL lid with the HL surface loop decreases the activity of the enzyme against triolein (Faustinella et al., 1992). Henderson et al. (1993) have also shown that the distribution of polar residues on the loop is crucial as the replacement of the hydrophobic isoleucine residue by the more polar threonine results in the production of an inactive enzyme and type I HLP in a subject.

The function of the LPL carboxy terminal domain is not well defined yet but it has been suggested that it may be involved in determining substrate specificity and mediating lipid binding (Lookene and Bengtsson-Olivecrona, 1993). These workers have reported that the removal by chymotrypsin cleavage of the 58 C-terminal residues of LPL produces an enzyme which can hydrolyse water-soluble tributyrin but not chylomicrons, and which cannot bind to chylomicrons. Pre-incubation of LPL with antibodies directed to the C-terminus has a similar effect (Wong et al., 1994). Furthermore, a truncated natural mutant of LPL (Trp382 to stop, see Table 1.2) is enzymatically inactive and causes type I HLP (Gotoda et al., 1991a). Kozaki et al. (1993) have generated a series of deletion mutants for the carboxy terminus and have shown that the removal of as little as 14 amino acids markedly decreases LPL mass and activity (approximately 25-30% of control levels). The lower mass is not associated with a decrease in mRNA and may be due to lower protein stability. This is compatible with the suggestion that the C-terminus plays a role in the formation of the active LPL homodimer (Santamarina-Fojo and Dugi, 1994). Finally, the ability of LPL to bind to
the LRP has been localised to the C-terminus of LPL between residues 313-448 (Nykjaer et al., 1994; Williams et al., 1994)

**1.4.5.2 Determination of LPL-specific properties with domain exchange experiments.**

An alternative approach for the investigation of LPL functional domains involves the construction of LPL/HL chimaeras containing the N-terminus of LPL linked to the C-terminus of HL or vice versa. These studies provide a molecular basis for the understanding of functional differences between LPL and HL. Experiments have confirmed that the apo CII binding site resides in the amino terminus of LPL (Davis et al., 1992) and that the kinetic parameters for each enzyme are also determined by this region. Antibodies specific for the LPL C-terminus blocked triolein hydrolysis by the HL/LPL chimaera, further confirming the role of this domain in lipid binding (Wong et al., 1991). An interesting result has emerged from the work of Davis and colleagues (1992). They report that the higher affinity of LPL for heparin when compared to HL appears to be determined by the C-terminus even though heparin binding appears to be dependent on N-terminus residues (Ma et al., 1994b). Lastly, interaction between the N- and C-terminal domains has been suggested to affect the affinity for phospholipids (Davis et al., 1992), rather than an individual domain. This is because both the LPL/HL and HL/LPL showed higher phospholipase activity than HL. Such an interaction might occur between the N-terminus of one LPL monomer and the C-terminus of the other. These are proposed to represent a functional unit in the model of Wong et al. (1994) with the C-terminal domain binding and presenting the lipid substrate to the catalytic domain.

**1.5 The LPL gene and its product: control of expression and tissue-specific regulation.**

A complex pattern of regulation is emerging where tissue-specific expression is modulated by developmental, hormonal and dietary changes. The regulation mechanisms
reported vary widely depending on the cell type used and occur both at the transcriptional and post-transcriptional levels (reviewed by Braun and Severson, 1992).

1.5.1 Control of LPL gene transcription - Cis regulatory elements and transacting transcriptional regulators.

The LPL 5’ flanking region has been studied extensively since the gene was isolated and characterised (Kirchgessner et al., 1989a,b; Deeb and Peng, 1989). Examination of the sequence upstream of the transcription start site has revealed a TATA box at position -27 and two CCAAT motifs at -65 and -506 (Deeb and Peng, 1989). The latter may mediate the binding of NF-1, an enhancer-binding transcription factor. Three octanucleotide sites (OCT-1, ATTTGCAT) which recognise the transcription factor OTF-1 are also present (Hua et al., 1991; Previato et al., 1991). Enerback et al. (1992) have identified two elements, termed Lpalpha and Lpbeta, which are important for inducing the transcription of the LPL gene during adipocyte differentiation. Additional sequences of potential importance include a thyroid hormone response element (Enerback and Gimble, 1993) and a silencer element which binds as yet unidentified proteins (Tanuma et al., 1995). The relevance of these sequence motifs to tissue-specific is still unknown.

1.5.2 Post-transcriptional regulation.

A large body of evidence suggests that the modulation of LPL activity may occur primarily at the post-transcriptional level (Braun and Severson, 1992). Changes in mRNA levels have been detected during development in the rat (Semenkovich et al., 1989a). Other studies have reported alterations of mRNA levels under certain pathophysiological conditions, but these were usually too small or too slow to account for the variation observed in LPL activity (Olivecrona and Bengtsson-Olivecrona, 1993).

A large number of factors have been reported to influence tissue LPL. These include diet composition, fasting/fed state, insulin levels and diabetes, age and exercise.
Diabetes is usually associated with a decrease in both adipose and cardiac tissue LPL although a distinct mechanism appears to be involved for each tissue (Braun and Severson, 1992;). Insulin increases LPL activity in 3T3 adipocytes while decreasing new LPL synthesis (Semenkovich et al., 1989b) but it has no effect on isolated cardiac myocytes (Braun and Severson, 1992). Feeding and fasting regulate adipose and muscle LPL in a reciprocal fashion. In the rat, fasting increases heart muscle LPL and decreases adipose tissue LPL, an effect rapidly reversed by re-feeding. These changes do not involve LPL synthesis and seem to be mediated via a pool of inactive intracellular LPL molecules (Doolittle et al., 1990) since they are associated with changes in glycosylation of LPL. Other studies in animals (Semb and Olivecrona, 1989) and humans (Ong and Kern, 1989) have similarly failed to observe variation in mRNA levels or LPL synthesis, and have concluded that LPL regulation occurs mostly at the post-translational level.

1.5.3 Regulation of LPL hydrolytic activity.

In addition to post-transcriptional control of LPL synthesis, plasma LPL activity itself appears to be regulated. A well-known element in this process is the presence of apo CII. More recently, the roles of FFA and apo CIII as negative regulators of LPL activity have been investigated and re-evaluated.

1.5.3.1 Apolipoproteins CII and CIII.

In 1970, La Rosa et al. reported the isolation of an essential activator of LPL activity in human plasma. This activating factor was localised to VLDL and stimulated LPL obtained from a variety of sources including rat and human plasma and milk (Havel et al., 1973). The 79 amino acid glycoprotein, apo CII (Jackson et al., 1977), bound non-covalently to LPL and dramatically increased the hydrolysis of emulsified lipids in vitro while its absence in vivo led to type I HLP (section 1.2.1.2). It has been estimated
that normal apo CII levels are well in excess of the amount necessary to achieve maximal activation of LPL (Saheki et al., 1991). While the significance of this excess capacity is unknown, it has recently been shown that a proportion of plasma apo CII 'stored' in HDL cannot efficiently activate LPL (Goldberg et al., 1990b; Tornoci et al., 1993).

The molecular mechanism through which apo CII exerts its effect is still poorly understood. There is no evidence for binding of the co-factor prior to the interaction with the substrate (the Tg molecule) as both are localised to the same particle. Vainio et al. (1983) have proposed that apo CII might induce a conformational change in LPL, thereby reorienting the enzyme at the lipid-water interface. Its binding site on LPL is believed to be located in the N-terminal domain (section 1.4.3) with the bond being mediated at least in part by electrostatic interactions involving the C-terminus of apo CII (Kinnunen et al., 1977; Cheng et al., 1990; Bruin et al., 1994a). The removal of the charged terminal tripeptide (residues 77-79, GEE) from a fully active fragment of apo CII (55-79) and a naturally occurring premature termination at position 69 both abolish the activation of LPL by apo CII (Kinnunen et al., 1977; Connelly et al., 1987).

One of the early observation made regarding the regulation of LPL activity was its inhibition in vitro by apo CIII from Tg-rich lipoproteins (Brown and Baginsky, 1970). Apo B turnover studies performed by Ginsberg et al. (1986) in two sisters deficient in apo CIII showed that VLDL conversion to IDL and LDL was markedly increased in such patients, suggesting an inhibitory role for apoCIII in vivo. These authors could not measure the receptor-mediated uptake of these rapidly metabolised VLDL due to their very low level. More recently, overexpression of human apoCIII was shown to cause hypertriglyceridaemia with accumulation of large VLDL particles in a transgenic mouse model (Aalto-Setala et al., 1992). It was proposed that impairment of both lipolysis and uptake mechanisms was involved in mediating the hyperlipidaemic phenotype, through alterations of the particle surface and displacement of the receptor ligand, apo E. Surprisingly, overexpression of apo CII in the same model also leads to hypertriglyceridaemia, possibly due to the displacement of apo E (Shachter et al., 1994).
1.5.3.2 End-product regulation by FFA.

*In vitro* experiments have revealed that LPL has a built-in regulation mechanism for its hydrolytic activity. In the absence of albumin to remove released FFA from the reaction site, FFA bind and stabilise LPL. This in turn decreases the enzyme’s affinity for lipid droplets, apoCII and HSPG (Olivecrona and Bengtsson-Olivecrona, 1987; Olivecrona et al., 1989). If it occurred *in vivo*, FFA binding might provide a sensitive mechanism for the local regulation of LPL activity. In this model, when local cellular needs for FFA have been met, further lipolysis is prevented both by blocking access to the substrate and displacing LPL from the area. Two sets of observations can be put forward in support of this model. Firstly, tissue LPL activity generally exceeds the rate of cellular FFA uptake (Vernon and Clegg, 1985; Olivecrona et al., 1989), making possible the local accumulation of FFA. Secondly, in experiments with healthy volunteers, a small amount of LPL activity was shown to be released from the endothelial surface following the ingestion of a test fatty meal (Peterson et al., 1990). The increase in plasma LPL activity paralleled the rise of plasma FFA rather than total triglycerides and was modest compared to that induced by the injection of heparin (20-50 fold lower)(Hultin et al., 1992). While LPL released by FFA can apparently relocate to a different site on the capillary endothelium, and particularly to tissues that do not synthesise LPL, heparin-released LPL appears to be mainly cleared and degraded by the liver (Wallinder et al., 1984; Hultin et al., 1992).

1.6 Detection of novel variation in DNA: the use of SSCP.

The advent of DNA amplification through the polymerase chain reaction (Saiki et al., 1986) has played a crucial role in the successful search for causative mutations and their detection, allowing rapid and systematic screening of DNA. Single-strand conformation polymorphism (SSCP) is one technique which has been combined with
PCR for this purpose and will be reviewed briefly in this section.

The single-strand conformational polymorphism technique developed by Orita et al. (1989a) is a method suited for the detection of variation in PCR products of 150-300 nucleotides by separation of DNA fragments on an acrylamide gel matrix. Under non-denaturing conditions, a single strand of DNA folds back on itself due to intra-strand base-pairing between short complementary segments (Ainsworth and Rodenhiser, 1994). For any given sequence of 150-300 nucleotides, it is likely that several sequence-dependent conformations are theoretically possible. However, few will be thermodynamically favourable and even the stablest conformation is usually relatively heat labile, with several unpaired loops. As a first consequence, loosely folded single-stranded DNA will generally migrate more slowly than double-stranded DNA in a gel matrix. Secondly, owing to their complementary base composition, the two strands of a DNA duplex will adopt distinct conformations that will result in different migration rates. Thirdly, single base substitutions which alter local base-pairing are presumably sufficient to change the shape of the folded molecule such that this causes a mobility shift relative to the native strand. If the mutant and the normal strands are electrophoresed over a sufficiently long distance, the difference in mobility will become apparent. A schematic diagram of the expected band patterns for SSCP analysis is shown in Fig. 1.6.
Fig. 1.6 Predicted band patterns for SSCP and heteroduplex analysis. Two bands are expected for homozygous samples and four for heterozygous samples. DNA single strands are indicated as 'ss' and undenatured/re-annealed DNA is shown as ds (double-stranded). Homozygosity for the normal allele is denoted as NN, homozygosity for the mutant allele as MM, and heterozygosity as NM. A heteroduplex pattern, which sometimes accompanies SSCP is shown for one heterozygote pattern and is marked by an asterisk.

A typical protocol for SSCP detection is to heat-denature a radiolabelled PCR product and load the single-stranded sample onto a 40cm, non-denaturing acrylamide gel (sequencing gel size) for a long run with low constant current settings. When this is completed, the DNA single stands can be visualised by autoradiography. Sequencing is then performed on samples exhibiting a variant pattern to identify the mutation. Non-isotopic application of the method in combination with silver-staining has also been reported (Ainsworth and Rodenhiser, 1994).

The PCR sample can be diluted prior to denaturation to decrease the probability of re-annealing in the early stages of the electrophoretic process. Some renaturation will
nevertheless usually occur, weakening the signal associated the single stands. However, this may actually improve the detection power of the method. If the sample originates from a heterozygous individual, heteroduplexes will be formed upon re-annealing, creating an unpaired 'bubble' at the site of the mismatch. This has been shown to retard the migration of the double-stranded product which will then appear as a doublet. The presence of glycerol in the gel and a higher acrylamide to bisacrylamide ratio, which increases the gel pore size, have been shown to increase the resolution power of the system (Orita et al., 1989b). Additionally, the temperature of the gel obviously needs to be carefully controlled because the single-strand conformation is heat labile.

The power, simplicity and effectiveness of SSCP as a screening method have been amply demonstrated by the identification of causative mutations in a variety of diseases. These include conditions as diverse as autosomal dominant retinitis pigmentosa (Dryja et al., 1991), Tay-Sachs disease (Ainsworth and Rodenhiser, 1994), familial hypercholesterolaemia (Gudnason et al., 1993) and LPL deficiency (Hata et al., 1990b; Reina et al., 1992). However, the specific gel conditions (length of run, addition of glycerol etc.) required to achieve maximal resolution need to be determined empirically. It is also not possible to predict the effect of a specific mutation on mobility. Finally, the cost of amplification primers can become prohibitive when screening a very large gene because the technique can only be applied to relatively short segments.

1.7 Hypothesis and aims of this thesis.

Over the past few years, evidence has been accumulating that FCHL, the commonest hyperlipidaemia in humans (Grundy et al., 1987), probably represents a constellation of disorders at the molecular level rather than a single entity (Kwiterovich, 1993b). It has been shown by Brunzell and colleagues that a subset of patients with FCHL have a partial deficiency in LPL activity and mass (Babirak et al., 1992). In parallel, a proportion of obligate heterozygotes for LPL deficiency (one defective allele)
display mild hypertriglyceridaemia (Wilson et al., 1990) or a lipid pattern similar to FCHL subjects (Babirak et al., 1989). These results have led to the hypothesis that defects in the LPL gene might contribute to the etiology of hyperlipidaemia, and FCHL in particular.

The principal objectives of this thesis are as follows:

a) to identify and characterise mutations in the structural gene coding for lipoprotein lipase present in individuals with primary type I HLP from the UK and Europe
b) to determine whether the mutations identified through objective a) or other variants are present in a selected group of patients with CHL and low LPL activity
c) to establish the functional significance of selected mutations identified through objectives a) and b)
d) to investigate the contribution of these mutations to the development of hyperlipidaemia and CHD in large samples comprising healthy and affected individuals

These objectives will be achieved in the following manner:

a) SSCP will be used to detect mutations in the coding exons of LPL in subjects with type I HLP and CHL and the nature of the mutations determined by direct sequencing (chapters 3 and 4)
b) rapid screening methods will be used to ascertain the frequency of mutations in healthy men from the UK, in hyperlipidaemic individuals from the Netherlands and Sweden with their control groups and in MI sufferers from Sweden, Ireland and France with their control groups (chapter 4)
c) the functional significance of several mutations will be confirmed by in vitro mutagenesis followed by expression in a transient assay system (chapter 5)
d) lipid, lipoparticle or apolipoprotein levels in carriers identified through the screening procedure will be compared to levels in non-carriers (chapter 6).

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2 MATERIALS, METHODS AND STUDY SUBJECTS.

2.1 Materials

All chemicals were obtained from BDH, Gibco-BRL or Sigma unless otherwise stated. Restriction enzymes and biochemicals were obtained from Pharmacia, Sigma, BRL, Promega or Anglian Biotechnology. Suppliers of kits are given in the text.

Radiochemicals, Hybond-N and X-ray film were obtained from Amersham. Tissue culture media was obtained from Gibco-BRL and plastics from Nunc or Falcon.

Oligonucleotides were either synthesised on a Pharmacia Gene Assembler (Pharmacia, Uppsala, Sweden), or they were ordered from Severn Biotech Ltd. (Kidderminster, UK.), Oswell Scientific Ltd, (Edinburgh, UK.), Advanced Biotechnology Centre (Charing Cross and Westminster Hospital Medical School, London, UK.) and Genosys Ltd (Cambridge, UK).

The constituents of standard solutions referred to in this thesis (20xSSC, 20xSSPE, 10xTAE, 10xTBE and TE buffer) are given in Maniatis et al. (1982).
2.2 Study subjects.

2.2.1 Subjects screened for new LPL mutations.

2.2.1.1 Subjects with type I or type V hyperlipoproteinaemia (HLP).

Twenty unrelated probands diagnosed with type I HLP, based on triglyceride levels (generally > 20mmol/L at time of diagnosis) and the presence of chylomicrons in fasting plasma, or type V HLP with LPL deficiency, were recruited for this study. Most patients (17/20) were identified in infancy or childhood, either through routine blood sampling or due to recurrent episodes of abdominal pain and vomiting. In older subjects, secondary causes for hyperlipidaemia were ruled out. DNA from these individuals was used for the studies described in chapter 3. Details of clinical presentation, with biometrical and biochemical data, is presented in Table 3.1.

Five of the subjects were patients at the Institute of Child Health, London, 4 at the Royal Infirmary, Manchester, 4 at the Lipid Clinic, St. Thomas' Hospital, London, 3 at the Middlesex Hospital Lipid Clinic, London. The remaining four probands were recruited via paediatricians and GPs in the UK (1), Sweden (2) (Lithell et al., 1978) and Italy (1). Nine of the patients were of English descent, 7 were from the Indian subcontinent, 2 were Swedish and 2 were of Mediterranean descent.

2.2.1.2 Individuals with low LPL activity

Fifteen patients with combined hyperlipidaemia recruited at Charing Cross Hospital, London as part of a larger study (see section 2.2.2.5) and ten Swedish individuals taking part in a case-control study of MI before the age of 45 (see section 2.2.2.1) were also screened for new mutations/common variants in the LPL gene. Low LPL activity (in the lower third of the distribution) was used as the selection criterion.
2.2.2 Population samples screened for LPL variants

DNA samples from 2992 subjects originating from the UK (England, Northern Ireland and Scotland), France, Sweden and the Netherlands were used in the course of the studies presented in chapter 4. The various groups are briefly described below and their general characteristics are summarised in Table 4.7.

2.2.2.1 Swedish patients and controls I

This first sample consisted of Swedish patients and controls participating in a case-control study of myocardial infarction at a young age (<45 yrs). The two subgroups will further be referred to as Young MI (YMI) patients and controls. Data on lipid, lipoprotein, lipase activity and haemostatic variables have been presented elsewhere (Hamsten et al., 1986, 1987; Peacock et al., 1992).

The group of patients consisted of 143 individuals from Stockholm county who were recruited as described in Hamsten et al., (1986) and had suffered an MI before the age of 45. Patients with serum cholesterol levels above 9.5 mM or a diagnosis of familial hypercholesterolaemia, diabetes or porphyria were excluded from the lipid analysis. DNA studies were performed on a sample of 100 of these patients (76 men, 24 women). They were age-matched with 93 randomly selected, healthy male residents of Stockholm county that were free of clinical signs of CAD.

2.2.2.2 Swedish patients and controls II

A second group of randomly selected Swedish male individuals aged between 40 and 50 years old were included in our studies. They had participated in a community-based study on hypertriglyceridaemia in Stockholm county and were divided as follows:
65 hypertriglyceridaemic individuals (fasting serum Tg above 95th percentile, 3.1 mmol/l) and 60 age-matched normotriglyceridaemic individuals (Tg < 3.1 mmol/l). They will be referred to as Swedish HTG and Swedish NTG respectively. A detailed description of this sample can be found in Asplund-Carlson and Carlson, 1994.

2.2.2.3 Dutch combined hyperlipidaemics

A sample of 240 patients (149 males and 91 females) with combined hyperlipidaemia (chol and Tg values above the 95th percentile) were recruited and followed at the Lipid Research Clinic of the University of Amsterdam and had a mean age of 47.2 yrs. This group will be called Dutch CHL. Familial hypercholesterolaemia was ruled out on clinical grounds and familial dysbetalipoproteinaemia was excluded by determination of the apo E genotype.

2.2.2.4 Dutch controls

A companion control group of 190 normolipidaemic, healthy Dutch males, age-matched with the Dutch CHL patient group, was recruited independently for a risk factor study. Individuals with plasma lipids above the 95th percentile for age and gender were excluded from our studies.

2.2.2.5 Charing Cross Hospital combined hyperlipidaemics

Forty-one consecutive patients (30 males and 11 females with age ranging from 27-69 yrs) with combined hyperlipidaemia (chol > 6.5 mM, fasting TG > 2.2 mM) were recruited from the Lipid Clinic of Charing Cross Hospital, London for a study of the relationship between lipoprotein lipase and hepatic lipase activities, and combined
hyperlipidaemia. They will be further referred to as CX CHL. All had a family history of hyperlipidaemia or CHD and/or personal history of CHD. Lipid and lipoprotein data concerning this sample has recently been published (Seed et al., 1994) and lipoprotein lipase activity data will be presented in section 4.1. In this highly selected sample, 38 individuals had a family history of hyperlipidaemia, 32 had a family history of CHD and 26 had clinical manifestations of CHD. Patients with familial HTG (fasting TG > 8mM), diabetes mellitus or thyroid disease were excluded. Familial dysbetalipoproteinaemia was probable in one individual based on apo E genotype.

2.2.2.6 General population controls from the UK

This group consisted of 773 male subjects aged 40 to 64 attending two general practices: 360 men were from the Camberley area in Southern England and 413 men from the St. Andrews district in Scotland. All had been recruited as part of the Northwick Park Heart Study II of risk factors for heart disease and were free of clinical signs of disease at the time of entry into the study. Part of the lipid and haemostatic data pertaining to the Camberley group has been published (Humphries et al., 1994) while the St. Andrews material was generously made available to us by Dr. George Miller.

2.2.2.7 ECTIM case-control study

This sample included 1430 male subjects, aged 25 to 64 years, participating in the ECTIM multi-centre case-control study (Etude Cas-Témoins sur l’Infarctus du Myocarde)(Parra et al., 1992). The subjects originating from four MONICA-WHO centres (Belfast, Lille, Strasbourg and Toulouse) were subdivided as follows: 404
subjects from Belfast (202 controls and 202 cases), 371 from Toulouse (220 controls and 151 cases), 223 from Lille (155 controls and 68 cases) and 432 from Strasbourg (211 controls and 221 cases). All individuals included in the ECTIM study had four grandparents born in Europe (French centres) or Ulster (Belfast subjects) and their families had been residing locally for at least two generations. All cases had survived a myocardial infarction three to nine months prior to enrolment in the study. The controls were drawn from the local electoral rolls and age-matched with their related patient group. The aim of this study was to investigate the large difference in CHD incidence between France and Northern Ireland. One method used to achieve this objective involved the comparison of DNA polymorphisms and their association with measured levels of risk factors for MI (lipoproteins and coagulation factors) in groups with differing risks of MI.

2.2.3 Family studies

2.2.3.1 Type I hyperlipoproteinaemia

In addition to the twenty type I probands, DNA samples from 25 relatives of these individuals (from 8 families) were obtained for study. These were relatives of the five Institute of Child Health probands, of St.Thomas’ Hospital proband MA, of Swedish proband LA and of the Italian proband CB. Partial lipid and LPL activity data was available for 18 of these individuals.
2.3 Biochemical data: lipid and LPL measurements

Full lipid and lipoprotein data from Swedish studies I and II, Dutch CHL and controls, UK general population controls and ECTIM case-control study was made available to us by Drs. Anders Hamsten (Sweden), John Kastelein (The Netherlands), George Miller (UK men from Camberley/ St. Andrews) and François Cambien (ECTIM study). For the CX CHL study, cholesterol and triglyceride levels were determined enzymatically using a BM/Hitachi 717 analyser with Boehringer Mannheim reagent kits (Boehringer Mannheim UK, Lewes). Lipoproteins (HDL) were separated by precipitation of other lipoproteins with dextran sulphate/magnesium chloride (Warnick et al., 1982). These analyses were performed by Miss Emer Doherty at Charing Cross Hospital.

2.3.1 Post-heparin plasma LPL activity and mass

The work presented in this thesis is based on samples obtained from a variety of sources. As a consequence, some of the key data generated prior to or in the course of the present studies have been obtained by a number of methods. This is the case for PH-LPL activity and mass measurements in human subjects where three activity assays based on different principles were used. For LPL mass, two related assays were used. LPL activity is expressed in milliunits (mU)/ml which corresponds to nmol of free fatty acids released/min/ml. LPL mass is expressed in ng/ml.

2.3.1.1 Protocol for plasma collection

Ethical approval for the heparin injection was obtained from the appropriate institutional review boards. Fasted blood samples were collected in 10 ml Na-EDTA
tubes 15 minutes after an intravenous heparin injection. A dose of 50 or 100 U heparin/kg body weight was used depending on the studies (50U: YMI study, 100U: Swedish NTG/HTG, CX CHL, UK controls’ study). The blood was then centrifuged at 2,000 rpm and the separated plasma snap-frozen and kept at -70°C until analysis.

2.3.1.2 Methods for LPL activity and mass measurement in humans I

PH-LPL activity and mass were determined by Dr. John Brunzell at the University of Washington in Seattle for 5 patients with type I HLP and their available relatives, according to Peterson et al. (1992). Plasma samples were added to a phosphatidylcholine-stabilised radiolabelled triolein emulsion and the released free fatty acids were extracted and counted in a beta scintillation counter. LPL activity was obtained by subtracting HL activity from total lipolytic activity. Selective measurement of HL activity was achieved by specific inhibition of LPL by 5D2 Mab.

A sandwich ELISA assay was used to estimate LPL dimer mass, the proposed active species. The assay was based on the property of an LPL monomer-specific Mab (5F9) which is presumed to bind to an antigenic site normally hidden in the native LPL dimer but exposed by monomerisation. MAb 5F9 was used as the coating antibody, and horseradish peroxidase-labelled MAb 5D2 (which recognises an external epitope on monomer and dimer species) for detection. Dimer mass was calculated as the difference between LPL monomer mass with and without denaturation by guanadinium chloride.

2.3.1.3 Methods for LPL activity and mass measurement in humans II

Post-heparin plasma LPL activity was determined directly by first inhibiting plasma HL with anti-HL IgGs (Karpe et al., 1992) in samples from the English
(Camberley) controls (section 2.2.2.6) and the Swedish NTG/HTG study (section 2.2.2.2). The substrate was a tritiated triolein emulsion stabilised with Intralipid™.

LPL mass for the English controls and in vitro produced LPL was determined using a solid phase sandwich ELISA assay with a chicken polyclonal antibody to capture the antigen and monoclonal antibody 5D2 as the detection antibody (Vilella et al., 1993). These assays were performed in the laboratory of Drs. Gunilla and Thomas Olivecrona, Umeå, Sweden.

**2.3.1.4 Method for LPL activity measurement in humans III**

For samples from the Charing Cross and the Swedish YMI studies, LPL activity was measured as described by Nilsson-Ehle and Ekman (1977) and Baginsky and Brown (1979). LPL activity was measured using lysophosphatidylcholine-stabilised glycerol tri{9,10(n)-^H}oleate in the presence of heat-inactivated serum (30 mins, 56°C) as a source of apoC II, after inhibition of HL with sodium dodecyl sulphate (SDS). HL activity was measured in the absence of serum activator using 1 mol/L sodium chloride to inhibit LPL. Liberated radioactive oleic acid was counted after extraction (Belfrage and Vaughan, 1969). All values obtained were within the linear range of the assay.

For the CX CHL study, samples were taken at 5, 10, 20, and 30 minutes post heparin by Dr. Mary Seed (Charing Cross Hospital) and measurement of LPL and HL activities was performed by Dr. Dave Vallance (Royal Free Hospital). Reference ranges were obtained following heparin administration to 23 normolipaemic adults (M=11, F=12). The efficiency of the extraction procedure was checked for each batch of measurements using [9,10^-3H]-oleic acid. Blanks were run for each assay by adding the extraction mixture immediately after addition of serum to the substrate. To ensure that
full inhibition of the enzyme was achieved with the appropriate inhibitor, a "zero activity" tube was assayed for each of the patient's samples using the HL specific substrate and SDS-treated post-heparin plasma. Under these conditions, residual lipase activity was less than 3.5mU/ml.

2.3.1.5 Activity, stability and mass of LPL produced in vitro

Samples of LPL produced in vitro by transfection of COS cells (section 2.14) were assayed for activity either by us or Dr. Gunilla Olivecrona. The method used by Dr. Olivecrona was the same as that described in section 2.3.1.3, with the exception that inhibition of HL was not necessary. Our protocol, drawn from Nilsson-Ehle and Schotz (1976), is outlined below.

A concentrated substrate emulsion was first prepared by mixing 36mg phosphatidylcholine dissolved in benzene (100mg/ml), 660mg triolein and 3500kBq (94.6μCi) 3H triolein (Amersham) in 10ml glycerol. The preparation was sonicated for 5 minutes while on ice. The emulsion was then kept at room temperature for several weeks, needing vortexing only prior to use.

On the day of the assay, 1 vol of the emulsion concentrate was mixed with 4 vols BSA solution (3% BSA in 0.2M Tris pH 8.0) and 1 vol inactivated serum as a source of apo CII, then vortexed vigorously for 30 seconds. The substrate preparation was then mixed 1:1 with the sample to be assayed (generally 100μl each), vortexed 15 seconds and incubated for 1 hr at 37°C. Free fatty acids were extracted by adding 1.05ml borate buffer (6.2g boric acid, 13.8g potassium carbonate per litre, pH 10.5) and 3.15ml extraction solution (chloroform/methanol/heptane, 1.25:1.45:1) and vortexing 30-40 seconds. The aqueous (methanol/water) phase was separated by centrifugation, 1ml was
pipetted into a vial containing 9ml scintillation fluid and the radioactivity counted. Enzyme activity in the sample, expressed in mU/ml (1mU = 1nmole fatty acid released per minute) was calculated based on the formula found in Nilsson-Ehle and Schotz (1976):

$$\text{Activity (mU/ml)} = \frac{\text{net cpm}}{\text{60 mins}} \times \frac{1}{\text{sp. act.}} \times 3 \times 3.224$$

In this equation, net cpm refers to the radioactivity in 1ml of upper phase. This is corrected for the radioactivity in mock transfected samples and multiplied by 10 to take into account the sample volume assayed (100μl). Specific activity (sp.act.) is expressed in cpm per nmole triglyceride. The last two factors represent respectively the molar ratio of fatty acid released to triglyceride hydrolysed (3) and a combined correction factor for the total volume of the aqueous phase and the percent recovery of oleic acid in the extraction system (2.45ml / 0.76 = 3.224). Specific activity of the substrate was converted from 252 dpm/nmole triolein (94.6μCi/ 825μmoles) to 63 cpm/nmole assuming 25% counting efficiency. Samples were assayed in triplicates.

The stability of LPL activity was tested by pre-incubating samples at 37°C for variable periods of time prior to the assay. These samples were assayed in duplicates, due to the limited amount of material.

LPL mass from in vitro experiments was determined by the same method as described in section 2.3.1.3 by Dr. Gunilla Olivecrona.

### 2.3.2 Heparin-Sepharose profiles and stability assays of plasma samples

Plasma samples and transfected cell media were collected for LPL analysis by affinity chromatography on heparin-Sepharose columns (Ostlund-Lindqvist and Boberg, 1977). Briefly, 10ml samples were injected into the column and 1-ml fractions eluted
(1ml/minute) at 4°C with increasing salt gradient concentration. Lipase activity and mass in individual fractions were measured as described above (section 2.3.1.3).

In plasma samples, fractions with maximum LPL activity and no HL activity as determined by the mass/activity profile were used to test the stability (resistance to denaturation) of LPL activity. This was achieved by pre-incubating fractionated samples at 37°C in the presence of 0.75M guanamidium-HCl prior to performing the assay (Osborne et al., 1985). All the assays outlined in this section were performed by Dr. Olivecrona.

2.4 DNA analysis.

2.4.1 DNA isolation from whole blood

Blood was collected in 10ml Na-EDTA tubes and kept frozen at -20°C. Genomic DNA was extracted by the salting-out method (Miller et al., 1989). Five to 10ml whole blood were mixed with ice-cold sucrose lysis buffer (0.32M sucrose, 10mM Tris-HCl pH 7.5, 5mM MgCl2, 1% Triton X-100) to a final volume of 30ml in a polypropylene tube and spun at 10,000 rpm at 4°C in a Sorvall RC-5 centrifuge equipped with an SA-600 rotor for 10 minutes. The supernatant was carefully decanted, the pellet was resuspended in lysis buffer and centrifuged as above. This step was usually repeated a third time to obtain as white a pellet as possible. The pellet was then resuspended in 3ml nuclei lysis buffer (10mM Tris-HCl pH8.2, 0.4M NaCl, 2mM Na2EDTA), to which 0.2ml of 10% SDS and 150-200μl of 10mg/ml Proteinase K were added. The mixture was incubated overnight on an orbital shaker at 37°C.

The next morning, 1.5ml of a saturated NaCl solution (approx. 6M) was added and mixed well by inversion for 10-15 seconds to precipitate the proteins. The resulting
turbid solution was spun at 10,000 rpm at room temperature as before and the clear supernatant carefully removed (avoiding the pellet) to a clean 30ml tube. Absolute ethanol (15ml) was added slowly to create an interface at which the DNA becomes insoluble. The fibrous material is condensed by gentle swirling, removed from solution using a sterile Pastette, transferred to an Eppendorf vial and dissolved in 1ml TE buffer.

2.4.2 Isolation of plasmid/phagemid DNA from bacteria

2.4.2.1 Miniprep

DNA was extracted using the alkali lysis method (miniprep) from 1.5ml transformed JM109 or BMH 71-18 mutS overnight cultures as described in the 'Altered Sites in vitro mutagenesis system technical leaflet (Promega UK, Southampton). Briefly, the cells were pelleted and lysed in ice-cold buffer (50mM glucose, 10mM EDTA, 25mM Tris-HCl, pH 8.0). The bacterial cell membranes, chromosomes and proteins were removed by precipitation with freshly prepared 0.2M NaOH, 1% SDS followed by 3M potassium/5M acetate solution at pH 4.5-5.5 and centrifugation. The phagemid/plasmid DNA was extracted with phenol/chloroform/isoamylalcohol (IAA) 25:24:1, ethanol precipitated and the pellet was resuspended in TE and treated with 10μl of 10mg/ml DNase-free pancreatic RNase A at 37°C for 10 minutes. The DNA was stored at -20°C.

2.4.2.2 Midiprep

To obtain sufficient material for the purification of inserts (section 2.6), pAlter phagemid DNA was prepared from 20ml overnight cultures. The solutions used were the
same as for the miniprep procedure described above. The cell pellet was thoroughly resuspended in 1.44ml lysis buffer to which 160µl lysozyme (Boehringer) (20mg/ml lysis buffer) was added. The mixture was incubated on ice for 10 minutes. Freshly prepared NaOH/SDS solution was added, mixed very gently to lyse the cells and incubated on ice for 5 minutes. The mixture was neutralised by the addition of the potassium acetate solution and again incubated on ice for 30 minutes. This was followed by centrifugation at 10,000rpm in a Sorvall RC5 or the equivalent equipped with an SA-600 rotor, at 4°C for 25 minutes. The supernatant was filtered through tissues and transferred to fresh centrifuge tubes. After the addition of 4.8ml 2-propanol and incubation at room temperature for 30 minutes, the sample was spun at 12,000rpm for 30 minutes. The supernatant was discarded and the pellet resuspended in 0.4ml TE buffer plus 4µl RNase A. After 15 minutes, proteins were removed by extracting once with phenol/chloroform/IAA (25:24:1 by volume) and once with chloroform/IAA (24:1 by volume). Forty µl 3M Na-acetate was added and the DNA was ethanol precipitated (2.5 vol) overnight at -20°C. The pellet was washed with 70% ethanol, and left to dry in air for a few minutes. The phagemid DNA was resuspended in 50µl sterile water.

2.4.2.3 Maxiprep

Large scale preparation of plasmid DNA was performed for plasmids required for transfection experiments. One mc1061/p3 ampicillin and tetracyclin resistant colony, harbouring a mutant pcDNAI vector, was inoculated into 10mls of LB medium containing 30µg/ml ampicillin/7.5µg/ml tetracyclin and incubated overnight at 37°C. One ml of this culture was used to inoculate 500ml of the same medium as previously and the culture incubated overnight. Plasmid DNA was rapidly extracted and purified with
the Circleprep kit (BIO 101 Inc., California) according to manufacturer's instructions. The resulting DNA was quantified spectrophotometrically and stored at -20°C.

2.4.3 Synthesis and isolation of single-stranded phagemid DNA

This procedure was a modified version of the protocol accompanying the mutagenesis kit (Promega UK, Southampton).

A tetracycline resistant colony, previously transformed with a vector containing the replication origin of a single-strand DNA bacteriophage (phagemid pAlter, Promega) into which a 2.4kb LPL fragment had been inserted (section 2.14), was used to inoculate 2mls of LB broth containing 15μg/ml tetracyclin and incubated overnight at 37°C. The next morning, 10mls of LB medium plus tetracyclin (in a 250-ml flask to insure maximal aeration) were inoculated with 100μl of the overnight culture and incubated until OD600 reached 0.4. At this point, the culture was infected with 80μl helper phage R408 (Promega mutagenesis kit) and grown overnight.

The cells were pelleted at 12,000g for 15 minutes at 4°C, and the supernatant removed and spun again to remove residual cells and debris. A solution of 20% PEG/2.5M NaCl was added to the culture supernatant (1ml PEG/NaCl per 3mls supernatant) and DNA precipitated at room temperature for 1 hour. The solution was spun again at 12,000g for 15 minutes and the supernatant discarded. Care was taken to remove all liquid using a piece of tissue. The pellet was then resupended in 0.6ml 20mM Tris, pH 7.5, transferred to a 1.5ml Eppendorf tube and extracted several times with an equal volume of phenol/chloroform/IAA and twice with chloroform/IAA. Finally, the phagemid DNA was ethanol precipitated and resuspended in TE.
Polymerase chain reaction (PCR) amplification of LPL exons 2-9 was achieved on a Cambio machine in the plate mode, using oligonucleotide primers on either side of individual exons (sequences from Oka et al., 1990, Wion et al., 1987, and Devlin et al., 1990). Wherever the nature of the sequence allowed it, the primers’ 5’ end was located 30-35 bases from the intron/exon boundary. However, intron sequence data being very limited, this was not always possible. The 5’ exon 6 primer was entirely within the exon and the 3’ exon 6 primer was in fact about 50 bases downstream from the boundary, while the following primers straddled the intron/exon border: 3’ exon 3, 3’ exon 4, 5’ exon 8 and 3’ exon 9. The sequence of these amplification primers and the size of the expected product are given in Table 1. The reactions were carried out in Eppendorf tubes in standard buffer supplied by Gibco-BRL (10X = 500mM KCl, 100mM Tris-HCl pH 8.3, 2mM each dNTP, 0.01% gelatin) (Saiki et al 1986; Mullis and Faloona 1987) with 1μl DNA, 100ng each primer, 5% W-1 and 0.5U Taq polymerase (Gibco-BRL, UK) per 50μl reaction, at a final MgCl₂ concentration of 1.7mM. The latter MgCl₂ concentration was chosen as it allowed good amplification of all exons with the same program and PCR block. A cocktail mix of all components of the reaction except DNA was prepared to minimize variability. The reaction volume varied from 50 to 100μl, depending on the subsequent use of the product. An equivalent volume of liquid paraffin was overlaid on the reaction mixture to prevent evaporation. Following denaturation at 98°C (1 minute except for the first cycle - 5 minutes at 97°C), and annealing at a final temperature of 55°C (reached in 5 steps, 70 - 65 - 60 - 58 - 55, over 6 cycles) for 1 minute, the nascent PCR product was extended at 72°C for 1.5 minutes (5 minutes for the first cycle). This was repeated over for 35-45 cycles.
Table 2.1 Oligonucleotide primers used for PCR amplification of LPL gene exons 2-9, *Pvu*II and *Hind*III polymorphisms. The expected size of the PCR fragment is also indicated.

<table>
<thead>
<tr>
<th>Exon/fragment</th>
<th>Left hand primer</th>
<th>Right hand primer</th>
<th>Fragment size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>5' CTCCAGTTAACCTCATATCC 3'</td>
<td>5' CACCACCCCAATCCACTC 3'</td>
<td>237</td>
</tr>
<tr>
<td>3</td>
<td>5' GCTTGTGTACATCTCTTCAG 3'</td>
<td>5' TCCCAGTCTTTACGTCCAC 3'</td>
<td>212</td>
</tr>
<tr>
<td>4</td>
<td>5' GCAGAACTGTAAGCACCCTT 3'</td>
<td>5' TGCTTTCTTTACGTAATTCTG 3'</td>
<td>163</td>
</tr>
<tr>
<td>5</td>
<td>5' TTTACAAATGTGTTTCCCTGC 3'</td>
<td>5' CTCTGCAAATCACCAGGAT 3'</td>
<td>258</td>
</tr>
<tr>
<td>6</td>
<td>5' GTGGACCAGCTAGTGGAAG 3'</td>
<td>5' GCATGATGAAATAGGACCTCC 3'</td>
<td>295</td>
</tr>
<tr>
<td>7</td>
<td>5' AAGATTGTACACATGTTGTT 3'</td>
<td>5' ACTGCTGCCATGATGACCGC 3'</td>
<td>194</td>
</tr>
<tr>
<td>8</td>
<td>5' TTTAGGCCTGAAGTTCCAC 3'</td>
<td>5' GGGGTTCTAAAGTGAAAG 3'</td>
<td>227</td>
</tr>
<tr>
<td>9</td>
<td>5' TGTTCCTAACGATGCTTAC 3'</td>
<td>5' GCACGACTGCTGACCTTC 3'</td>
<td>150</td>
</tr>
<tr>
<td><em>Pvu</em>II</td>
<td>5' ATGGCACCCATGTGAAGTGTG 3'</td>
<td>5' GTGAACCTTTCTGATATAGCTC 3'</td>
<td>440</td>
</tr>
<tr>
<td><em>Hind</em>III</td>
<td>5' TTTAGGCCCTGAAGTTCCAC 3'</td>
<td>5' CCCAGAATGCTCCAGC 3'</td>
<td>1250</td>
</tr>
</tbody>
</table>
For the detection of the *PvuII* and *HindIII* RFLP (see also section 2.8.1), amplification was performed essentially as outlined above with the following exceptions:
- the extension time was lengthened to 3 minutes for *HindIII* due to large product size (1.25 kb) with the Cambio machine in the phial mode
- the amplification procedure for *PvuII* was simplified to a two-step mode (denaturation at 98°C for 1 minute and annealing/extension at 58°C for 5 minutes).
The oligonucleotide primers used and PCR product sizes are also given in Table 1.

For the detection of the apoE polymorphism, a 216bp region in exon 4 of the apoE gene spanning both polymorphic sites was amplified using the following primers: 5' CTGGGCGCGGACATGGAGGACGT 3' and 5' GATGGCGCTGAGGCCGCTCG 3'. The buffer was similar as described above except that W-1 was replaced by 10% DMSO (final concentration). The amplification was carried out in the plate mode with a two-step protocol with denaturing at 95°C and annealing/extension at 65°C for 2.5 mins (Mailly et al., 1992).

### 2.6 Purification of DNA

When purification of DNA was required, two methods were used. In the early part of this work, purification of samples prior to direct sequencing (section 2.11.1) was achieved by binding PCR products to the 'GLASSMILK' silica matrix (Geneclean kit, BIO 101 Inc., La Jolla, Ca). This allowed the removal of unincorporated primers and nucleotides as well as DNA contaminants that may have been present. During cloning operations for *in vitro* mutagenesis of LPL (section 2.14), cDNA inserts were separated from the vector on agarose gels, visualised under UV light and insert bands cut out. The
agarose was dissolved at 55°C in 4M NaI for 5 minutes and inserts were purified with Spin-Bind buffers and DNA extraction units (Biozym, Netherlands). These eppendorf-fitting cartridges were suited for the purification from agarose and recovery of inserts, using a microcentrifuge. Both purification methods were carried out according to the respective manufacturers’ recommendations.

2.7 Sample preparation for SSCP

For SSCP analysis, amplification was performed as described above except that 0.2μL [α-32P]dCTP at 10Ci/μL, 3000mCi/mmol (Amersham, UK) was added to each sample. The label was added to the cocktail mix prior to aliquoting, after sufficient cocktail for at least two unlabelled samples had been removed. If amplification was found to occur in these unlabelled control samples, the reaction was assumed to have worked in all samples. An aliquot of the radioactive PCR products was then diluted five-fold in 0.1% SDS/10mM sodium-EDTA (Orita et al., 1989b) and kept frozen until required.

To separate the DNA single strands, 4μl from the PCR reaction was mixed with an equal amount of formamide dye buffer (100ml deionized formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, 20mM EDTA), denatured at 98°C for 2 minutes and snap-cooled on ice immediately. Loading onto the resolving gel (section 2.9.3) proceeded without delay.

2.8 Restriction enzyme digestion of DNA

Restriction enzyme digests were set up in 15 or 20μl volume for PCR products, 45μl volume for genomic DNA and 50μl volume for plasmid/phagemid DNA. For PCR
products, 10-15μl from the amplification reaction were mixed with 1.5-2μl of 10x digestion buffer and 8-10 units of the appropriate restriction enzyme. This was then incubated at 37°C for at least 4 hours, except for the enzyme TaqI where incubation was at 65°C as recommended by the manufacturer. Plasmid/phagemid digests were set up in the same manner but were only incubated for 1-2 hours. Genomic DNA (5 μg) was digested overnight with 30U PstI or StuI enzymes in a total volume of 45μl. For both plasmid and genomic DNA digests, one tenth of the volume was run on an agarose gel to check whether digestion was complete. In the event of incomplete digestion, more enzyme was added and the incubation was continued. In all samples, the volume of enzyme added did not exceed one tenth of the total sample volume. An appropriate volume of marker loading dye (0.25% bromophenol blue and/or 0.25% xylene cyanol, 25% Ficoll, 0.1M EDTA) was added to every sample prior to electrophoresis.

2.8.1 LPL polymorphisms or rare variants detected by restriction digests

For the PvuII RFLP, digestion of the 440-bp PCR product yielded two fragments of 330 and 110 bp respectively when the polymorphic site was present (P+ allele) or an unchanged 440-bp fragment when it was absent (P- allele). The presence of the HindIII polymorphism (H+ allele) was detected by the appearance of a 625-bp fragment upon digestion of the 1.25kb PCR product, unchanged in the absence of the site (H- allele).

To screen for the D9N substitution in exon 2 originally reported by Lohse et al. (1991), the 237 bp PCR product was digested with TaqI. In addition to the 179bp constant band, the digestion generated either a 52 or a 58bp band in the absence or presence of the mutation respectively, when its products were separated on a polyacrylamide gel.
The presence of the A158T or G188E mutations in exon 5 was detected by digesting the 258bp PCR fragment with \textit{PvuII} or \textit{AvaII} respectively. In individuals with A158T substitution, the PCR product remains unchanged following digestion, while only a smaller 210bp band was expected in the normal pattern, the 48bp remaining fragment being too small to be visible. Both bands were present in heterozygote individuals. The \textit{AvaII} digest yielded a 206bp fragment in homozygotes for the G188E mutation, in contrast to the 118 and 88bp bands found in normal individuals and the three-band pattern (88, 118 and 206bp) observed in carriers of the mutation.

The Y262H substitution in exon 6 destroyed a StuI restriction site, resulting in full-length PCR product (295bp) in place of the 210bp fragment normally detected after digestion.

The methods used for the resolution of the bands patterns associated with these mutations are described below.

\subsection*{2.8.2 Apo E polymorphism detected by restriction digest.}

The Cys/Arg substitutions at amino acid positions 112 and 158, which give rise to the three common plasma isoforms of apo E, were ascertained in a one-step procedure as outlined previously (Mailly et al., 1992). PCR products were digested with the restriction enzyme \textit{HhaI} and the fragments resolved on polyacrylamide gels as described below. The $\epsilon 4$ allele was identified by the presence of a cutting site, resulting in a 72bp fragment, in contrast to the more common 91bp band associated with the $\epsilon 3$ allele. For the $\epsilon 2$ allele, the loss of an \textit{HhaI} site resulted in a band of 83bp compared to the common 48bp fragment.
2.9 Gel electrophoresis of DNA

2.9.1 Agarose gel electrophoresis

For the separation of PCR products, agarose gels were made with 1.5-2% agarose in 1xTAE containing 1μg/ml EtBr. The solution (100mls) was boiled and allowed to cool before being poured into a gel former with a comb to form wells for loading DNA samples. The gel was left to set, the comb removed and the gel placed in a horizontal electrophoresis tank containing 1xTAE. The samples to be loaded were first mixed with one tenth volume 10x loading buffer. After sample loading, the gel was run at a suitable voltage to separate the DNA fragments and the resulting bands were then visualised using U.V. transillumination.

For the separation of digested genomic DNA, 300 mls of a 0.9% agarose solution were poured in a larger gel former and the gel allowed to set. Samples were run for 20 hours at 25V.

2.9.2 Denaturing polyacrylamide gel electrophoresis for DNA sequencing

Spacers (0.4mm) were placed between two clean plates (40 x 60cm), one of which had been siliconised using Repelcote (BDH, Lutterworth), and these were then taped together to prevent leakage of the acrylamide solution. Seventy-five mls of 6% acrylamide solution was prepared with 19:1 acrylamide:bisacrylamide solution, 42% urea and 1xTBE. The solution was warmed at 37°C to dissolve the urea. Polymerization was achieved by the addition of 75μl of a crosslinking agent, TEMED (NN N’N’-tetramethylethylenediamine) and 75μl of freshly made 40% ammonium persulphate solution. The solution was then poured carefully between the glass plates, avoiding the formation of bubbles. Two 0.4mm sharks tooth (24 wells each) combs were inserted
upside down and clipped in place to form a horizontal, 3-4mm deep groove at the top of the gel. Once the gel had set (approx. 1 hour) it was untaped, placed in a vertical electrophoresis apparatus and the combs were inverted with the teeth forming small wells at the top of the gel. The gel was pre-run in 1xTBE until warm (approx. 30-40 minutes) and the wells were flushed out to remove any urea which may have leached out of the gel. The gel was run at a constant power of 60W (maximum current 55mA, maximum voltage 2000V) until the required separation was achieved. After the appropriate time the gel was transferred to 3MM filter paper (Whatmann) and dried under vacuum at 80°C for at least 1 hour. Since the radioisotope used in sequencing was 35S, autoradiography was carried out for at least 16 hours (but usually a few days) at room temperature.

2.9.3 Non-denaturing polyacrylamide gel electrophoresis.

2.9.3.1 SSCP analysis

The plates were prepared as for sequencing. A 7.5% gel solution (75ml) was prepared for each gel with 10.7ml of 50%, 50:1 acrylamide:bisacrylamide solution, 7.5ml 10xTBE, 7.5ml glycerol and 49.3ml water. The gel was poured and processed as above. Denatured, 32P labelled samples were loaded onto the gel (which had not been pre-run) and electrophoresis was carried out at constant current (15mA) for 16-22 hours (16-18 hrs for fragments <200bp, 18-22 hrs for fragments 200-300 bp). The following day, the gel was transferred to 3MM Whatman paper and dried as described above. Autoradiography was carried out at -70°C for a minimum of 16 hours with an intensifying screen.
2.9.3.2 Resolution of small DNA fragments generated by restriction digests

For effective separation of DNA fragments of less than 150bp, two procedures were used. In the first instance, clean glass plates (20x20cm) separated by 1mm spacers and a 20-tooth comb were clipped together and the edges taped to prevent leakage. Fifty mls of a 10% acrylamide solution was prepared with 19:1 acrylamide:bisacrylamide solution and 1xTBE. TEMED and ammonium persulphate (100μl) were added and the gel poured, avoiding trapped air bubbles. Once the gel had set, the tape was removed and the gel clipped in a vertical electrophoresis tank (Cambridge Electrophoresis, Cambridge) with 1xTBE. The comb was removed carefully and wells were flushed prior to a short pre-run and to sample loading. Samples were run usually for 2-3 hours at 35mA. Two gels could be run simultaneously.

The second system utilised, the Hoefer 'Mighty Small' vertical electrophoresis apparatus, allowed the convenient pouring of up to 6 gels (80 x 70 x 1mm) in a perspex gel caster. Gel sandwiches comprising glass plate and and aluminium plate and 1mm spacers were assembled and stacked up vertically in the mould. The gel solution was poured in from the top, after which a flexible 15-well teflon comb was inserted in all the gel sandwiches. The composition of the gel solution was as above with only 7.5ml/gel required. Polymerisation occurred within 30 minutes. Gels were detached from the mould and each other and clipped, two by two, on Hoefer vertical electrophoresis units and the buffer chambers filled with 1xTBE. The combs were gently pulled out and the gels equilibrated by pre-running 10-15 minutes. Samples were run for 1 to 1.5 hour at 35mA.

2.9.3.3 Silver staining of polyacrylamide gels
Visualisation of DNA bands on polyacrylamide gels was achieved by a modification of the silver-staining procedure of Merril et al. (1981). All solutions except the developer could be kept at room temperature for months and approximately 100mls of each solution was required per gel (250mls for developer). Gels were fixed for 5 minutes in 10% ethanol, followed by oxidation for 2 minutes in 1% nitric acid. After a brief rinse in distilled water, gels were immersed in a silver nitrate solution (2g/l) for 20-30 minutes and rinsed again 2-3x with distilled water. Precipitation of silver was achieved by first rinsing gels in freshly made developing solution (30g anhydrous sodium carbonate, 0.5ml formaldehyde per liter) until the solution turned yellow-brown (30-40sec) and then soaking in developer as long as required for the appearance of the bands, which was generally 5-10 minutes. Finally, the reaction was stopped in 10% acetic acid (5 minutes) and rinsed well with distilled water prior to drying.

2.10 Detection of DNA variation by hybridisation

2.10.1 Blotting and detection of N291S mutation by hybridisation with allele-specific nucleotides (ASOs)

Exon 6 PCR products were run on 1.5% agarose gels. These were denatured for 30-60 minutes in 1.5M NaCl/0.5M NaOH after which a Hybond N+ membrane (Amersham, UK), Whatman 3MM paper (pre-wetted in 2XSSC) and absorbent paper (3-4 cm thick), all cut down to gel size, were added to one side of the gel. Air bubbles were smoothed out after the addition of each layer. The mount was turned over and the procedure repeated for the other side of the gel. A glass plate and a weight (approx. 0.5kg) were placed on top and transfer allowed to proceed for 4-18 hours, using the denaturing solution in the gel as the transfer buffer. The membranes were subsequently
rinsed in 2xSSC/0.1M Tris-Cl pH 7.5 and baked at 80°C for 2 hours to fix the DNA.

ASOs (TGACTTTTATGTACTCTCA for the Asn allele and GACTTTACTGTACTCTCA for the mutant Ser allele) were labelled at the 5' end with [\(\gamma\)^32P] ATP (Amersham, UK) and T4 polynucleotide kinase (Gibco BRL, Paisley, UK) to a specific activity of approximately 0.1\(\mu\)Ci/pmol and separated from unincorporated nucleotides in a G25 Sephadex spun column (Maniatis, 1982).

Filters were hybridised for 3-4 hours in 5x SSPE/ 0.5% SDS/ 5x Denhardt's solution with approx. 5ng/ml of each oligo per cm² of membrane, at 37°C in a rotating oven (Bachofer GmBH, Germany), then washed at 44°C in excess 5xSSPE/0.1% SDS for 10 minutes. Autoradiography was carried out for 4-24 hours at -70°C using intensifying screens.

### 2.10.2 Southern blotting

Following electrophoresis, 20x25cm agarose gels containing PstI or StuI digested genomic DNA were denatured for 1 hour in 1.5M Nacl/0.5M NaOH after which the bottom 5cm were trimmed off. The DNA was then transferred to a solid support by the conventional Southern blotting method (Southern, 1975). Briefly, for each gel, a 20x20cm plastic support was placed in a tray containing 20xSSC (about 2cm deep) and covered with 3MM Whatman paper wetted and dipping in the same solution so as to act as a wick. A denatured gel was carefully placed on the wick, wells down, to avoid trapping air bubbles. A piece of nylon membrane (Hybond-C, Amersham, ), pre-wetted pieces of 3MM Whatman paper and a 10cm stack of paper towels, all cut down to gel size, were placed on the gel in that order, with care taken again not to trap air bubbles. The transfer was allowed to proceed for 20 hours after which the membrane was rinsed.
briefly in 3xSSC and baked at 80°C for 2 hours to fix the DNA. The membrane was then rolled up in 50-ml plastic tubes and pre-hybridized for 4 hours in phosphate-SDS buffer (0.5M PO₄, 7% SDS).

2.10.3 cDNA probe preparation and hybridisation of genomic blots.

Phagemid pAlter containing the wild-type LPL sequence was used to transform JM109 bacteria and DNA was extracted using the midiprep method (section 2.4.2.2). The 2.4 kb LPL cDNA insert was cut out of the vector by XbaI/PstI restriction digest, separated from the vector on a 2% low-melting point agarose gel containing EtBr and purified as outlined in section 2.6. The cDNA probe was labelled (expected specific activity 0.7-2.6 dpm/µg probe) by the method of Feinberg and Vogelstein (1983) using 25-100ng insert, 5µl [α³²P] dCTP (800Ci/mm) and the solutions provided in the Boehringer Mannheim Random Primed DNA Labeling kit.

A 1.0kb fragment of the cDNA was also prepared for hybridisation by digestion of the 2.4 kb insert with StuI. The bands (0.4, 0.8 and 1.2kb) were visualised under long wave U.V. light and the appropriate band cut out from the agarose gel. This was weighed and an amount of water representing 3x the weight was added. The mixture was then boiled for 5 minutes to melt the agarose, aliquoted and stored at -20°C. The probe was then labelled as described above.

Separation of labelled DNA from nucleotides was achieved with a Sephadex G50 column equilibrated with 3xSSC and the peak fractions counted. Generally 10⁶ cpm of the probe were denatured by boiling and added per ml of hybridisation solution [phosphate-SDS buffer (0.5M PO₄, 7% SDS)]. The pre-hybridisation solution was removed, replaced by the radioactive solution and hybridisation was carried out at 65°C.
for 48 hours. The blots were then washed for several hours as follows: four rinses at room temperature in 1xSSC, 0.1% SDS, one wash (2 hours) at 65°C in 3xSSC, 0.1% SDS and one wash (15-30 minutes) at 65°C in 1xSSC, 0.1% SDS. Autoradiography was done at -70°C with intensifying screens for several days.

2.11 Sequencing of LPL variants.

2.11.1 Direct sequencing of PCR products (1st method).

Direct sequencing of variants detected by SSCP was carried out using one of the primers used in the amplification reaction. The PCR product was purified using the GeneClean II kit (Bio101, La Jolla, CA) and then sequenced by the dideoxy method essentially as described by Green et al. (1989) et al. using modified T7 polymerase (Sequenase, United States Biochemical Corp.). Primer-template annealing was carried out in a total volume of 7μl 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 for 10 mins then snap-cooled on dry-ice. A labelling mixture was made, containing 5μCi α[^35]dATP (Amersham, UK), 1μl 0.1M dithiothreitol, 0.45μl 10% NP-40 and 0.15μl Sequenase enzyme (diluted 1:8 in Sequenase buffer,) and TE buffer, to a total volume of 4.5μl for each sample to be sequenced. Four μl of this mixture were added to each annealed primer/template, following which 2μl of this new mixture were quickly dispensed to each of four tubes containing 2μl of "A", "C", "G" and "T" termination mixes respectively. The termination mixes contained 80μM each of dCTP, dGTP, and dTTP and 8μM of the appropriate ddNTP, except for the "A" mix which contained 80μM of dCTP, dGTP, and dTTP and 0.08μM ddATP. After 5 mins of incubation at 37°C, reactions were chased with 2μl of a solution
containing 0.25μM each dNTP and 0.05%NP-40 and incubated for a further 5 mins at 37°C. Reactions were stopped with the addition of 4μl of stop solution (95% formamide/4% EDTA/0.5% bromophenol blue/0.5% xylene cyanol). Four μl of the final volume were boiled for 10 mins then immediately loaded onto a 6% polyacrylamide urea gel which was run as described in section 2.9.2. The gel was autoradiographed at room temperature for between 48 hours to a week, depending upon the strength of the signal, using Hyperfilm βmax (Amersham, UK).

2.11.2 Direct sequencing of PCR products (improved method).

For exons 5 and 6, two biotinylated primers (5' side of each exon) were manufactured to order. The sequence of the biotin-labelled exon 5 primer was the same as given in Table 2.1. It was used in combination with a new exon 5 3' primer, located downstream of the exon/intron border, designed from the sequence in Oka et al. (1990) as follows: 5' TAAGAGTCACATTTAATTCGC 3'. The sequence of the 5' exon 6 primer was also different than that listed in Table 2.1. It was located upstream of the intron/exon border and its sequence was as follows: 5' GTGGACCAGCTAGTGAAG 3'. The biotin-labelled primers allowed the purification of the PCR fragment with streptavidin-coated magnetic beads. Forty μl of PCR product were mixed with an equal volume of Dynabeads (Dynal AS, Norway) in a solution of 10mM Tris-HCl (pH7.5), 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 (pH7.5)/ 1mM EDTA /1.0M NaCl and then resuspended in 8μl 0.1M NaOH and incubated for 10 mins. After removal of this 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. This was annealed to the non-biotinylated primer used in the amplification with a primer:template molar ratio of approximately 25:1, and sequenced using modified T7 DNA polymerase following the manufacturer’s protocol (Sequenase sequencing kit, USB, Cleveland USA). Reactions were boiled for 2-3 mins before loading the gel. Gel running, drying and autoradiography was as in section 2.11.1, with the exception that a 24hr autoradiography period was often adequate due to the increased strength of the signal.

2.11.3 Sequencing of cloned DNA.

For verification of mutagenised constructs (see section 2.14), double-stranded plasmid DNA isolated by the mini or maxi prep methods was denatured in 0.2M NaOH, 0.2mM EDTA for 30 minutes at 37°C. The mixture was then neutralized by adding 0.1 volume of 3M sodium acetate (pH 4.5-5.5) and the DNA precipitated with 2-4 volumes of ethanol at -70°C for 15 minutes. The pelleted DNA was washed once with 70% ethanol and redissolved in 7µl distilled water. Sequencing was carried out as before, using a modified T7 DNA polymerase (Sequenase, USB, Cleveland USA) following the manufacturer’s recommended protocol. Three reverse primers complementary to the LPL cDNA sequence (Wion et al., 1987) were used:

- primer 1 (exon 7), 5’ ATGTTCTCACTCTCGGCC 3’ for LPL-291 plasmid
- primer 2 (exon 6), 5’ CCTGTAGGCTTACTTG 3’ for LPL-158 and -193 plasmids
- primer 3 (exon 2). 5’ GCCACGGACTCTGCTAC 3’ for LPL-9.
After running, the gel was autoradiographed at room temperature for between 24-48 hours, depending upon the strength of the signal, using Hyperfilm βmax (Amersham UK).

2.12 Bacterial strains: maintenance and preparation of competent cells.

All the strains used were grown at 37°C. The JM109 and BMH 71-18 mut S (repair deficient) strains needed for the mutagenic process were maintained on minimal plates (M-9) supplemented with 1mM thiamine-HCl (Promega mutagenesis technical leaflet). This selects for the presence of the F’ episome, which carries a nutritional requirement for growth and decreases the number of false positives. Cells were made competent for transformation as described in the 'Altered Sites' technical leaflet with MOPS/RbCl solutions.

The mc1061/p3 strain (Invitrogen corp., obtained from British Biotechnology Ltd) used for the amplification of the recombinant expression vector pcDNA1-LPL harbours the p3 plasmid which expresses a functional kanamycin resistance gene but defective ampicillin and tetracyclin resistance genes (amber mutations). Reversion rates of 5% and 1% respectively are observed for these mutations. To ensure efficient selection when transforming the cells with recombinant pcDNA1, competent cells must be prepared from non-revertant colonies. This was achieved by plating several kanamycin resistant colonies onto plates containing either ampicillin (30 μg/mL) or tetracycline (7.5 μg/mL) and picking a colony sensitive to these 2 antibiotics. Cells were then grown overnight in LB broth, a 0.5ml aliquot of which is added to 20ml LB and incubated until OD₆₀₀ reaches 0.13-0.15. The cells were subsequently pelleted, resuspended in 2 ml 0.1M MgCl₂, followed by re-centrifugation and resuspension in 2ml
0.1M CaCl₂. After 15 minutes incubation on ice, a final centrifugation step was performed followed by resuspension in 0.1M CaCl₂, 12% glycerol. This suspension was stored in 100µl aliquots.

### 2.13 Transformation of bacterial cells.

All transformations were performed as described in the Promega 'Altered Sites' technical manual. Briefly, the contents of the mutagenesis reaction along with DMSO (3µL /200µl cells) were added to BMH 71-18 mut S cells, followed by a 30 minute incubation on ice and a heat shock (42°C for 1 minute). The same protocol was used to transform JM109 cells with phagemid isolated from transformed BMH 71-18 mut S cells, or to transform mn1061/p3 cells with recombinant pcDNAI. Cells were then grown in the presence of the appropriate antibiotic.

### 2.14 Site-directed mutagenesis

*In vitro* site-directed mutagenesis was used to synthesise the Asn9, Arg193, Thr158 and Ser291 mutant alleles (Altered Sites™ mutagenesis system, Promega, Madison, Wisconsin). The mutagenesis procedure is illustrated in Figure 2.1. A 2.4 kb fragment of the LPL cDNA containing the entire coding sequence was inserted in the anti-sense orientation in the pAlter phagemid vector carrying a defective ampicillin resistance gene and single-stranded pAlter-LPL WT (wild type LPL sequence) DNA was produced as described above (section 2.4.3). The purified material was then annealed to the ampicillin repair primer and to one of the following mutagenic oligonucleotides:

- **D9N**, 5' ACTTTTCGATGTTGATAAAATCT 3';
- **A158T**, 5' TTAGGTCCAGTTGGATCGAGG 3';

99
S193R, 5'ATTCCAATGCCTCGACCAGGG3';
N291S, 5' TCTGACTTTAÇTGATCTCATA3'.

The mutant nucleotide is underlined. Second strand synthesis was performed according to the manufacturers' recommendations. Following two rounds of transformation with ampicillin selection in BMH7018 (repair-defective strain) and JM109 bacteria (Altered Sites™ mutagenesis system, Promega, Southampton, UK), phagemid DNA from resistant colonies was isolated by the miniprep procedure and checked for the presence of the mutation by direct sequencing.

After re-amplification, mutated and wild-type LPL inserts were excised from pAlter by digestion with XbaI/PstI and purified as described above (section 2.6). Inserts were then ligated into the linearised pcDNAI expression vector (Invitrogen Corp., San Diego, obtained from British Biotechnology Ltd) (digested with XbaI and PstI and purified) in the sense orientation. Ligations were carried out overnight at 4°C with a vector/insert ratio of 1:2 as described in the Promega manual. The resulting pcDNA-LPL9, -LPL158, -LPL193, -LPL291 and -LPLWT constructs had the LPL cDNA under the control of the CMV promoter and expressed the tyrosine tRNA suppressor gene (synthetic SupF gene). These constructs along with appropriate controls were used to transform mc1061/p3 bacteria as described before, and suppress amber mutations in the Amp and Tet genes of the p3 plasmid, following which, ampicillin and tetracyclin resistant colonies were selected. Large-scale purification of the plasmids for transfection was achieved with the Circle Prep kit (Bio101, La Jolla, CA) from 500 mL overnight cultures as outlined earlier.
Fig. 2.1 Schematic diagram for the site-directed mutagenesis procedure and expression of LPL variants in vitro. The lefthand side of the diagram is reproduced from the Promega pAlter mutagenesis system manual. pAlter is the mutagenesis vector and pcDNAI the expression vector.
2.15 Transient expression studies in COS B cells.

2.15.1 Maintenance and preparation of COS B cells

COS-B cells were obtained from Drs. Zonneveld and Pannekoek, (Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands) and grown in 75 or 225cm² flasks. Cells were maintained in Gibco D-MEM medium containing Na pyruvate and 1g glucose/L (Cat#041-02320) with FCS added to a final concentration of 10% for routine culturing of cells, but only 2% during transfection. Ciprofloxacine (Ciproxin, Bayer) was used as antibiotic (final conc. 10µg/mL) with medium change every 48 hrs and passaging every 3-4 days. Cells were kept at 37°C in a humidified incubator equilibrated with 5% CO₂ and 95% air.

Prior to transfection, cells were trypsinised, seeded in 60mm dishes (1-2 x 10⁵ cells)(unless otherwise specified) and allowed to attach. The transfection was carried out in dishes where cells were 50-80% confluent, usually 24 hours after seeding.

2.15.2 DEAE-Dextran transfection of Cos cells

The DEAE-dextran method (Selden et al., 1986) was used for transfection of COS B cells. Briefly, the DNA to be transfected was diluted in 1.25 ml DMEM medium with chloroquine 0.65mg/ml and then mixed 1:1 with a 0.6mg/ml DEAE-dextran/D-MEM chloroquine solution. Five micrograms of plasmid DNA were used per 60 mm dishes unless otherwise specified. In each experiment, one dish was mock-transfected with the DEAE-Dextran/D-MEM/chloroquine solution in the absence of DNA. The cells were washed with medium once and the transfection mixture then added for 3.5 hours. The cells were then shocked briefly (1 min) with 10% DMSO in PBS, washed once with PBS and allowed to recover with fresh media containing 10%FCS.
The media was changed again the next day.

Cell media was collected 3 days post-transfection, with heparin (20U/ml medium) being added to half of the dishes 2 hrs prior to collection, unless otherwise specified. Media samples were centrifuged to remove cell debris. The cell layer was detached from the dish by incubating 10 minutes with PBS/1mM EDTA followed by gentle pipetting. After centrifugation, the cells were resuspended in fresh PBS. All material was then snap-frozen and kept at -70°C until assayed.

2.16 Statistical analysis.

2.16.1 Allele frequencies.

The gene-counting method with a X2 test with Yates’ correction was used to compare the frequency of the Asn9 variant allele between the different groups. Confidence intervals for the percentages and the critical ratio z(c) with continuity correction were calculated according to Colton (1974) as:

\[
z(c) = \frac{x_L - \frac{1}{2} - x_S + \frac{1}{2}}{n_L} \quad \frac{1}{n_S} \sqrt{pq \left(\frac{1}{n_L} + \frac{1}{n_S}\right)}
\]

where \( p \) is the proportion of total study sample carrying the allele of interest, \( q = 1 - p \), \( n_L \) and \( x_L \), represent the total number of individuals in a group and the number carrying the allele of interest respectively in the group with the higher frequency, and \( n_S, x_S \), represent the same in the group with the lower frequency.

The estimate of relative risk (RR) of being a carrier was calculated by standard techniques. To compare RR between samples, the estimates of \( \log \) (RR) were weighted
2.16.2 Lipid, lipoprotein and LPL data.

Biometrical and plasma lipid data that had been obtained previously for each of the studies were used for comparison of carriers with non-carriers of the Asn9 and Ser291 variants. For the UK, Dutch and Swedish studies, all tests and transformations were performed using the SPSS statistical package. The t-test was used to compare lipid variables between carriers and non-carriers in the healthy men from the two UK studies. Triglyceride levels values were log-transformed prior to testing. A Mann-Whitney non-parametric test was used to compare LPL activities and mass between carriers and non-carriers of the Asn9 allele.

For the ECTIM study, the SAS software was used for statistical analysis. All data were adjusted for age, BMI and center unless otherwise specified. Means for lipid and lipoprotein traits were compared by an analysis of variance with case/control, carrier/non-carrier status as well as BMI as grouping factors. Interactions between groupings and dependent variables were also tested to assess the homogeneity of the association.

Statistical significance was considered to be at the 0.05 level.

2.17 Protein variation modelling

To evaluate the effect of given amino acid substitutions, the protein secondary structure was modelled using the DNAstar "Protein" computer program with a seven-
residue window. This program made secondary structure predictions using the Chou-Fassman algorithms. Hydrophobicity was estimated by the Hopp-Woods algorithm. Charge and hydrophobic moment (a measure of amphipathicity) were also calculated. A minimum segment of 100 amino acids on either side of the substitution site were analysed.

The position of amino acids of interest in native LPL was approximated using the proposed tridimensional structure of pancreatic lipase (Winkler et al., 1990) as drawn by Santamarina-Fojo (1992) and Lalouel et al. (1992).
3. LPL MUTATIONS IN TYPE I/TYPE V HYPERLIPOPROTEINAEMIA

A group of 20 probands with proven or suspected LPL deficiency were studied to examine the nature and distribution of LPL gene mutations. The individuals had either a type I hyperlipoproteinaemia phenotype or a type V profile with LPL activity deficiency. For simplicity, they will generally be referred to as the type I group.

3.1 Patient description

Thirteen of the individuals were of European origin, including two of Mediterranean descent (CB and MA), and seven were from the Indian sub-continent (TA, SAK, LD, RL, GM, KP, Wa). Consanguinity was recorded in only one family where the parents were first cousins (proband BB). The biochemical and clinical data pertaining to the individuals included in this study are presented in Table 3.1. No information was available for three of the individuals (RL, JLe, MVA). In the 17 remaining subjects, there was a wide range of fasting triglyceride and cholesterol levels (Tg: 18 - >100mM, chol: 5.7 - 16). Most probands (14/17) were diagnosed in childhood or infancy and 15/17 had experienced one or more classical symptoms associated with type I HLP (hyperchylomicronaemia, abdominal pain, hepatosplenomegaly, recurrent pancreatitis, fat intolerance, eruptive xanthomata). Three individuals (WG, LA and GM) were apparently asymptomatic until adulthood and four (PB, WG, GM, BS) predominantly showed a type V phenotype as adults (chylomicrons and VLDL increased in plasma).

Lipolytic activity was measured by Dr. John Brunzell, University of Washington, Seattle, for subjects TA, SAK, LD, PL and KP and obtained from medical records for the rest of the group. The absence or very low levels of post-heparin plasma...
Table 3.1 Clinical characteristics and lipid levels of the study subjects.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age at diagnosis</th>
<th>Clinical symptoms</th>
<th>Lipid levels (mmol/L)</th>
<th>Plasma LPL</th>
<th>Mutation*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chol</td>
<td>TG</td>
<td>Act. (mU/mL)</td>
</tr>
<tr>
<td>LA</td>
<td>25 yrs</td>
<td>Abd. pain, HTG</td>
<td>-</td>
<td></td>
<td>&gt;30</td>
</tr>
<tr>
<td>MA</td>
<td>6 yrs</td>
<td>CH, abd. pain</td>
<td>5.8</td>
<td>30.0</td>
<td>Neg.</td>
</tr>
<tr>
<td>MVA</td>
<td>childhood</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TA</td>
<td>3 months</td>
<td>HSM, anaemia, vomitting</td>
<td>-</td>
<td>&gt;100</td>
<td>2</td>
</tr>
<tr>
<td>SAK</td>
<td>6 months</td>
<td>HTG, HSM</td>
<td>-</td>
<td>&gt;21</td>
<td>&lt;10%</td>
</tr>
<tr>
<td>BB</td>
<td>childhood</td>
<td>Abd. pain, fat intolerance</td>
<td>16.0</td>
<td>50.0</td>
<td>Neg.</td>
</tr>
<tr>
<td>CB</td>
<td>7 yrs</td>
<td>HTG</td>
<td>6.1</td>
<td>16.1</td>
<td>0</td>
</tr>
<tr>
<td>PB</td>
<td>childhood</td>
<td>Abd. pain, fat intolerance</td>
<td>-</td>
<td>-</td>
<td>&gt;20.0</td>
</tr>
<tr>
<td>EC</td>
<td>4 yrs</td>
<td>HTG, abd.pain, Xanth.</td>
<td>9.0</td>
<td>18.8</td>
<td>-</td>
</tr>
<tr>
<td>LD</td>
<td>1 week</td>
<td>HTG</td>
<td>10.5</td>
<td>48.0</td>
<td>0</td>
</tr>
<tr>
<td>WG</td>
<td>39 years</td>
<td>AP, Xanth.</td>
<td>16.3</td>
<td>51.0</td>
<td>-</td>
</tr>
<tr>
<td>GL</td>
<td>childhood</td>
<td>CM, AP</td>
<td>5.7</td>
<td>20.9</td>
<td>0</td>
</tr>
<tr>
<td>RL</td>
<td>childhood</td>
<td>Abd. pain, HTG</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PL</td>
<td>2 months</td>
<td>Xanth., vomiting attacks</td>
<td>14.1</td>
<td>26.0</td>
<td>Neg.</td>
</tr>
<tr>
<td>JLa</td>
<td>2 yrs</td>
<td>Xanth., HSM, abd. pain</td>
<td>-</td>
<td>&gt;30</td>
<td>0</td>
</tr>
<tr>
<td>JLe</td>
<td>?</td>
<td>unknown</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GM</td>
<td>46 yrs</td>
<td>AP, NIDDM</td>
<td>8.6</td>
<td>43.0</td>
<td>0</td>
</tr>
<tr>
<td>KP</td>
<td>2 weeks</td>
<td>CH, HSM (slight)</td>
<td>-</td>
<td>&gt;100</td>
<td>-3</td>
</tr>
<tr>
<td>BS</td>
<td>childhood</td>
<td>Abd. pain, AP, Xanth.</td>
<td>8.6</td>
<td>17.1</td>
<td>0</td>
</tr>
<tr>
<td>W</td>
<td>?</td>
<td></td>
<td>16.4</td>
<td>34.0</td>
<td>-</td>
</tr>
</tbody>
</table>

HTG = hypertriglyceridaemia, CH = chylomicronaemia, HSM = hepatosplenomegaly, Xanth = eruptive xanthomata, AP = acute pancreatitis episode(s), Abd. = abdominal, NIDDM = non-insulin-dependent diabetes mellitus, NEG = negligible. * Mutations written in lower case letters were found in heterozygous form, those in capital letters in homozygous form (valid only for this table).
2 Although PH plasma from this individual could not be analysed, two HTG siblings were shown to have virtually no LPL activity or mass, while LPL mass was undetectable in the mother's plasma samples.
3 No post-heparin LPL activity data was available for this proband, but there was evidence for low LPL activity (father) or mass (mother) in her parents.
LPL activity (PH-LPL) were noted in 12 of the 17 patients for whom data was available (Table 3.1). Ten had no LPL activity and one (SAk) had levels approximately 10% of the mean LPL activity determined for controls (Dr. J. Brunzell, personal communication). For individual LA, LPL activity measured in skeletal muscle tissue was found to be 17 and 8% of the activity measured before and after clofibrate therapy respectively in moderately hyperlipidaemic individuals (Lithell et al., 1978). Abnormal LPL was suspected in two other subjects (subjects TA and KP) on the basis of low activity and mass measured in the plasma of relatives (Table 3.2). Deficiency in apo CII, a rarer cause of type I HLP, could be ruled out in three of the six remaining probands (EC, WG and Wa) based on apparently normal CII (amount and charge) on IEF analysis.

LPL dimer mass and total mass (monomer plus dimer species) was available for three probands (SAk, LD, PL) and suggested heterogeneity in the molecular basis of LPL deficiency in these individuals (Table 3.2). Subject SAk had increased levels of LPL dimer and total mass, with low post-heparin LPL activity, implying that the enzyme had very low specific activity. In contrast, LPL dimer mass was undetectable in PL’s plasma despite high levels of total LPL mass, pointing at the instability of the dimer species, while proband LD and an affected brother showed low levels of either LPL species, suggesting a decrease in the production/secretion of the protein.

Acute pancreatitis was not associated with the highest Tg levels, possibly because the reported lipid levels were not necessarily measured on the day of the acute event (Table 3.1). In the 11 individuals where both the exact age of onset and Tg were known, there was no obvious relationship between these two variables. In two individuals where residual LPL activity was reported (LA and SAk), Tg levels were in the middle of the
range, higher than in individuals who had no detectable LPL activity.

Table 3.2 Post-heparin LPL activity and mass in relatives of LPL deficient individuals.

LPL measurements for relatives of probands TA, SArk, LD, PL and KP and are presented below with each row representing a family. When available, data for the probands is shown in bold typescript. LPL activity, dimer mass and total mass (monomer + dimer) was measured by Dr. J. Brunzell as described in section 2.3.1

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>LPL activity (nmol/min/ml)</th>
<th>LPL dimer mass (ng/ml)</th>
<th>Total LPL mass (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sister TA</td>
<td>1</td>
<td>5</td>
<td>43</td>
</tr>
<tr>
<td>Brother 1 TA</td>
<td>15</td>
<td>12</td>
<td>50</td>
</tr>
<tr>
<td>Brother 2 TA</td>
<td>3</td>
<td>1</td>
<td>39</td>
</tr>
<tr>
<td>SArk</td>
<td>21</td>
<td>779</td>
<td>1562</td>
</tr>
<tr>
<td>Mother SArk</td>
<td>38</td>
<td>69</td>
<td>-</td>
</tr>
<tr>
<td>LD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Father LD</td>
<td>6</td>
<td>15</td>
<td>-</td>
</tr>
<tr>
<td>Mother LD</td>
<td>34</td>
<td>17</td>
<td>-</td>
</tr>
<tr>
<td>Brother LD</td>
<td>0</td>
<td>36</td>
<td>40</td>
</tr>
<tr>
<td>PL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Father PL</td>
<td>4</td>
<td>0</td>
<td>376</td>
</tr>
<tr>
<td>Mother PL</td>
<td>95</td>
<td>318</td>
<td>-</td>
</tr>
<tr>
<td>Sister PL</td>
<td>40</td>
<td>18</td>
<td>-</td>
</tr>
<tr>
<td>S</td>
<td>36</td>
<td>87</td>
<td>-</td>
</tr>
<tr>
<td>Father KP</td>
<td>20</td>
<td>180</td>
<td>-</td>
</tr>
<tr>
<td>Mother KP</td>
<td>82</td>
<td>44</td>
<td>-</td>
</tr>
<tr>
<td>Normal range$^1$</td>
<td>100-340</td>
<td>80-310</td>
<td>-</td>
</tr>
</tbody>
</table>

3.2 Detection of the G188E substitution

To determine the molecular basis of their hyperchylomicronaemic phenotype, DNA from the study subjects was initially screened for the G188E substitution (G to A substitution at nucleotide 818) as this mutation has been reported to account for 25% of mutant LPL alleles (Monsalve et al., 1990). Following PCR amplification of exon 5, samples were digested with AvaiI, yielding a single 206 bp fragment in individuals homozygous for the mutation, two fragments of 118 and 88 bp in unaffected individuals or all three bands in heterozygotes (Fig. 3.1). Five subjects out of 20 (PL, PB, BB, JLe GL) were found to be homozygous while two heterozygotes for the mutation were identified (JLa, MA) in this group of type I patients. All were of European origin. Thus, the A allele (Glu188) accounted for 12/26 (46%) European mutant alleles and 12/40 (30%) alleles overall in this group.

3.3 Genotyping for PvuII and HindIII polymorphisms

To examine the ancestry of the mutation, a simple haplotype analysis was carried out using the HindIII and PvuII polymorphisms in the LPL gene. The five G188E homozygotes (BB, JLe, PL, GL and PB) were also homozygous for the cutting allele of the HindIII (+/+)(625 bp approx.) and the non-cutting allele of the PvuII (-/-)(440 bp) polymorphic sites. The two G188E heterozygous individuals (JL and MA) were homozygous for the HindIII + allele but showed heterozygosity at the PvuII site (440 and 330 bp fragments) (Fig. 3.2). The data from G188E homozygotes shows that the mutation is carried on a chromosome with a P- H+ haplotype. Two haplotypes are present in G188E heterozygotes, P- H+ and P + H+, and this suggests that the former identifies a chromosome common to all individuals, which carries the mutation and is identical by descent.
Fig. 3.1 Detection of the G188E substitution. Exon 5 was PCR amplified and digested with AvaiI. The mutation (G818 to A) abolishes the AvaiI restriction site. The three possible genotypes are represented above. Lane 1: uncut PCR, 258bp. Lane 2: homozygous subject for the mutation. Lane 3: heterozygous subject. Lane 4: normal individual. Lane 5: kb ladder.

Fig. 3.2 Detection of the PvuII and HindIII polymorphisms in individuals carrying the G188E substitution. Panel A - PvuII: presence of the cutting site (P+), 330bp; absence of the cutting site (P-), 440bp. Lane 1: kb ladder, lane 2: P+ P- individual, lane 3: P+ P+ individual. Panel B: Presence of the cutting site in all subjects (625bp). Subjects are referred to by the same initials as in the text.
3.4 Detection of new mutations using SSCP analysis.

In order to detect other mutations that may be present in the subjects, SSCP analysis was used following exon by exon amplification of the LPL coding region (exons 2-9). Subject EC was not included in this analysis and subject GM was only studied for exon 5 due to the unavailability of DNA at this stage of the study. Both the denatured single-stranded DNA and the undenatured PCR products were examined. Variant single-strand or double-strand patterns were clearly identifiable in PCR fragments from exons 3, 4, 5, 6 and 8 (Table 3.3). These patterns were highly distinctive, specific and reproducible. The exon 5 PCR fragment was shown to have the most variant patterns (six) in single-stranded DNA, which were detected in samples from subjects BS, SAK, LA (LAnd), MVA, PL and MA (SSCP panels, Fig. 3.3). The latter two had been included as positive controls since they were known to be respectively homozygous and heterozygous for the G188E substitution previously detected by AvaII digest of exon 5 PCR (Fig. 3.1 and Fig.3.3, panels A and E). In samples from BS, LA and MVA, a variant band was present in addition to the two normal bands, suggesting that these individuals were heterozygous for an LPL mutation. In contrast, the normal pattern was absent from lane SAK (sample SAK), implying that this individual might be homozygous for a mutation in exon 5. In exon 6, three subjects had extra bands, Wa, WG and CB (Fig.3.4, panel A) and two of the variant patterns were seen in both the single and double-stranded area of the gel (subjects Wa and CB) while the other was only apparent in the double stranded DNA fragment (subject WG). For exons 3, 4 and 8, variation in single-strand patterns was observed in two individuals, RL (exon 3, homozygous pattern) and MVA (exon 4 and 8, both heterozygous patterns)(Fig. 3.5, panel A, B and C).
Fig. 3.3 Detection of mutations in LPL exon 5 by SSCP and identification by sequencing or restriction digest. Patients are identified as in the text. N = normal; U = undigested. Only single-stranded DNA is shown for SSCP.
Fig. 3.3 - continued
Fig. 3.4 Detection of mutations in LPL exon 6 by SSCP and identification by sequencing.
Patients are identified as in the text. N = normal individual. Both single-stranded and double-stranded DNA bands are shown.
Fig. 3.5 Detection of mutations in LPL exons 3, 4 and 8 by SSCP and identification by sequencing. Patients are identified as in the text. N = normal. Only single-stranded DNA is shown.
Sequencing of the fragments exhibiting a variant pattern revealed nucleotide changes at positions 511 (T - G), 609 (G - A), 727 (G - A), 804 (C - G), 818 (G - A), 832 (A - C), 875 (C - T), 1006-1007 (CT dinucleotide deletion), 1127 (A - G), 1157 (T - C) and 1163 (T - C) (Table 3.3, Figs. 3.3, 3.4 and 3.5). Nine of these changes were predicted to cause amino acid substitutions (511, W86G; 727, A158T; 804, H183Q; 818, G188E; 832, S193R; 875, P207L; 1127, N291S; 1157, M301T; 1163, L303P) while the G to A substitution at position 609 did not, as it occurred at the wobble third position of a codon (Glu118). The 2-bp deletion in exon 6 was arbitrarily assigned to the first of three consecutive CT dinucleotides and resulted in a frame shift. This is expected to lead to the replacement of the Leu-Leu dipeptide (aa 252-253) by Val-Glu followed by a premature termination codon (Table 3.3, Fig. 3.4). The nature of the variant pattern detected in exon 8 (MVA) has not been determined by sequencing analysis and its possible functional significance remains unknown. Since a large proportion of reported mutations occurred in the central segment of LPL, direct sequencing of exon 5 and 6 was undertaken in samples from the eight individuals (TA, EC, LD, WG, JL, GM, KP and BS) in whom one or both mutant alleles had yet to be identified. By this approach, only one additional mutation was discovered, a T to C substitution at nucleotide 1157, predicted to result in a Met for Thr substitution at position 301 (M301T) (Fig. 3.6).

Overall, only 4 of the 11 identified defects had been reported elsewhere (H183Q, Tenkanen et al., 1994; G188E, Monsalve, et al., 1990; P207L, Ma et al., 1991; A291S, Ma et al., 1993c). Seven of the single-base changes were transitions and three were transversions. Only one of the variants occurred at a highly mutable CpG dinucleotide (nt 875, P207L). Four of the mutations affected a restriction enzyme cutting site:
A158T, G188E, S193R and P207L eliminated PvuII, AvaII, Sall and BsiYI sites respectively. All the novel sequence variants reported in this chapter excluding M301T were confirmed by sequencing the second strand or digesting the relevant PCR fragment with an appropriate restriction enzyme.

Fig. 3.6 Identification of the T1157 to C mutation by direct sequencing. This base change results in a Met to Thr substitution at position 301 in subject GM.
Table 3.3 LPL gene mutations identified in 20 probands with proven or possible LPL deficiency.

<table>
<thead>
<tr>
<th>Location of mutation</th>
<th>Nucleotide</th>
<th>Nature of defect</th>
<th>Amino acid</th>
<th>Number of alleles</th>
<th>Homozygote/Heterozygote</th>
<th>Ethnic origin/descent</th>
<th>Detection by SSCP</th>
</tr>
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<tbody>
<tr>
<td>Exon 3</td>
<td>T511-C*</td>
<td>Trp86 - Gly</td>
<td>2</td>
<td>1/0</td>
<td>Indian subcontinent</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Exon 4</td>
<td>G609 - A₁</td>
<td>No change</td>
<td>1</td>
<td>0/1</td>
<td>Swedish</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Exon 5</td>
<td>G727-A*</td>
<td>Ala158 - Thr</td>
<td>2</td>
<td>1/0</td>
<td>Pakistani</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Exon 5</td>
<td>C804-G</td>
<td>His183 - Gln</td>
<td>1</td>
<td>0/1</td>
<td>English</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Exon 5</td>
<td>G818-A</td>
<td>Gly188 - Glu</td>
<td>12</td>
<td>5/2</td>
<td>Engl./Irish/Greek</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Exon 5</td>
<td>A832-C*</td>
<td>Ser193 - Arg</td>
<td>1</td>
<td>0/1</td>
<td>Swedish</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Exon 5</td>
<td>C875-T</td>
<td>Pro207 - Leu</td>
<td>1</td>
<td>0/1</td>
<td>Swedish</td>
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<td></td>
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<tr>
<td>Exon 6</td>
<td>Δ1006-1007*</td>
<td>Val-Glu-Ter254</td>
<td>2</td>
<td>1/0</td>
<td>Italian</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Exon 6</td>
<td>A1127-G</td>
<td>Asn291 - Ser</td>
<td>1</td>
<td>0/1</td>
<td>English</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Exon 6</td>
<td>T1157-C*</td>
<td>Met301 - Thr</td>
<td>1</td>
<td>0/1</td>
<td>Indian subcontinent</td>
<td>No</td>
<td></td>
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<tr>
<td>Exon 6</td>
<td>T1163-C*</td>
<td>Leu303 - Pro</td>
<td>2</td>
<td>1/0</td>
<td>Indian subcontinent</td>
<td>Yes</td>
<td></td>
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<tr>
<td>Exon 6/Intron 7</td>
<td>2kb insertion #</td>
<td>-</td>
<td>3</td>
<td>0/3</td>
<td>English/Swedish/Greek</td>
<td>No</td>
<td></td>
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<tr>
<td>Exon 8</td>
<td>SSCP</td>
<td>?</td>
<td>1</td>
<td>0/1</td>
<td>Swedish</td>
<td>Yes</td>
<td></td>
</tr>
</tbody>
</table>

* Novel mutation identified in this study
# From Langlois et al., PNAS, 1989.
₁ This base change appears to be a common polymorphism as it is present in 6/27 Swedish individuals (R.Peacock, personal communication)
3.5 Detection of large gene defects by Southern blotting.

The occurrence of major insertions or deletions at the LPL gene locus in this cohort was examined by Southern blotting and hybridization with the full-length LPL cDNA. Genomic DNA (5µg) from type I individuals was digested with *StuI* as described in section 2.8) On the blot, an additional band of approx. 4.7 kb was detected in lanes MA, EC and JC (EC's mother), along with the constant 7.4, 6.1, 4.4, 3.4 and 2.7kb bands present in all lanes including the control lane (Fig. 3.7a). The band pattern and the position and size of the extra band was very similar to the description by Langlois et al. (1989) of a 2kb insertion mutation in a young man with LPL deficiency, except that all the fragments detected here were larger by approximately 0.5kb. This was probably due to differences in the size-marker standard curve. To confirm the nature of the defect, DNA was digested with *PstI*, then blotted and hybridised with a 1.0 kb *StuI* fragment of the probe corresponding to nucleotides 1-1036 and covering exons 1-6. In individuals LA (for whom the signal was undetectable on the *StuI* blot), MA, EC and JC, an extra 8.5kb band was visible in addition to 11-11.5kb and 6.6kb bands (Fig. 3.7b). The presence of an additional fragment detected with a second restriction enzyme reinforced the suggestion that these individuals were heterozygous for an insertion or deletion. Furthermore, the probe localised the defect 5' of exon 7. This pattern is completely consistent with the previously reported 2 kb insertion/inversion in exon 6/intron 6 in patients of European descent (Langlois et al., 1989; Devlin et al., 1990). The three subjects in our study who carried this defect were of Swedish (LA), Greek (MA) and English (EC) descent respectively (Table 3.1). A second extra band (5.1 kb, double asterisk) was present in sample MA with the *StuI* digest (Fig.3.7a) but not with the *PstI* digest. Since this individual is already heterozygous for another known
functional mutation in the LPL gene, it is likely that this band represent a neutral previously unreported polymorphism and it was not investigated further.

Fig.3.7a. Detection of a 2.0kb insertion by StuI digest. Genomic DNA (5 μg) was digested with StuI, transferred to a membrane by Southern blotting and hybridised with the 'full-length' cDNA probe (2.4kb) covering all coding exons as outlined in the methods section. C: control. EC and MA: type I patients. JC: mother of subject EC. The single asterisk shows the additional 4.7kb band corresponding to the fragment with the 2.0kb insertion (Langlois et al., 1990). The double asterisk indicates the presence of a probable StuI polymorphism in subject MA.
Fig. 3.7b. Detection of a 2.0kb insertion by PstI digest. Genomic DNA (5 μg) was digested with PstI, transferred to a membrane by Southern blotting and hybridised with a partial cDNA probe (1.0kb) covering coding exons 1-6 as outlined in the methods section. C: control. EC, LA and MA: type I patients. The single asterisk shows the additional 8.5kb band corresponding to the fragment with the 2.0kb insertion (Langlois et al., 1990).
3.6 Positional analysis of the mutations.

To gain better understanding of the impact of the mutations identified in section 3.3, their position was determined in relation to the known LPL functional domains. We used the published tridimensional model of PL (Winkler et al., 1990) on which key LPL structures had been superimposed (Santamarina-Fojo, 1992). The true position of the residues in native LPL may differ slightly from this representation. The position of disulfide bonds, the heparin-binding domain (aa292-302) and the active-site triad (Ser132, Asp156, His241) were used as guides, along with schematic diagrams of conserved and variable elements (Derewenda and Cambillau, 1991). To determine further whether the residues were located on the outside of the protein or buried within, the ribbon PL model drawn by Lalouel et al. (1992) based on the Winkler structure proved to be very useful.

The nine point mutations causing amino acid substitutions were positioned on the PL model structure represented on Fig.3.8. All the mutations were located in the large, catalytic N-terminal domain (aa 1-334). Recent data reviewed by Lalouel et al. (1992) and presented by Derewenda and Cambillau (1991) suggests that the tightly packed, central β-sheet and α-helices of this domain are very sensitive to alterations. Therefore, even apparently conservative mutations in this region are likely to affect LPL function.

Trp86 has its bulky side chain apparently packed against a short stretch of hydrophobic residues, Ala98-Gly99-Tyr100. The residue is conserved in human HL, PL and LPL and a Trp to Arg substitution at this position has been shown to inactivate the enzyme (Ishimura-Oka et al., 1992a). Trp86 is also very close to one of three active site loops (Gln91 - Pro95) thought to be involved in substrate binding (van Tilbeurgh et al., 1994) and may itself interact with the acyl chain of the triglyceride. Although its effect
may be expected to be less severe than W86R, it is possible that the replacement of tryptophan by glycine may force a rearrangement of hydrophobic residues in the vicinity or disrupt van der Waals interactions. This may in turn change the orientation of the substrate-binding loop and leading to less efficient presentation of the substrate.

Residue 158 appears to be located in a fold, in close proximity to both Asp156 and Ser132, two members of the catalytic triad. Ala158 is part of a proposed substrate-binding loop (residues 157-160) and may display hydrophobic interactions with the triglyceride substrate (van Tilbeurgh et al., 1994). Ala158 is also part of a strictly conserved segment in all lipases (TGLDPA) (Derewenda and Cambillau, 1991; Bensadoun, 1992) and other substitutions in this region at positions 154, 156 and 157 have been shown to completely abolish activity (Ma et al., 1992a, Bruin et al., 1992, 1993). Therefore, the presence of the polar threonine residue at this position is expected to markedly affect LPL function, probably by hindering the access of the substrate to the catalytic site or by weakening the strength of the binding. Alternatively, the hydroxyl group of Thr158 may interfere with the charge relay system of the triad by disrupting the alignment of Ser132, Asp156 and His 241. However, this possibility is difficult to evaluate in the absence of precise information regarding the orientation of the Thr side chain.
Fig. 3.8 Positioning of the LPL gene mutations identified in this study on the 3D model of human PL (Winkler et al., 1990). The mutations are indicated by shaded dots on the structure, next to the one-letter code for each mutation, in relation to the catalytic triad amino acids (dots with crosses) and the glycosylation site (Asn43). Internal hydrophobic segments where mutations have been identified are shown by a discontinuous line (short dashes) whereas the disulfide bridges are indicated by long dashes.
The H183Q substitution was recently identified in a proband of Swiss/Russian
descent (Tenkanen et al., 1994) and shown to lead to an inactive enzyme. This residue
appears to be in close contact with the helix containing His241 in a densely packed
region. Haubenwallner et al. (1993) have recently reported that a conservative
substitution with respect to charge but not to size at position 180 (Asp to Glu) abolishes
activity, probably due to steric hindrance. By examining the helix sequence in the
vicinity of His183 and assuming compatible orientation of side chains, it is possible to
envisage an ionic interaction with Glu242. This bond would be disrupted by the
substitution with Gln and may alter the topology of the catalytic triad.

Residues 188 and 193 are located on the third proposed substrate-binding loop
element (Arg187 - Ile196), under the lid structure which is predicted to rotate away upon
interfacial activation. Although Ile194 is the only residue thought to interact directly with
the substrate (van Tilbeurgh et al., 1994), it has been proposed that the loop segment
forms a hydrophobic groove. If this model is correct, the replacement of serine by the
bulkier, charged asparagine residue would be expected to effect a major change in the
local structure and restrict substrate access to the catalytic site. By analogy with the
analysis of Derewenda and Cambillau (1991) for the G188E mutation, electrostatic
interactions with Arg187 and Glu227 (part of the lid structure) can be postulated. These
interactions could modify the shape of the protein such as to make the LPL dimer
unstable, as the G188E and G195E substitutions have been shown to do (Hata et al.,
1992). Alternatively, the attraction to Glu227 may increase the rigidity of the structure
and thus interfere with the displacement of the lid covering the catalytic site.

Amino acid positions 207, 301 and 303 are not in close proximity to catalytic
triad residues but rather are part of the last two parallel β-strands of the N-terminal
domain. The closely packed \( \beta \)-sheet structure of the N-terminal provides the stable frame needed to maintain the alignment of the catalytic triad residues. The substitutions at positions 301 and 303 both involve the replacement of bulky hydrophobic residues by smaller and less hydrophobic (in the case of Thr) ones, which may lead to weaker interactions between strands. This loss of rigidity of the \( \beta \)-sheet structure may perturb the linear arrangement of the catalytic triad. For its part, the substitution at position 207 involves the replacement of a proline residue, an imino acid which can adopt a limited number of conformations (MacArthur and Thornton, 1991). The presence of a leucine residue may introduce some unwanted flexibility in the region, again possibly disturbing the delicate alignment of the triad.

Finally, residue 291 is located on a protruding loop extending away from the catalytic site in the PL model. However, this segment (aa290-300 in LPL) is poorly conserved between LPL and PL so that its structure in LPL may not be accurately predicted by the PL model (Persson et al., 1989, Derewenda and Cambillau, 1991). The residue is still likely to be on the surface of the normal protein because asparagine has a polar side chain. Moreover, it is surrounded by positively charged residues from two segments which are involved in heparin-binding (aa279-283 and 292-300) (Hata et al., 1993). Ma et al. (1993c) recently reported that the Asn to Ser substitution had half-normal activity \textit{in vitro}. Residue 291 does not appear to lie in close proximity to the active site so that its effect on enzyme activity might be indirect. It is also possible that altered hydrogen bonding decreases the stability of the LPL dimer.

The 2-nt deletion in exon 6 is expected to result in a truncated protein lacking part of the catalytic domain (aa 254-334) including a putative heparin-binding site and
the whole C-terminal lipid binding domain. This protein could not be anchored and stabilised on the cell surface via interactions with HSPG and would probably be rapidly degraded.

3.7 Relationships between mutations and phenotype.

To examine the possible relationship between the phenotype and the underlying molecular defect, an attempt was made to associate LPL levels, Tg levels at presentation and the severity of the disorder with particular mutations. Since the clinical symptoms were recorded by different clinicians without a standardised questionnaire and may not be directly comparable, age at diagnosis was used as a crude index of the apparent severity of the disorder.

Given the large variation in lipid levels between individuals and owing to the incompleteness of the data, it was difficult to detect an overall pattern in this relatively small group. There was no clear relationship between high Tg levels (>40mmol/l) and a particular mutation (Table 3.1). To investigate whether homogeneity at the molecular level resulted in specific clinical phenotype, the seven subjects who carried the G188E substitution (5 homozygotes and 2 Hheterozygotes) were examined more closely. There was a wide range of Tg (20-50mmol/l) and chol (5.7-16mmol/l) levels in these subjects, but all had been diagnosed early in life and had been symptomatic. This is consistent with the demonstration that the Glu188 protein is an inactive, unstable enzyme (Hata et al., 1992). Accordingly, we found that LPL mass was associated exclusively with the inactive monomer form in the plasma of one individual homozygous for the G188E substitution (subject PL , Table 3.2).

Attempts were next made to group patients with functionally related mutations.
Mutations shown previously to completely abolish LPL activity both in vivo and in vitro such as H183Q, G188E, P207L (Tenkanen et al., 1994; Emi et al., 1990b; Ma et al., 1991) were associated with an early diagnosis. This is in contrast to two mutations where residual LPL activity has either been demonstrated in vitro (N291S, Ma et al., 1993c; Table 3.1 and section 5.3) or has been detected in the patient’s skeletal muscle tissue (S193R, Table 3.1). The two probands bearing these mutations (WG, LA) were diagnosed well into adulthood. Subject LA was heterozygous for the S193R substitution, but his second mutation, a 2kb insertion, was presumed to be a null allele, based on published data (Langlois et al, 1989, Devlin et al.,1990). Consequently, it is assumed that any residual activity observed in this individual is attributable to the S193R protein.

In a third proband with low plasma LPL activity (SAk), diagnosis was made in early childhood. However, the clinical symptoms reported for this subject and an older affected sibling appeared relatively mild (no documented pancreatitis or xanthomas) with only moderate adherence to dietary recommendations.

An alternative approach was to group mutations according to their position in the structure. Substitutions near the catalytic triad (A158T, H183Q), in the loop/lid segment (G188E, S193R) or in conserved β-strand segments distant from catalytic triad (W86G, P207L, M301T) were compared for effect on LPL activity where available and for age at diagnosis. No simple patterns emerged from this analysis. There was heterogeneity within the groups with regard to LPL activity and apparent severity of the disease. In particular, one mutation from each of the three groups occurred in an individual with late diagnosis (A158T, S193R and M301T) and there was residual LPL activity reported for individuals carrying the A158T and S193R mutations, which belonged to the catalytic triad and loop/lid classes respectively.
3.8 Summary and brief discussion.

A total of 13 mutations (12 potentially functional, 1 silent substitution) have been detected in the LPL gene of a cohort of 20 individuals with type I HLP. Eight of these were identified in UK residents, emphasizing the molecular heterogeneity underlying LPL deficiency in this country. This is consistent with studies from the United States and from Europe where over 40 mutations have now been reported (reviewed by Lalouel et al., 1992). Nine point mutations which altered amino acids accounted for 23 alleles, there was one small homozygous deletion (2bp), and one 2.2kb insertion present in 3 heterozygous subjects. The G188E substitution was the only point mutation found in more than one individual and accounted for 12 of those alleles. Excluding the uncharacterised mutation in exon 8 and the silent substitution in exon 4, it is predicted that 28 of the 40 mutant alleles in this group have been identified. Six of the mutations (W86G, A158T, S193R, M301T, L303P and A1006-1007) have not been reported previously. As expected, the mutations were not randomly distributed along the coding sequence. The majority of mutations occurred in exons 4-6 (11/13) which encode the central catalytic domain and are highly conserved amongst lipases (Hide et al., 1992).

Haplotype analysis for the G188E mutation showed that it was carried on a P-H+ chromosome. This is likely to be the same haplotype which was previously identified in other Caucasian patients with the G188E mutation (Monsalve et al., 1990; haplotype 1: PvuII-, HindIII+, BamHI-). Taken together, these results further support the contention that the mutation is borne on a single chromosome identical by descent in all individuals.

One noteworthy observation is that the A158T substitution identified in a proband of Pakistani origin abolished the same PvuII restriction site as the P157A mutation found
in a Dutch pedigree (Bruin et al., 1992). This emphasises the necessity to verify the precise molecular nature of the mutation when screening individuals with different genetic backgrounds.

No clear associations were found between the position of the mutation and the severity of the disease or Tg levels. This is not surprising in view of the small number of subjects used in this analysis, the crudeness of the severity index and the enormous range of serum Tg values among the patients. In addition to differences in genetic background between individuals which may obviously influence the expression of the disease, environmental factors may have obscured the analysis. In several cases, Tg levels at presentation were not available. Given the good response to dietary treatment observed in type I subjects and the likely variation in the degree of compliance to dietary recommendations, the values may not have been accurately reflecting the magnitude of the impaired Tg clearance. In addition, the extent of the molecular heterogeneity underlying the disorder (with only one mutation accounting for a significant number of alleles) necessitated the grouping of mutations using rather arbitrary criteria. Unsurprisingly, a clearer pattern emerged when LPL activity itself was examined in relation to severity but data from only eight individuals was available for this analysis.

3.8.1 Efficacy of SSCP as a first line detection method.

Our screening approach was based on the detection of variable patterns in single-stranded DNA from amplified exon sequence. The data presented here confirms the validity and effectiveness of this method in screening for biologically important variation. The variant single-stranded DNA patterns found were reproducible and highly specific, allowing the detection of a particular mutation directly by visual inspection of
the autoradiograph. Mutations were detected even if they occurred relatively close to the end of the PCR fragment such as the silent substitution at Glu118 (<40 bases from the end). Eight of the nine mutations identified in exons 5 and 6 were detected by SSCP, with direct sequencing of both exons revealing the existence of only one additional mutation in exon 6 (M301T, subject GM). DNA from this subject was not actually examined by SSCP for exon 6 so that it is still possible that the mutation would have been detected. This suggests that most variants can be ascertained by SSCP. Moreover, the analysis was performed under a single set of conditions in the present work and it is known that the sensitivity of the method could be increased further by varying the glycerol concentration or the length of the electrophoretic run and the temperature at which it is carried out (Orita et al., 1989a).

3.8.2 Unidentified mutations in the cohort.

The twelve defects presumed to affect the protein sequence identified in this study accounted for 28/40 alleles in these individuals with type I or type V HLP. In probands TA, LD and KP, no mutations were found while only one mutant allele was identified in subjects EC, WG, JLa, GM and BS. Several reasons can explain the failure to identify all the mutant alleles in this cohort. Firstly, although SSCP was shown to be very sensitive, it is conceivable that it will miss a small proportion of point mutations. According to Sheffield et al. (1993) and Cotton (1993), 80-90% of point mutations are detected on average in fragments ranging from 150-200 bases. Moreover, heterozygous small deletions (50-200bp) which would not be detected by Southern blotting may result in non-amplification by PCR and an apparent normal pattern. Also, the overall figure (28/40) includes four alleles from two individuals not fully screened by SSCP (GM and
EC) where additional mutations may have been found. Secondly, small segments of the LPL coding sequence (up to 20 nucleotides plus one intron/exon border for exons 3, 4, 6 and 9) were not covered by SSCP screening, due to the constraints on primer design. It has been estimated that 15% of mutations responsible for human genetic diseases occur in the vicinity of mRNA splice junctions (Krawczak et al., 1992) and it is noteworthy that two splice-site mutations have already been reported in the LPL gene. Therefore, it could well be that a small number of such mutations have been missed. In addition, sequences not coding for amino acids in mature LPL were not examined for variation in this study. These comprise the promoter region, the 5' untranslated region/signal peptide encoded by exon 1 and the 1.9kb 3' untranslated region. A third obvious consideration is that any missed mutation may itself account for several alleles. Finally, it cannot be excluded that apoCII deficiency, known to account for a small proportion of cases with type I HLP, rather than LPL deficiency may actually be responsible for the disease in at least one of the individuals where CII deficiency was not ruled out.
4. IDENTIFICATION OF COMMON LPL VARIANTS AFFECTING LPL ACTIVITY IN NORMOLIPIDAEMIC AND HYPERLIPIDAEMIC POPULATIONS.

It was suggested by Brunzell and colleagues that partial LPL deficiency due to a defect in the LPL gene might be responsible for a sizeable proportion of cases with familial combined hyperlipidaemia (FCHL) (Babirak et al., 1989, 1992). However, not all carriers of a mutation in the LPL gene present with hyperlipidaemia in the fasting state. A dietary challenge (fat load) might be required for the expression of dyslipidaemia (Miesenbock et al., 1993) and other factors such as age and obesity may further modulate this expression (Wilson et al., 1990). Moreover, the studies in chapter 3 revealed (not unexpectedly) considerable molecular heterogeneity at the LPL gene locus in the UK. In view of these considerations, two approaches were used to examine the 'Brunzell' hypothesis. Firstly, exon by exon SSCP analysis was performed on samples from individuals with combined hyperlipidaemia (CHL) and low LPL activity (Seed et al., 1994) to identify variants in the LPL structural gene. Such an analysis would have the potential to uncover mutations previously found in type I subjects if those were present in CHL subjects, as well as to detect novel, CHL-specific mutations. Secondly, a rapid screening strategy, based on the amplification of pooled DNA samples, was developed and applied to the detection of rare LPL variants in a large number of subjects. Two mutations were studied by this method: one variant identified in the CHL/low LPL group (D9N) and the G188E substitution, the commonest mutation in our cohort of type I subjects.

Finally, the prevalence of another variant (N291S) was investigated in normolipidaemic individuals from the UK (Camberley and St. Andrews groups, section
2.2.2.6) using PCR amplification and allele-specific oligonucleotides (ASOs). The N291S substitution had been identified in an individual with type I HLP (Chapter 3) and was reported to be present and relatively common in the Swedish YMI controls (R. Peacock, personal communication). I am indebted to Dr. Rachel Fisher and Le Ahn Luong for screening the St. Andrews' cohort.

4.1 LPL activity in individuals with CHL.

To test the hypothesis put forward by Brunzell and colleagues that partial LPL deficiency may cause FCHL, a group of 41 consecutive subjects (30 males, 11 females) with CHL (chol > 6.5mmol/l and Tg > 2.2mmol/l) was recruited from Charing Cross Hospital Lipid Clinic and post-heparin LPL activity (PHLA) measured. The characteristics of the group of patients are presented in Table 4.1. A family history of CAD and/or hyperlipidaemia was reported for 93% of the patients and 64% had clear clinical manifestations of premature CAD. Patients in this selected group thus had a high likelihood of having FCHL. Hypertension was not over-represented in this group (13%). The lipid and lipoprotein results illustrated the mixed nature of the hyperlipidaemia with significant elevation in total cholesterol, triglycerides, and apo B and a reduction in HDL compared to a group of 23 healthy normolipidaemics (Table 4.2). These findings are typical of the lipid profile characterising FCHL (reviewed by Grundy et al., 1987). The apoE genotype was determined in the 41 subjects as described (section 2.8.2) and the frequency of all three alleles was similar to that found in a UK control population (0.19 vs 0.17) (Snowden et al., 1991). A representative gel showing the three common apoE alleles is presented in Fig.4.1.
Figure 4.1 Determination of apoE genotypes by PCR. PCR-amplified DNA samples were digested with restriction enzyme Hhal and fragments were separated on a 10% acrylamide gel. M: 1 kb size marker. The bands corresponding to each allele are shown on the left of the gel while their size and the size of the marker bands are indicated on the right.
Table 4.1 Clinical characteristics of combined hyperlipidaemic patients from the CX-CHL study.

<table>
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<th>CHARACTERISTICS</th>
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</tr>
</thead>
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<tr>
<td>MALE:FEMALE</td>
<td>30:11</td>
</tr>
<tr>
<td>% CAD</td>
<td>64</td>
</tr>
<tr>
<td>MEAN AGE (RANGE)</td>
<td>50 (27-69)</td>
</tr>
<tr>
<td>MEAN AGE OF ONSET OF CHD</td>
<td>44 (30-55)</td>
</tr>
<tr>
<td>MEAN BMI* (kg/m²)</td>
<td>26.8 ± 0.5</td>
</tr>
<tr>
<td>% SMOKERS</td>
<td>24</td>
</tr>
<tr>
<td>% EVER SMOKERS</td>
<td>45</td>
</tr>
<tr>
<td>% HYPERTENSIVE</td>
<td>13</td>
</tr>
<tr>
<td>% FAM HX CAD ~</td>
<td>78</td>
</tr>
<tr>
<td>% FAM HX HLP^</td>
<td>62</td>
</tr>
</tbody>
</table>

* = Body mass index  
~ = Family history of coronary heart disease  
^ = Family history of hyperlipidaemia

Table 4.2 Lipid and lipoprotein results [mean ± sd] in the CX-CHL patient group.

A group of laboratory controls is shown for comparison. Values are expressed in mmol/l (chol, trig and HDL-chol) or in mg/dl (apo B and apo Al).

<table>
<thead>
<tr>
<th></th>
<th>CHOL</th>
<th>TRIG</th>
<th>HDL</th>
<th>HDL(M)</th>
<th>HDL(F)</th>
<th>Apo B</th>
<th>Apo A1</th>
</tr>
</thead>
<tbody>
<tr>
<td>N=41</td>
<td>8.2 ± 1.1</td>
<td>4.15± 1.22</td>
<td>0.90 ± 0.23</td>
<td>0.88± 0.25</td>
<td>0.94± 0.20</td>
<td>171± 30</td>
<td>122± 20</td>
</tr>
<tr>
<td>N=23</td>
<td>4.9 ± 0.9</td>
<td>0.94± 0.30</td>
<td>1.30 ± 0.24</td>
<td>1.16± 0.24</td>
<td>1.36± 0.22</td>
<td>96± 30</td>
<td>127± 43</td>
</tr>
</tbody>
</table>

* = Median  
M = Male  
F = Female
The results of assays for post-heparin LPL activity (PHLA) are summarized in Table 4.3 and Figure 4.2. As shown in Figure 4.1, plasma PHLA in patients and controls increased rapidly after heparin administration with the maximum activity occurring between 20-30 minutes post heparin. The 5-minute PHLA was significantly lower (p<0.01) than that at 10 minutes. Although PHLA continued to rise from 10 to 30 minutes there was no significant difference in activities for the 10, 20 and 30 minute samples and thus the value of the 10-minute sample was used for most comparisons between groups. There was no statistically significant sex difference in PHLA activity in the control or patient groups. In the patient group, LPL activity at 10 mins PH was significantly lower in both males and females compared with the male and female control groups (Table 4.3, 52 vs 66, p<0.05 in males, 43 vs 63, p<0.01, in females). Overall, 15 of 41 individuals (36.6%) in the patient group had a 10-minute LPL activity below 45.1nmol/min/ml (mU/ml), the 10th percentile value as determined in control individuals using the SPSS program (mean: 64 mU/ml).

4.2 Relationship between lipase activity and lipid traits.

The pairwise relationships between BMI, LPL activity and lipid traits was examined in the whole patient group and in the males only and the correlation results are presented in Table 4.4. HDL-chol and Tg measured on the day of the LPL assay were negatively correlated, with the relationship strongest in the male group (r=-0.26, p=0.05 vs r=-0.37, p=0.025 females excluded). LPL activities at 10, 20, and 30 minutes were significantly correlated with each other. A weak positive correlation was detected between LPL activity and BMI although this only reached statistical significance using the value for LPL activity measured at 30 mins post-heparin (r=0.33, p < 0.02).
Fig. 4.2 Post-heparin LPL activity in patients from the CX-CHL study and laboratory controls over time. The asterisk indicates statistically significant differences. The activity is expressed in mU/ml. Males and females combined. T-test used for comparisons.

Table 4.3 Post-heparin LPL activities in males and females from the CX-CHL study and in laboratory controls with time. The activity is expressed in mU/ml (1 mU = 1 nmole FFA released per minute). A two-tailed t-test was used to compare LPL activities at 5, 10 20 and 30 minutes post-heparin.

<table>
<thead>
<tr>
<th>Lipoprotein lipase activity (S.E.M.)</th>
<th>5'</th>
<th>10'</th>
<th>20'</th>
<th>30'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls M (n = 11)</td>
<td>43 (4)</td>
<td>66 (4)</td>
<td>71 (4)</td>
<td>78 (4)</td>
</tr>
<tr>
<td>Controls F (n = 12)</td>
<td>45 (4)</td>
<td>63 (5)</td>
<td>72 (5)</td>
<td>75 (5)</td>
</tr>
<tr>
<td>Patients M (n = 30)</td>
<td>37 (3)</td>
<td>52 (3)</td>
<td>61 (4)</td>
<td>65 (5)</td>
</tr>
<tr>
<td>Patients F (n = 11)</td>
<td>28 (3)</td>
<td>43 (4)</td>
<td>56 (8)</td>
<td>67 (9)</td>
</tr>
</tbody>
</table>

1 Significantly different from female controls, p < 0.01
2 Significantly different from male controls, p < 0.05
Table 4.4 Pairwise relationships between BMI, lipid traits and PHLA in patients from the CX-CHL study. Correlation coefficients were computed either for the whole group (All, n = 41) or for the male patients only (Males, n = 30). A p-value is shown only for significant results. The lipid traits examined were cholesterol, triglycerides and HDL-cholesterol measured in a blood sample obtained immediately prior to the heparin injection. LPL activity at 10, 20 and 30 minutes PH was indicated as LPL10, LPL20 and LPL30.

<table>
<thead>
<tr>
<th></th>
<th>BMI</th>
<th>Chol</th>
<th>Tg</th>
<th>HDL-c</th>
<th>LPL10</th>
<th>LPL20</th>
<th>LPL30</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI</td>
<td>1.00</td>
<td>0.08</td>
<td>-0.11</td>
<td>0.09</td>
<td>0.07</td>
<td>0.21</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
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<td>0.01</td>
<td>0.06</td>
<td>-0.03</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p=0.003</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tg</td>
<td></td>
<td>1.00</td>
<td>-0.026</td>
<td>-0.16</td>
<td>-0.21</td>
<td>-0.06</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p=0.05</td>
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<td></td>
</tr>
<tr>
<td>HDL-c</td>
<td></td>
<td>1.00</td>
<td>-0.21</td>
<td>-0.05</td>
<td>-0.004</td>
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</tr>
<tr>
<td>LPL10</td>
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<td>1.00</td>
<td>0.77</td>
<td>0.54</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p&lt;0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPL20</td>
<td></td>
<td></td>
<td>1.00</td>
<td>0.74</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p&lt;0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>BMI</th>
<th>Chol</th>
<th>Tg</th>
<th>HDL-c</th>
<th>LPL10</th>
<th>LPL20</th>
<th>LPL30</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI</td>
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<td>0.23</td>
<td>-0.26</td>
<td>0.16</td>
<td>0.04</td>
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</tr>
<tr>
<td>Chol</td>
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<td>1.00</td>
<td>0.57</td>
<td>0.02</td>
<td>-0.17</td>
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</tr>
<tr>
<td>Tg</td>
<td></td>
<td>1.00</td>
<td>-0.36</td>
<td>-0.17</td>
<td>-0.27</td>
<td>-0.06</td>
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</tr>
<tr>
<td></td>
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<tr>
<td>HDL-c</td>
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<td>-0.28</td>
<td>0.01</td>
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</tr>
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<td></td>
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<td>LPL20</td>
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<td></td>
<td>1.00</td>
<td>0.73</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p&lt;0.001</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
LPL activity was not significantly correlated with any of the lipid traits on the day of the lipase assay although a weak inverse relationship was apparent with Tg at 10 and 20 minutes post-heparin (r = -0.16 and -0.21 respectively, NS).

4.3 Mutation detection by SSCP analysis in subjects with CHL and low LPL activity.

Screening of LPL exons 2-6 and 8-9 for mutations was undertaken using SSCP in a group of 25 hyperlipidaemic subjects (15 from the CX CHL group plus 10 from the Swedish YMI study) selected on the basis of low LPL activity. The CX CHL individuals had LPL activity at or below the 10th percentile of the healthy control group, while the Swedish YMI subjects were the ten with the lowest activity out of 100 patients and controls for whom LPL activity was available (five patients and five controls). Biometrical and biochemical data for these individuals is presented in Table 4.5. Mean LPL activity for the 15 CX-CHL individuals was about half of that measured in healthy controls (35 vs 63 mU/ml). The apoε4 allele was overrepresented in this subset compared to the whole CX-CHL group (0.30 vs 0.19, Table 4.5) but the difference was not significant.

A variant pattern for exon 2 (Fig.4.3a) was detected in three individuals, two from the combined hyperlipidaemic, low lipase group from Charing Cross Hospital (subjects AH and PR) and one from the low lipase patient group from the Swedish YMI study (subject 2053). No pattern differences in any other exons were detected for these three individuals. Direct sequencing of exon 2 revealed a G to A transition at position 280 (sequence numbering according to Wion et al., 1987)(Fig.4.3b), predicted to result in the substitution of asparagine for aspartic acid at amino acid residue 9.
Fig. 4.3a Single strand conformation polymorphism in a 237 bp PCR product containing exon 2 of the LPL gene. The arrow shows the presence of a doublet in two samples (Swedish YMI 2053 and CX-CHL PR).

Fig. 4.3b Direct sequencing of LPL exon 2 showing a G to A substitution at nucleotide 280. This creates the Asp to Asn substitution at position 9 of the polypeptide chain.
Table 4.5 Individual lipid, lipoprotein and post-heparin LPL activity (PHLA) in the 25 individuals selected for SSCP analysis. Subjects identified by initials were part of the CX-CHL study while those identified by a number originate from the Swedish YMI study (patient ID begin with a 2, control ID with a 4). PHLA was measured with different assays in different laboratories for the two groups and are not directly comparable.

<table>
<thead>
<tr>
<th>ID</th>
<th>Age (yrs)</th>
<th>Chol (mmol/l)</th>
<th>Tg (mmol/l)</th>
<th>HDL-chol (mmol/l)</th>
<th>ApoE genotype</th>
<th>PHLA (mU/ml)</th>
<th>CX YMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>M A</td>
<td>36</td>
<td>9.8</td>
<td>4.0</td>
<td>1.00</td>
<td>3/2</td>
<td>40</td>
<td>-</td>
</tr>
<tr>
<td>M B</td>
<td>47</td>
<td>7.8</td>
<td>3.1</td>
<td>1.19</td>
<td>3/3</td>
<td>42</td>
<td>-</td>
</tr>
<tr>
<td>E B</td>
<td>60</td>
<td>7.9</td>
<td>5.8</td>
<td>0.88</td>
<td>4/3</td>
<td>30</td>
<td>-</td>
</tr>
<tr>
<td>E C</td>
<td>47</td>
<td>8.2</td>
<td>3.0</td>
<td>1.05</td>
<td>3/3</td>
<td>31</td>
<td>-</td>
</tr>
<tr>
<td>D C</td>
<td>61</td>
<td>6.9</td>
<td>3.9</td>
<td>0.88</td>
<td>4/4</td>
<td>45</td>
<td>-</td>
</tr>
<tr>
<td>P C</td>
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<td>8.5</td>
<td>5.3</td>
<td>0.90</td>
<td>4/4</td>
<td>20</td>
<td>-</td>
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<tr>
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<td>3.6</td>
<td>0.70</td>
<td>3/2</td>
<td>33</td>
<td>-</td>
</tr>
<tr>
<td>M F</td>
<td>56</td>
<td>6.8</td>
<td>2.8</td>
<td>0.92</td>
<td>3/3</td>
<td>43</td>
<td>-</td>
</tr>
<tr>
<td>Az H</td>
<td>44</td>
<td>7.9</td>
<td>3.8</td>
<td>1.02</td>
<td>4/3</td>
<td>27</td>
<td>-</td>
</tr>
<tr>
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<td>5.1</td>
<td>0.89</td>
<td>3/3</td>
<td>32</td>
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<tr>
<td>G M</td>
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<td>8.9</td>
<td>5.1</td>
<td>0.43</td>
<td>3/2</td>
<td>40</td>
<td>-</td>
</tr>
<tr>
<td>Y M</td>
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<td>8.6</td>
<td>2.1</td>
<td>0.80</td>
<td>4/3</td>
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<td>-</td>
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<tr>
<td>P R</td>
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<td>3.0</td>
<td>1.08</td>
<td>3/2</td>
<td>35</td>
<td>-</td>
</tr>
<tr>
<td>S R</td>
<td>26</td>
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<td>3.4</td>
<td>1.25</td>
<td>3/2</td>
<td>30</td>
<td>-</td>
</tr>
<tr>
<td>C R</td>
<td>46</td>
<td>9.3</td>
<td>3.5</td>
<td>1.34</td>
<td>4/4</td>
<td>33</td>
<td>-</td>
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<tr>
<td>2001</td>
<td>46</td>
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<td>9.1</td>
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<td>2042</td>
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<td>1.06</td>
<td>4/3</td>
<td>-</td>
<td>46</td>
</tr>
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<td>2053</td>
<td>47</td>
<td>8.0</td>
<td>1.6</td>
<td>1.37</td>
<td>4/4</td>
<td>-</td>
<td>49</td>
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<tr>
<td>2062</td>
<td>48</td>
<td>6.7</td>
<td>3.3</td>
<td>0.95</td>
<td>4/2</td>
<td>-</td>
<td>41</td>
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<tr>
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<td>45</td>
<td>7.0</td>
<td>7.3</td>
<td>0.68</td>
<td>3/3</td>
<td>-</td>
<td>41</td>
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<tr>
<td>4001</td>
<td>53</td>
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<td>1.7</td>
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<td>3/2</td>
<td>-</td>
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<td>4008</td>
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<td>1.6</td>
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<td>3/3</td>
<td>-</td>
<td>48</td>
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<tr>
<td>4032</td>
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<td>2.8</td>
<td>0.92</td>
<td>4/3</td>
<td>-</td>
<td>35</td>
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<td>4052</td>
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<td>7.9</td>
<td>2.8</td>
<td>1.26</td>
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<td>-</td>
<td>45</td>
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<td>4.0</td>
<td>1.15</td>
<td>-</td>
<td>-</td>
<td>51</td>
</tr>
</tbody>
</table>

1 PHLA is expressed in mU/ml where 1mU is equal to 1nmol fatty acid released per min. Mean activity was 72 and 92mU/ml for Swedish YMI patients and controls respectively (Peacock et al., 1992) and 50mU/ml for the CX-CHL subjects (Seed et al., 1994).
The presence of the G-A transition at position 280 was predicted to abolish a TaqI restriction site, resulting in the production of a larger 58bp variant fragment, compared to the wild-type 52bp fragment, upon digestion of the exon 2 PCR product with this enzyme (Fig. 4.4a).

Interestingly, this substitution had been identified previously in a Caucasian subject with LPL deficiency who was homozygous for two mutations, D9N and Y262H (Lohse et al., 1991). In vitro mutagenesis experiments showed that the Y262H mutation resulted in the production of an inactive enzyme and it was concluded that this defect was responsible for the phenotype. A small decrease in activity was noted with the D9N mutant plasmid in vitro (S. Fojo, personal communication to Prof. Humphries) but this was not investigated further. The Y262H mutation was not present in the three carriers of the D9N substitution from this study (see section 4.5). However, the presence of the D9N substitution in the proband studied by Lohse et al. (1991) suggested that this might be a common variant.

One other variant was detected in exon 4 in from two individuals and was not investigated further as the gel pattern was very similar to that seen for the silent G to A substitution at position 609 (section 3.3). It was assumed to represent the same variant based on the fact that all the LPL mutations detected in the current study exhibited distinct SSCP patterns. No variation was apparent in exons 3, 5, 6, 8 and 9. This was unlikely to be due to lack of sensitivity of the method as samples with known mutations were included as positive controls in the gels and were always detected.

4.4 Development of a rapid screening strategy for LPL variants.

To estimate the frequency of relatively rare polymorphic variants (<5%) in large
groups of hyperlipidaemic and healthy individuals, a rapid screening strategy was
developed based on the pooling of DNA samples prior to amplification. DNA samples
were pooled together, amplified and digested with the appropriate enzyme. Individual
samples from positive pools (i.e where at least one sample showed the presence of the
rare fragment) were then re-amplified and re-digested for final identification of carriers.
This method has the sensitivity to identify a carrier individual from a pool of ten samples
(1 mutant allele out of 20) for the D9N substitution (Fig.4.4b) and from a pool of twenty
samples (1 mutant allele out of 40) for the G188E substitution (Fig.4.5).

![Diagram](image.png)

**Fig.4.4** Schematic representation of LPL exon 2 and *Taq*I digest of exon 2PCR product for
the detection of carriers of the Asn9 variant.
a) location of the variant (dotted arrow) and constant (filled arrow) *Taq*I sites in exon2 and 52,
58 and 179 bp fragments following *Taq*I digest and separation on 10% polyacrylamide gel.
Horizontal arrows indicate the position of the primers used for amplification.
b) detection of the variant 58bp TaqI fragment in pooled DNA samples - TaqI digest after amplification of DNA samples containing Asn9 carrier DNA mixed with increasing amounts (vol:vol) of Asp9 DNA. N = Asp9 homozygote; C = Asn9 carrier individual; 1:1, 1:4, 1:9 = Asn9 and Asp9 DNA mixed 1:1, 1:4 and 1:9 respectively.

Fig. 4.5 Detection of the G188E substitution in pooled DNA samples. DNA from a carrier individual was mixed with normal DNA 1:1, 1:4, 1:9 and 1:19, amplified and digested with Avall. C = G188E carrier individual.
To determine the optimal number of samples per pool, the total number of PCR reactions (pooled DNA PCRs plus individual PCRs for samples from positive pools) to be carried out was calculated as a function of the number of samples per pool and the frequency of the variant (Table 4.6). The following equation was used:

$$\text{PCR}_T = \left(\frac{n}{4} - p\right) + (p \times f \times n)$$

where $\text{PCR}_T$ equals the total number of PCRs to be carried out, $n$ is the number of samples in the study, $p$ represents the pool size and $f$ the estimated carrier frequency for the variant under study. This formula assumes that no pools will contain more than one sample carrying the mutation (a reasonable assumption with a low frequency polymorphism) and that the amplification failure rate is negligible. From our initial estimates of the frequency of the Asn9 variant, based on results from twenty pools of five samples (two positives), pools containing five or six samples were in the optimal range.

<table>
<thead>
<tr>
<th>Number of samples in pool</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
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<tbody>
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<td>990</td>
<td>900</td>
<td>861</td>
<td>849</td>
<td>855</td>
</tr>
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<tr>
<td>5</td>
<td>1350</td>
<td>1350</td>
<td>1401</td>
<td>1479</td>
<td>1575</td>
</tr>
<tr>
<td>6</td>
<td>1470</td>
<td>1500</td>
<td>1581</td>
<td>1689</td>
<td>1815</td>
</tr>
</tbody>
</table>

Table 4.6 Total number of PCRs needed for the identification of carriers using a DNA pooling strategy (based on 3000-sample study).
4.5 Population screening for the Asn9 variant (D9N) I.

Patients from England (CX CHL, section 2.2.2.5), two groups from Sweden (Swedish HTG, 2.2.2.2; Swedish YMI, section 2.2.2.1) and one from Holland (Dutch CHL, 2.2.2.3), selected on the basis of being either hyperlipidaemic or having suffered a myocardial infarction before the age of 45, were screened as described in section 4.4. Each patient group had a comparison group of general population controls (Camberley general population controls, section 2.2.2.6; Swedish HTG controls, section 2.2.2.2; Swedish YMI controls, section 2.2.2.1; Dutch control group, section 2.2.2.4) and the lipid levels and LPL activity (where available) of these groups are summarized in Table 4.7. The two Dutch samples were screened by Paul W.A. Reymer at the Academic Medical Centre, Amsterdam. A total of 37 Asn9 carriers were identified, including the three that had been identified by SSCP analysis. As shown in Table 4.8, in the four control groups the frequency of carriers ranged from 1.6-2.5% (average 2.5% and 95% CI 1.1-3.2%) with no statistically significant evidence for heterogeneity in frequency between the groups \((X^2 = 0.31, p = 0.9)\). One individual in the English general population (Camberley) group was homozygous for the allele coding for Asn9. Two additional carriers were identified in the CX-CHL group in addition to the two detected by SSCP. In each of the patient groups, the frequency of carriers was roughly twice as high as compared to their matched control group. The frequency was increased four-fold (9.8 vs 2.5%) when the CX-CHL sample was compared to the English general population controls. The two study groups from Sweden gave similar frequency differences, with prevalence increased almost two-fold in YMI patients compared to their healthy counterparts (4.0 vs 2.2%) and more than three-fold in hypertriglyceridaemic subjects compared to normotriglyceridaemic individuals (5.0 vs 1.6%).
Table 4.7 General description of Uk, Dutch and Swedish samples screened for the D9N substitution.

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>% Male</th>
<th>Age (S.E.M.)</th>
<th>BMI (S.E.M.)</th>
<th>Chol (mmol/l) (S.E.M.)</th>
<th>Tg (mmol/l) (S.E.M.)</th>
<th>LPL act* (S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>General practice South East England</td>
<td>360</td>
<td>100</td>
<td>54.6 (0.16)</td>
<td>26.6 (0.17)</td>
<td>5.80 (0.05)</td>
<td>1.97 (0.07)</td>
<td>-</td>
</tr>
<tr>
<td>General practice Scotland</td>
<td>413</td>
<td>100</td>
<td>56.2 (0.18)</td>
<td>26.2 (0.18)</td>
<td>5.76 (0.05)</td>
<td>1.96 (0.09)</td>
<td>-</td>
</tr>
<tr>
<td>Swedish YMI study - controls</td>
<td>93</td>
<td>100</td>
<td>40.5 (0.40)</td>
<td>24.6 (0.26)</td>
<td>6.09 (0.12)</td>
<td>1.48 (0.14)</td>
<td>92.1 (3.6)</td>
</tr>
<tr>
<td>Swedish normolipidaemic subjects</td>
<td>61</td>
<td>100</td>
<td>45.4 (0.40)</td>
<td>24.7 (0.35)</td>
<td>5.63 (0.10)</td>
<td>1.22 (0.05)</td>
<td>286.1 (11.1)</td>
</tr>
<tr>
<td>Dutch normolipidaemic subjects</td>
<td>190</td>
<td>100</td>
<td>46.0 (0.65)</td>
<td>25.0 (0.22)</td>
<td>5.55 (0.06)</td>
<td>1.26 (0.03)</td>
<td>-</td>
</tr>
<tr>
<td>Combined hyperlipidaemcs - Charing Cross Hosp.</td>
<td>41</td>
<td>71</td>
<td>51.2 (1.59)</td>
<td>29.2 (0.62)</td>
<td>8.05 (0.27)</td>
<td>4.15 (0.21)</td>
<td>49.7 (2.7)</td>
</tr>
<tr>
<td>Swedish YMI study - patients</td>
<td>100</td>
<td>76</td>
<td>40.4 (0.38)</td>
<td>26.4 (0.35)</td>
<td>7.24 (0.14)</td>
<td>2.61 (0.19)</td>
<td>72.3 (4.1)</td>
</tr>
<tr>
<td>Swedish hypertriglyceridaemic subjects</td>
<td>60</td>
<td>100</td>
<td>44.9 (0.47)</td>
<td>27.8 (0.47)</td>
<td>6.80 (0.15)</td>
<td>4.54 (0.20)</td>
<td>277.7 (10.1)</td>
</tr>
<tr>
<td>Dutch combined hyperlipidaemics</td>
<td>240</td>
<td>62</td>
<td>47.2 (0.85)</td>
<td>26.2 (0.18)</td>
<td>8.14 (0.08)</td>
<td>2.96 (0.09)</td>
<td>-</td>
</tr>
</tbody>
</table>

* LPL activity in mU/ml, which corresponds to 1 nmol of fatty acid released /min/ml.
† From Seed et al. (1994), LPL activity in healthy controls = 64.3 ± 3.1 (mU/ml).
Table 4.8 Frequency of Asn9 carriers in patients with hyperlipidaemia or premature myocardial infarction and controls of Dutch, English and Swedish origin, and relative risk of being a carrier.

<table>
<thead>
<tr>
<th></th>
<th>Asn9 : Asp9 *</th>
<th>% of Carriers</th>
<th>Companion Control Group</th>
<th>Asn9 : Asp9 ‡</th>
<th>% of Carriers</th>
<th>Relative Risk §</th>
</tr>
</thead>
<tbody>
<tr>
<td>CX - CHL</td>
<td>4 : 37 ‡ ‡</td>
<td>9.8</td>
<td>9 : 351 †</td>
<td>2.5</td>
<td>4.22</td>
<td></td>
</tr>
<tr>
<td>Swedish YMI</td>
<td>4 : 96</td>
<td>4.0</td>
<td>2 : 91</td>
<td>2.2</td>
<td>1.94</td>
<td></td>
</tr>
<tr>
<td>Swedish HTG</td>
<td>3 : 57</td>
<td>5.0</td>
<td>1 : 60</td>
<td>1.6</td>
<td>3.16</td>
<td></td>
</tr>
<tr>
<td>Dutch CHL</td>
<td>11 : 229</td>
<td>4.9</td>
<td>3 : 187</td>
<td>1.6</td>
<td>2.99</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 : 689</td>
<td>2.1**</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Percentage of carriers in patients do not differ between studies 4/37 v 4/94 v 3/57 v 11/229 p = 0.46.
† Percentage of carriers in controls do not differ between studies 9/351 v 2/91 v 1/60 v 3/87 p = 0.89.
§ Test for heterogeneity of RR X² 0.53 p=0.89
† ‡ One individual homozygous
* Number of Asp9 homozygous individuals (wild-type allele) compared to number of carrier individuals for the Asn9 variant
** Weighted relative risk
In the Dutch CHL group the frequency was three-fold higher compared to the Dutch controls (4.9 vs 1.6%). There was no statistically significant difference (p=0.91) between studies in the relative risk of being a carrier in patients compared with controls.

An additional 413 healthy men from a general practice in St. Andrews, Scotland were screened by. This was to ensure that a sufficient number of carrier individuals would be identified for a meaningful assessment of the impact of the variant in general population controls (chapter 6). Their characteristics were similar to the English control group and 18 carriers (including one homozygote) were identified. The frequency of the carriers in this group was thus 4.4% which is higher (but not significantly $X^2 = 1.74$ by gene counting, $p > 0.2$) than the frequency in the English group.

4.5.1 Screening for the Y262H mutation.

In view of the report from Lohse et al. (1991) where the D9N and Y262H mutations coexisted on the same chromosome in a type I subject, Asn9 carriers were screened for the presence of the Y262H mutation. This was achieved by amplifying exon 6 of LPL with the primers described in Table 2.1 and digesting the PCR product with the enzyme StuI. The T to C transition at position 1039 would be expected to abolish the unique StuI site in this fragment, leaving the 295bp PCR product unchanged, while digestion of the normal allele should yield 213 and 82bp fragments. Thirty of 37 Asn9 carriers from the initial screening were tested and all showed the complete loss of the 295bp PCR product, indicating that none of them had the Y262H substitution (Fig. 4.6).
4.6 Population screening for the Asn9 variant II - the ECTIM study.

A total of 1430 subjects from the ECTIM study (section 2.2.2.7) were screened for the LPL-Asn9 mutation as described in section 4.4, of which 642 had suffered a myocardial infarction (cases) and 788 were recruited as general population controls. In all, 44 carriers were identified, 22 in each of the case and control groups (Table 4.9). Data from the three French centres (Lille, Strasbourg and Toulouse) were combined and compared to the Belfast sub-groups. The overall LPL-Asn9 carrier frequency in the controls was 2.8% (95% CI 1.7-3.9) and this was similar in the Belfast and French centres (2.0% and 3.1% respectively). The frequency of carriers was slightly higher in
the combined cases from both countries at 3.4% (95% CI 2.0-4.8) (Belfast, 2.5% and France, 3.8%), but this difference was not statistically significant.

Table 4.9 Frequency of D9N substitution carriers in the ECTIM study sample by centre and case/control status.

<table>
<thead>
<tr>
<th>Centre</th>
<th>Cases (frequency %)</th>
<th>Controls (frequency %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Northern Ireland - Belfast</td>
<td>5/202 (2.5%)</td>
<td>4/202 (2.0%)</td>
</tr>
<tr>
<td>France Lille</td>
<td>3/68 (4.4%) All: 17/440 (3.8%)</td>
<td>4/155 (2.5%) All: 18/586 (3.1%)</td>
</tr>
<tr>
<td>Strasbourg</td>
<td>7/221 (3.2%)</td>
<td>7/211 (3.3%)</td>
</tr>
<tr>
<td>Toulouse</td>
<td>7/151 (4.6%)</td>
<td>7/220 (3.2%)</td>
</tr>
</tbody>
</table>

4.7 Haplotype analysis in carriers of the Asn9 variant.

Genotypes for the *PvuII* (intron 6) and *HindIII* (intron 8) polymorphic sites were determined for 34/37 carriers of the Asn9 variant identified from the screening described in section 4.5 (all groups excluding the Scottish sample). Using 28 individuals for whom unequivocal haplotypes could be determined, two major Asn9 carrier haplotypes differing at the *PvuII* site were identified (Table 4.10a). Seven individuals were P-P/-H+H+ homozygotes (non-cutting for *PvuII*, cutting for *HindIII*) and thus carried the Asn9 variant on a P- H+ haplotype, while 4 individuals were found to be homozygous for the P+H+ haplotype. In a further 12 individuals who were heterozygous for the
*PvuII* restriction site, either haplotype could carry the Asn9 variant. Of the remaining five subjects, at least two appeared to have a third, rarer carrier haplotype (P- H-). For one of these individuals, a conservative assignment to a P- H- carrier haplotype was made on the basis that the accompanying haplotype P+ H- had not been observed in any other carriers.

Genotypes for the *PvuII*, *HindIII* and Serine447/Stop polymorphisms were available for 43/44 carriers identified from the ECTIM study. Thirteen individuals showed homozygosity at all three sites, allowing me to determine with certainty the Asn9 carrier haplotype. Two common carrier haplotypes differing at the *PvuII* site were observed, P+ H+ Ser and P- H+ Ser (Table 4.10b). In a further 27 carriers where both haplotypes could be identified unambiguously, the genotypes were compatible with the mutation being on one of these two haplotypes, which are likely to correspond to the P- H+ and P+ H+ haplotypes seen in the first screening study (section 4.5). The rarer P- H- carrier haplotype observed in two individuals from the Camberley and Dutch subgroups was only present in combination with a common haplotype (P- H+ Ser) in eight French ECTIM carriers and could not be ascertained unequivocally. In these ECTIM carriers, the P- H- haplotype could be further divided into two classes based on the presence of Ser (2 subjects) or Stop accompanying P- H- (5 subjects)(Table 4.9b). This information was unavailable for the unequivocal P- H- carrier (Camberley subject) but the individual who was assigned the P- H- haplotype (Dutch subject) was homozygous for the Ser allele. Thus, while it is still possible that the Asn9 variant is carried on the P- H- Stop haplotype, it appears more likely to reside on a P- H- Ser chromosome.
Table 4.10 Genotypes for PvuII (P) and HindIII (H) and predicted haplotypes carrying the Asn9 variant.

a) UK, Dutch and Swedish studies

<table>
<thead>
<tr>
<th>Genotypes (n)</th>
<th>Possible Haplotypes</th>
<th>Asn9 Carrier Haplotype (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P -/- H -/-(1)</td>
<td>P - H - P - H -/ P + H -</td>
<td>P - H - (2)</td>
</tr>
<tr>
<td>P +/- H -/-(1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P -/- H ++/ (7)</td>
<td>P - H +</td>
<td>P - H + (7)</td>
</tr>
<tr>
<td>P +/- H ++/ (4)</td>
<td>P + H +</td>
<td>P + H + (4)</td>
</tr>
<tr>
<td>P +/- H ++/ (12)</td>
<td>P - H +/ P + H +</td>
<td>P - H + or P + H + (12)</td>
</tr>
<tr>
<td>P -/- H +/- (3)</td>
<td>P - H +/ P - H -</td>
<td>P - H + or P - H - (3)</td>
</tr>
</tbody>
</table>

b) ECTIM study

<table>
<thead>
<tr>
<th>Genotypes PvuII HindIII Ser-Stop</th>
<th>No</th>
<th>Unabiguous or possible LPL-Asn9 Haplotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>- / - +/- +/+ +/+</td>
<td>6</td>
<td>P- H+ Ser</td>
</tr>
<tr>
<td>+/- +/+ +/+</td>
<td>7</td>
<td>P+ H+ Ser</td>
</tr>
<tr>
<td>+/- +/+ +/+</td>
<td>20</td>
<td>P- H+ Ser or P+ H+ Ser</td>
</tr>
<tr>
<td>- / - +/- +/- +/-</td>
<td>5</td>
<td>P- H+ Ser or P- H- Stop*</td>
</tr>
</tbody>
</table>

* Likely haplotype since Stop in strong allelic association with H- (Peacock et al., 1992).

n = number of individuals
4.7.1 Nature of the base change for the three carrier haplotypes.

The G to A transition at position 280 has occurred at a potentially mutable CpG dinucleotide (shown in bold typescript) with the likely mechanism being the deamination of the cytosine residue on the complementary strand and replacement by thymine:

```
5'  ATC [GAC] → ATC [AAC]
    TAG CTG    TAG TTG '5
   Ile 8  Asp9    Ile8  Asn9
```

The mutation was originally characterised in two individuals by sequencing, both carrying the P+ H+ haplotype. Thereafter, its presence was detected by digesting the exon 2 PCR product with TaqI (recognition sequence TCGA). The finding that the mutation, as detected by restriction digest, was carried on three distinct haplotypes suggested that it might have occurred more than once. It is obvious from the sequence that the cytosine residue in the coding (top) strand could also be deaminated, resulting in a silent substitution:

```
5'  ATC [GAC] → ATT [AAC]
    TAG CTG    TAG TTG '5
   Ile 8  Asp9    Ile8  Asn9
```

Therefore, DNA from five individuals with unequivocal carrier haplotypes was amplified and sequenced for this area (two each with the P+ H+ and P-H+ haplotypes and one with the P-H- haplotype). There was no evidence for heterogeneity at this site and all the samples showed the same G to A base change that had been identified originally. A representative sequencing from each haplotype class is shown in Fig.4.7.
Fig. 4.7 Identification of the D9N substitution in carriers with different HindIII genotypes. All three carriers as well as the control were from the UK normolipidaemic sample. The exon 2 3’ primer was used as the sequencing oligo and the autorad was reversed to facilitate the reading.
4.8 Population screening for the Gly188 to Glu substitution.

The rapid strategy described in section 4.4 was utilised to determine the frequency of the G188E substitution in the general population as well as in MI sufferers. This mutation was the only common mutation present in the type I cohort studied in chapter 3 where it accounted for nearly half of the mutant alleles (12/26) in white Caucasians. Therefore, it represented a good candidate to test the hypothesis that mutations at the LPL gene locus may be involved in causing FCHL. Screening of all samples from the Swedish YMI and ECTIM cases/control studies as well as from the Camberley controls group led to the identification of only three carriers. Two of these carriers were healthy men from the Camberley general practice and one was a female Swedish YMI patient. No carriers were detected in the ECTIM sample.

4.9 Population screening for Asn291 to Ser substitution.

The A to G transition at nt1127 in exon 6 causing the substitution of Asn by Ser at aa291 identified in a patient with type I HLP (N291S, chapter 3)(Mailly et al., 1992) was subsequently detected by Dr. Rachel Peacock in 12 Swedish YMI study subjects (6 controls and 6 patients). Several of these individuals had levels of LPL activity close to or well above the group mean (R. Peacock, personal communication, Ph.D. thesis, 1994). While this work was in progress, Ma and co-workers (1993) showed that the same mutation caused a reduction in LPL activity and mass in vitro and was present in 2/15 patients with type III HLP but not in 150 control individuals. They proposed that a mutation in the LPL gene might well be the second-hit necessary for the development of type III HLP.

To reconcile these conflicting results, the frequency of the N291S mutation was
ascertained in the St. Andrews (screened by Dr. Rachel Fisher and Le Ahn Luong in our laboratory) and Camberley general population controls, as well as in the CX-CHL group. Samples were screened using PCR amplification of exon 6 and detection with ASOs as described in section 2.10.1. The three possible patterns, Asn/Asn, Asn/Ser and Ser/Ser corresponding to the presence of A, A/G or G respectively at position 1127 are represented in Fig. 4.8. The mutant Ser allele was present in healthy individuals from the UK. Ten subjects out of 360 (2.8%) were found to be carriers for this substitution in the Camberley group, while the corresponding figure for the St. Andrews sample was 14 carriers out of 413 (3.4%), for a combined frequency of 3.1%. No homozygotes were found. In addition, two subjects from the CX-CHL group were found to be carriers (2/41, 4.9%). These individuals did not have low PHLA and had not been included in the SSCP analysis.

Fig. 4.8 Detection of the N291S substitution by ASOs. Amplified PCR product (exon 6, 295bp) was separated on agarose and transferred by double blotting to Hybond-N membranes. One filter was hybridised with each of the two allele-specific oligoprobes and the two resulting autoradiographs aligned. C: positive control, 560bp amplified fragment of plasmid LPL291 prepared by site-directed mutagenesis to express the Ser allele (see methods for further details). Lanes 1, 4: heterozygous carriers with both Asn/Ser alleles. Lanes 2, 3 and 5: homozygous individuals for the 'normal' Asn allele.
4.10 Summary and brief discussion.

A major objective of this thesis was to investigate whether the reported low LPL activity in some patients with FCHL (Babirak et al., 1992) was due to mutations in the structural gene coding for LPL such as those causing type I HLP in individuals homozygous for the defects. While a definite diagnosis of FCHL can only be established by extensive family studies, the forty-one CHL subjects selected for this study had a high likelihood of having the disorder. More than a third of the individuals had low PHLA (10 minutes), confirming the findings of Babirak et al. (1992).

The lack of relationship between LPL and Tg levels was not altogether surprising as the sample was small. Moreover, this correlation is usually weak in individuals with raised Tg (Taskinen, 1987), possibly because of the frequent occurrence of obesity which increases the mass of LPL producing tissue. The increasingly stronger correlation between LPL activity and BMI with time PH probably reflects the fact that LPL activity originating from the adipose tissue pool becomes proportionately more important with time. This is in agreement with classical studies from Fielding and Fielding (1976) who showed that LPL activity from adipose tissue is released more slowly than from muscles.

SSCP analysis in samples from individuals with CHL and/or low LPL activity allowed the identification of a variant in the LPL gene which alters aspartic acid at position 9 to asparagine. With the exception of the silent substitution in the Glu118 codon, none of the mutations which had been identified in the type I HLP group were present. This results suggest that mutations in the LPL structural gene which cause type I HLP are unlikely to be major contributors for the decreased LPL activity observed in these CHL patients. It cannot be ruled out that mutations common to the two phenotypes may have been missed for the same technical reasons (primer design and
SSCP efficiency) as outlined in section 3.8.2 and exon 7 was not screened in the CHL/low LPL group. In addition, the promoter region of the LPL gene was not investigated in this study. Several key regulatory elements, including consensus sequences for transcription factors, have recently been identified upstream of the transcription start site in the LPL gene (Previato et al., 1991; Enerback et al., 1992). Mutations in these regulatory sites might prevent or diminish the normal response to metabolic signals.

The D9N variant was found to be relatively common. The frequency of the carriers of the mutation in the healthy population in the South of England, Holland and Sweden ranged between 1.6-2.5%. The presence of the mutation in individuals from four different countries in Europe suggests that it is likely to be present in other caucasian groups and further studies are needed for a more accurate estimate of the frequency. The frequency of carriers of the mutation is consistently twice as high in Swedish MI patients and Swedish, English and Dutch hyperlipidaemic cases than in the respective general population control groups. Although it is not valid to combine data from the different patient groups since the selection criteria are different, the consistently higher frequency in the patient groups strongly suggests that the Asn9 variant may contribute to the development of hyperlipidaemia. Two alternative mechanisms can be envisaged for this: 1) the Asn9 allele may directly affect LPL function by decreasing plasma LPL activity or mass which may lead in turn to increased lipid levels. 2) the Asn9 allele is a marker in linkage disequilibrium with a functional mutation in or close to the LPL gene.

Three unequivocal haplotypes (of which two are common) have been identified in Asn9 heterozygotes and all three carried the same base substitution, suggesting that
the mutation may have occurred more than once. This is a likely possibility considering that a mutable CpG dinucleotide is involved (Cooper and Youssoufian, 1988) and recurrent mutations have been observed previously in the LPL gene (Henderson et al., 1991). The presence of the Asn9 mutation on multiple haplotypes due to separate mutational events would strengthen the hypothesis that the mutation itself is functional. The reason is that the repeated, independent and detectable association of a neutral Asn9 allele with a second (functional) mutation causing the increasing frequency in the hyperlipidaemic groups is highly unlikely. Alternatively, it cannot be ruled out that recombination events upstream of the PvuII or HindIII polymorphic sites may have generated the different haplotypes. Such events would preserve the phase between the Asn9 variant and a functional mutation located in the promoter region for example. Studies using highly polymorphic markers (dinucleotide repeats)(Narcisi et al., 1993) might indicate whether such an event has occurred. Other studies, assessing the biological significance of the Asn9 mutation, are also needed to resolve the issue. Such experiments, combining in vitro mutagenesis with transient expression of the mutant protein in a mammalian cell line, will be presented in the next chapter.

The N291S mutation was common in the two samples of healthy men from the UK with a combined frequency of 3.1%. This is lower than the frequency determined in the YMI Swedish study (6.6%, Rachel Peacock, Ph.D. thesis). In contrast, Ma et al. (1993c) did not detect the variant in their 150 normolipidaemic individuals, where four or five carriers might have been expected based on the UK frequency. This is an unlikely event by chance alone (p < 0.01) and it may reflect underlying population differences in the frequency of this variant. An alternative explanation is that carriers of
the N291S variant may have been selected out of the control sample used by Ma et al. by exclusion of individuals with lipid levels above the 95th percentile for age and sex. As will be seen in chapter 6, the Ser allele is associated with moderate increases in lipid levels in the UK samples.
5. STRUCTURAL AND FUNCTIONAL IMPACT OF LPL GENE MUTATIONS: EXPRESSION STUDIES AND SECONDARY STRUCTURE MODELLING.

In this chapter, the construction by *in vitro* mutagenesis of four LPL mutants are described and their functional significance tested in a transient expression system. Two of these were rare mutations identified in subjects with type I HLP, Thr158 and Arg193 (chapter 3) and two were common LPL variants identified in normolipidaemic as well as hyperlipidaemic individuals, Asn9 and Ser291 (chapter 4). The latter was also observed in a subject with hyperchylomicronaemia (chapter 3). The synthetic LPL mutant vectors were transfected into CosB cells, and the cells were allowed to express and secrete the mutant proteins into the media. After three days, the culture media was collected and tested for the effect of the mutations on LPL activity or secreted mass. LPL activity and mass was measured as described in section 2.3.1.5. The preparation of the recombinant vectors, all transfection experiments for the Thr158, Arg193 and Ser291 mutants (including LPL activity assays), as well as the first transfection experiment for the Asn9 mutation were carried out in Dr. John Kastelein’s laboratory, AMC, Amsterdam, during two 2-month visits. The invaluable assistance of Dr. Taco Bruin in particular is gratefully acknowledged.

In addition, all the mutations identified in chapter 3 and 4 were examined using the DNASTar protein modelling software with regards to their impact on secondary structure. The structural predictions for each mutations were related to available information on LPL activity measured *in vitro* or *in vivo* to determine if these measures of structural change had any predictive value.
5.1 Synthesis of mutant LPL sequences.

A single-stranded LPL cDNA template for mutagenesis was produced by infecting JM109 bacteria previously transformed with recombinant pAlter-LPL-WT (normal LPL sequence) with bacteriophage R408 (section 2.4.3). A 2-μl aliquot of the ssDNA preparation was run on an agarose gel alongside a similar preparation provided by Dr. Taco Bruin (AMC, Amsterdam) and which had already been used successfully for the mutagenesis procedure (Fig.5.1). There was no apparent difference between the new preparation and the control preparation.

Following the mutagenesis procedure and transformation into JM109 bacteria as outlined in section 2.14, 6-8 ampicillin-resistant colonies were picked (and streaked on a fresh plate) for each of the mutants and used to inoculate 2ml cultures. Plasmid DNA was extracted by the mini prep method (section 2.4.2) and small aliquots (2-5% total preparation volume) were first digested with the restriction enzyme PvuII to ensure that the LPL insert was present. Three fragments of 3.05, 2.25 and 1.7kb were observed on the agarose gel, confirming the presence of the insert (Fig.5.2b). These corresponded to nt269-2523 and 2523-5570 of the vector sequence and nt725-2390 of the insert sequence. Two smaller fragments of 581 and 403bp were not visible due to RNA in the plasmid preparation. For the LPL158 recombinant plasmid, the mutagenesis process abolished the PvuII site at position 725, yielding a larger 2.1kb fragment instead of the 1.7kb fragment observed previously (Fig.5.2c).
Fig. 5.1 Preparation of single-stranded DNA (ssDNA) from pAlter-LPL. JM109 cells transformed with pAlter-LPL were infected with bacteriophage R408, grown overnight and ssDNA isolated as described in section 2.4. An aliquot was run on a 1% agarose gel. Lane 1: HindIII marker; lane 2: new ssDNA preparation; lane 3: control ssDNA preparation (kind gift from Dr. Taco Bruin).
Fig. 5.2 Restriction digest of pAlter-LPL recombinant vector with PvuII following the mutagenesis procedure. Samples were digested for 2 hours with 5 units of enzyme, run on 1% agarose gels and visualised with short wave uv light.

a) Linear map of pAlter-LPL recombinant vector.

b) Expected band pattern following digest of mutagenised pAlter-LPL9, 193 or 291 plasmid preparations, confirming the presence of the LPL insert. Lanes 1-4: control and mutated plasmid preps; lane 5: lambda HindIII marker.

c) Expected band pattern of digested pAlter-LPL158 after the mutagenesis procedure. Lane 1: λ HindIII marker; lanes 2-5: pAlter-158 plasmid; lanes 6-7: pAlter-LPLWT.
Although the mutagenesis system used has a high efficiency, not all the clones are expected to have the mutation. Therefore, the remainder of the plasmid isolated above was used to sequence the critical region containing the mutation using appropriate primers and clones bearing the expected nucleotide substitutions were identified. These were G280 to A (Asp9-Asn), G727 to A (Ala158-Thr), A832 to C (Ser193-Arg) and A1127 to G (Asn291-Ser) (Fig. 5.3). One positive colony for each of the four mutants was selected at random and used to inoculate a 20ml overnight culture. Plasmid DNA was isolated by the midi prep method (section 2.4.2), the LPL insert was cut out (XbaI/PstI digest) and ligated into the expression vector, pcDNA. The recombinant vectors, pcDNA-LPL9, -LPL158, -LPL193 and -LPL291 were amplified by transformation of mc1061/p3 bacteria. Ampicillin and tetracyclin-resistant colonies were picked and grown further in broth. Plasmid DNA was again isolated and the presence of the LPL insert verified by digesting the preparation with XbaI/PstI (Fig. 5.4). After further amplification and purification followed by re-sequencing of the critical region for each mutant, the vectors were ready for use in transfection of CosB cells.
Fig. 5.3 Sequencing of critical regions of LPL cDNA following the mutagenesis procedure. A: pAlter recombinant plasmid LPL193 isolated from JM109 bacteria; clones 1, 4 and 5 have A to C substitution. B: pAlter recombinant plasmid LPL291 isolated from JM109 bacteria; clone 1 contains A to G mutation. C: pcDNAI recombinant plasmid LPL9 isolated from mc1061 bacteria with G to A substitution. D: pcDNAI recombinant plasmid LPL158 isolated from mc1061 bacteria, showing G to A substitution.
Fig. 5.4 Restriction digest of pcDNA recombinant vector with XbaI and PstI. Samples were digested for 2 hours with 5 units of enzyme, run on 1% agarose gels and visualised with short wave uv light. Lane 1: kb ladder; lane 2: LPL-WT control (gift from Dr. Taco Bruin); lanes 3-4 and 6-10: LPL-193 and -291 plasmid preparations from ampicillin and tetracyclin-resistant colonies. Partially digested samples (lanes 6 and 10) show the 6.4kb fragment (vector + insert).

5.2 Expression studies for the Thr158 and Arg193 LPL mutations.

Recombinant plasmids pcDNA-LPL158 and -LPL193 were introduced into Cos-B cells in separate experiments, with pcDNA-LPL WT transfected in parallel dishes as a control. In the first experiment, plasmid pcDNA-LPL158 and -LPL WT were transfected into two 60mm dishes each and an additional dish was mock-transfected. After three days, heparin (20U/ml medium) was added to one dish for each of the constructs and to
the mock transfected dish. The cell medium (5mls) was harvested 2 hrs later and and a 0.1ml aliquot assayed for lipolytic activity against a phosphatidylcholine-stabilised triolein emulsion as described in the methods section (section 2.3.1.5). The rest of the medium was snap frozen and stored at -70°C until the LPL mass assay was carried out. Cells that had been transfected with the LPL WT construct expressed a 6-fold increase in LPL activity compared to the mock transfected cells (43.49 vs 6.95 mU/ml) without addition of heparin and a 13-fold increase (93.69 vs 6.95 mU/ml) with heparin. In contrast, cells that had been transfected with the LPL158 construct showed only a marginal increase in activity in their media (8.71 vs 6.95 mU/ml). However, fresh medium had an apparent activity similar to that found in medium from mock transfected cells. (Table 5.1, Blank vs Mock: 6.62 vs 6.95 mU/ml). This was unlikely to be due to inherent activity of the culture medium as it was mixed only briefly with the substrate and the free fatty acids extracted immediately. Rather, it is thought to be due to impurities in the commercial triolein preparation (Nilsson-Ehle and Schotz, 1976). Therefore, LPL activity levels associated with mutant and wild-type constructs were compared after subtracting the activity measured in the medium collected from mock transfected dishes. The corrected values suggested that LPL activity was released in the medium of cells transfected with the LPL158 plasmid following heparin treatment. The amount of activity was much lower than that measured in cells transfected with the normal LPL sequence (Table 5.1, 1.76 vs 86.74 mU/ml or 2% of WT). LPL activity was negligible in the medium of cells not treated with heparin.
Table 5.1 *In vitro* expression of Thr158 variant. LPL activity in COS cell medium 3 days post-transfection with pcDNA-LPL158 (2μg/ml). LPL activity was measured with or without addition of heparin (20U/ml) 2 hrs prior to collection. The activity associated with mock-transfected cells has been subtracted from activity values obtained from cell media for both constructs with and without heparin and is shown along with the value obtained from a blank assay (media only) for comparative purposes.

<table>
<thead>
<tr>
<th>Construct/Treatment ( +/- heparin)</th>
<th>LPL activity (mU/ml)</th>
<th>% control*</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPL158 / - hep</td>
<td>0.10</td>
<td>Neg.</td>
</tr>
<tr>
<td>LPL158 / + hep</td>
<td>1.76</td>
<td>2.02</td>
</tr>
<tr>
<td>LPL WT / - hep</td>
<td>36.54</td>
<td>-</td>
</tr>
<tr>
<td>LPL WT / + hep</td>
<td>86.74</td>
<td>-</td>
</tr>
<tr>
<td>Blank</td>
<td>6.62</td>
<td>-</td>
</tr>
<tr>
<td>Mock</td>
<td>6.95</td>
<td>-</td>
</tr>
</tbody>
</table>

* Activity obtained with the LPL WT construct under the same media collection conditions i.e. with or without heparin.

Two transfection experiments were performed with plasmid pcDNA-LPL193 (Table 5.2). In the first instance, a small-scale experiment was carried out where COS cells were seeded in a 6-well plate and heparin (5U/ml medium) was added to the medium 20 hrs prior to collection (one well for each construct). This was in accordance with the standard protocol followed in Dr. J.Kastelein’s laboratory. The medium (1.5ml) was collected and tested only for LPL activity since the amount recovered was insufficient for a mass assay. LPL activity was readily detected in the medium of cells transfected with the WT construct with 23.79 and 34.10 mU/ml in untreated and
heparin-treated cells respectively. LPL activity in wells transfected with the mutant vector was slightly higher than background activity measured in mock-transfected cells (6.03 and 6.16 mU/ml without and with heparin respectively vs. 5.60 mU/ml in mock). After subtracting the background mock activity, very low levels of LPL activity reaching 1.6% and 1.8% respectively of values measured with the control plasmid with or without heparin treatment were obtained. LPL activity was 30% higher in the medium of LPL WT cells treated with heparin compared to untreated cells (-hep, 23.79 vs +hep, 34.10 mU/ml). The corresponding increase was 23% in cells transfected with LPL193 (-hep, 0.43 vs +hep, 0.56).

In the second transfection experiment with pcDNA-LPL193, 60mm dishes were used and heparin was added 2 hrs prior to collection of the medium at a concentration of 20U/ml. For both the control and mutant constructs, there was a two-fold increase in released LPL activity associated with the heparin treatment (3.56 and 111.72 with heparin vs 1.61 and 51.27 without), a much greater increase than that observed in the first transfection (Table 5.2). This suggested that a larger dose of heparin (20 vs 5U) combined with a shorter interval between heparin addition and medium collection (2 vs 20 hrs) allowed the recovery of higher activity. This suggests that LPL is inactivated relatively rapidly in these cultures, although inter-experiment differences in cell number and transfection efficiency also probably influenced the result. Activity was still much lower in dishes transfected with plasmid LPL193 compared to LPLWT, reaching approximately 3% of control levels (Table 5.2). Again, the remainder of the medium was saved for LPL mass measurement. Unfortunately, this assay failed for both the LPL158 and 193 media with the assay blanks and standard curve samples yielding high or inconsistent values (not shown).
Table 5.2 *In vitro* expression of Arg193 variant. LPL activity in COS cell medium 3 days post-transfection with pcDNA-LPL193 and pcDNA-LPL WT (2µg/ml) in two separate transfection experiments -T1 (transfection 1) and T2 (transfection 2). LPL activity was measured with or without addition of heparin prior to collection. Heparin was added either 20 (5U/ml, transfection 1) or 2 hrs (20U/ml, transfection 2) before collection. The activity associated with mock-transfected cells has been subtracted from all other activity values and is shown for comparative purposes.

<table>
<thead>
<tr>
<th>Plasmid/Treatment (+/− heparin)</th>
<th>LPL activity (mU/ml)</th>
<th>% control*</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1 LPL193 / - hep</td>
<td>0.43</td>
<td>1.8%</td>
</tr>
<tr>
<td>LPL193 / + hep</td>
<td>0.56</td>
<td>1.6%</td>
</tr>
<tr>
<td>LPL WT / - hep</td>
<td>23.79</td>
<td>-</td>
</tr>
<tr>
<td>LPL WT / + hep</td>
<td>34.10</td>
<td>-</td>
</tr>
<tr>
<td>Mock / + hep</td>
<td>5.60</td>
<td>-</td>
</tr>
<tr>
<td>T2 LPL193 / -hep</td>
<td>1.61</td>
<td>3.1%</td>
</tr>
<tr>
<td>LPL193 / + hep</td>
<td>3.56</td>
<td>3.2%</td>
</tr>
<tr>
<td>LPL WT / - hep</td>
<td>51.27</td>
<td>-</td>
</tr>
<tr>
<td>LPL WT / + hep</td>
<td>111.72</td>
<td>-</td>
</tr>
<tr>
<td>Mock / + hep</td>
<td>8.5</td>
<td>-</td>
</tr>
</tbody>
</table>

*Activity obtained with the LPL WT construct under the same media collection conditions i.e. with or without heparin.
5.3 Expression of Ser291, a common variant close to the proposed heparin-binding domain.

5.3.1 LPL activity

The pcDNA-LPL WT expression plasmid was mutagenised as described above to synthesise the Ser291 allele (pcDNA-LPL291). Two transfection experiments were carried out with these constructs, differing in the timing of the heparin addition and in the quantity added. Two 60mm dishes per construct were transfected, with heparin being added to one dish per construct prior to collection of the media. For the first transfection, heparin (5U/ml) was added 20 hrs prior to media collection. LPL activity was detected in the media of cells transfected with either construct but was consistently lower for the LPL291 construct (Table 5.3). In the absence of heparin, the activity in the LPL291 dish amounted to 50.7% of the activity detected in the LPL WT dish. When heparin was added to the media, measured activity levels rose, as expected, for both constructs but the increase was greater with the LPL WT plasmid (1.5 vs. 1.1 fold increase). LPL activity measured in the LPL291 dish was 39.6% of that observed with the control, due to this larger proportional increase in LPL activity released by heparin in LPL WT transfected cells. Coupled with the knowledge that residue 291 is in close proximity to a putative heparin-binding domain, this preliminary result suggested that the mutation might decrease the ability of LPL to bind HSPG at the cell surface.

Based on the results from the transfections with LPL193 (preceding section), a second experiment was performed where a larger dose of heparin (20U/ml) was added 1 hr prior to collection of the media. Consistent with the earlier result, LPL activity associated with the LPL291 plasmid was lower than the levels measured with the control.
plasmid, both with or without addition of heparin (no heparin, 21.5 vs 67.7 mU/ml; with heparin, 60.9 vs 154.9 mU/ml)(Table 5.3). In contrast with the previous experiment however, heparin caused a larger proportional increase in LPL activity in the media of LPL291 transfected cells compared to the control plasmid (LPL291, 2.8 fold increase vs LPL WT, 2.3 fold increase). Accordingly, activity for LPL291 recovered in the presence of heparin relative to the control was higher than in the absence of heparin (39.3 vs 31.7%, Table 5.3). Differences in transfection efficiency between heparin-treated and untreated dishes might have accounted at least in part for these conflicting results but LPL mass data, which could have clarified this issue, was not available. Therefore, an indirect approach was taken to try and shed light on this question.

Table 5.3 In vitro expression of Ser291 variant. LPL activity in COS cell medium 3 days post-transfection with pcDNA-LPL291 and pcDNA-LPL WT (2μg/ml) in two separate transfection experiments. T1 (transfection 1) and T2 (transfection2). LPL activity was measured with or without addition of heparin prior to collection.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Construct/ + or - heparin</th>
<th>LPL activity (mU/ml)</th>
<th>% control*</th>
<th>Effect of heparin (fold increase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>LPL291/ -hep</td>
<td>11.2</td>
<td>50.7</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>LPL291/ +hep</td>
<td>12.7</td>
<td>39.6</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>LPL WT/ -hep</td>
<td>22.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>LPL WT/ +hep</td>
<td>32.1</td>
<td>-</td>
<td>1.5</td>
</tr>
<tr>
<td>T2</td>
<td>LPL291/ -hep</td>
<td>21.5</td>
<td>31.7</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>LPL291/ +hep</td>
<td>60.9</td>
<td>39.3</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>LPL WT/ -hep</td>
<td>67.7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>LPL WT/ +hep</td>
<td>154.9</td>
<td>-</td>
<td>2.3</td>
</tr>
</tbody>
</table>

*Activity obtained with the LPL WT construct under the same media collection conditions, i.e. with or without heparin.
5.3.2 Stability of LPL291 enzyme activity.

The stability of the lipolytic activity collected in post-transfection media was evaluated by measuring activity following a pre-incubation (denaturation) step of increasing duration. Two experiments were performed. Firstly, media samples were incubated at 37°C and aliquots were removed after 30, 60 or 120 mins to be assayed for LPL activity. For both the LPL WT and LPL291 constructs, LPL activity in the media decreased with time. However, the loss of activity was proportionally more rapid for the LPL291 enzyme with 24.5% of its initial activity remaining after 120 mins compared to 60.0% for LPL WT (Fig.5.5a). Consistent with this data, the percent LPL291 activity relative to LPL WT fell from 39.3 to 15.9%. The half-life for the Ser291 enzyme was determined directly from the graph as 47 minutes. This was not possible for LPL WT since this point had not yet been reached by the end of the experiment, but the half-life of LPL WT activity was calculated as 162 minutes, assuming a simple exponential decay curve. This was done using the 120 mins data point to solve the equation $0.6 = 1 - (x)^2$, where 0.6 is the remaining activity, x is the proportion of activity remaining after one hour and 2 hrs is the time point corresponding to 60% of the initial activity (exponent in the equation). The value determined for x was then used to solve the equation at 50% remaining activity. A second set of activity decay curves was obtained by incubating media samples in mildly denaturing conditions at 37°C for up to 60 mins. Incubation of samples in urea at a final concentration of 0.5M accelerated the activity loss for both the control and Ser291 enzymes. Residual activity after 30 mins incubation was 40% of the initial level in LPL291 media compared to 62% for the same incubation period in non-denaturing conditions (Fig.5.5a and b). Media from LPL WT cells retained 45% of its initial activity after 60 mins incubation in denaturing conditions compared to 69.4% in
Fig. 5.5 Stability of LPL activity in media of LPL WT and LPL291 transfected cells. Each sample was assayed in duplicate. Decay is presented as % total activity (0 time point) for each construct. The histogram below each graph shows the activity obtained with the mutant plasmid LPL291 relative to the LPL WT for each time point.

a) Decay curve of LPL activity assayed without chemical denaturant
b) Decay curve of LPL activity obtained in the presence of 0.5M urea
non-denaturing conditions. Half-life estimates, as read from the graph, were 23 and 56 mins for the Ser291 and WT enzymes respectively, in the presence of urea. Using the ratio of half-lives for both experiments, these data show that the Ser291 substitution results in a 2.4 to 3.8 faster loss of activity compared to the normal sequence.

5.4 The Asn9 mutation: a common variant with moderate effect on activity.

5.4.1 Secreted lipolytic activity.

The common Asn9 variant, identified in normolipidaemic controls and hyperlipidaemic subjects was constructed by site-directed mutagenesis as outlined above. Following transfection of the LPL9 and LPL WT constructs in COS cells, the media was collected and LPL activity and mass measured by Dr. Gunilla Olivecrona (Umea University, Sweden) as described in the method section. The 5D2 MAb, which recognises an epitope near aa400 of the mature protein, is assumed to have the same affinity for both the WT and Asn9 proteins. Data from three separate experiments are summarized in Table 5.4, where both activity and total mass (monomer and dimer) were measured in the medium after heparin addition (20U/ml, 2 hrs prior to collection). In transfection 1, a single dish was transfected with either construct. While LPL mass measured in the medium was similar for both constructs (only marginally lower for LPL9), LPL activity associated with cells transfected with the Asn9 construct was 27% lower than for the Asp9 cells, with a resulting 20% decrease in specific activity. As this was a modest decrease which could have been due to differences in chance, the experiment was repeated using two plates per construct. The same relative decrease in activity was observed with the Asn9 construct. LPL mass levels were very similar between the two constructs but markedly lower than for the first experiment. In the face of the minor activity decrease between transfections 1 and 2, this suggests that a much
larger proportion of the enzyme was in the active dimer form at the end of the second experiment. The reasons for these presumed differences in inactivation of the dimer are not known.

A third transfection experiment was carried out where seven dishes per construct were transfected, of which four were treated with heparin. LPL activity and mass data was obtained for individual dishes. In contrast to the first two experiments, lower activity in the four heparin-treated LPL9 plates compared to LPL WT (40% overall) was accompanied by a reduction in mass (32% overall)(Table 5.4) and mean LPL activity and mass were significantly lower in the medium of LPL9 transfected cells. There was a large range of activity and mass values for the LPL WT but variation was consistent as reflected by the similar specific activity levels obtained for all dishes of a construct.

Taken together, these results provide some evidence that the Asp9 variant has slightly reduced activity in vitro but the mechanism leading to this decrease is not clear. Both lower specific activity and overall reduction in secretion could be involved.

5.4.3 Heparin-Sepharose profile.

Media from three untreated (no heparin) plates from both constructs (from transfection 3) was pooled and passed through a heparin-Sepharose affinity column. This was done to separate inactive monomeric LPL protein from the active dimer as excess monomer protein is often associated with these in vitro assays. The resulting profile still showed decreased dimer mass and activity for the Asn9 construct (lower peaks), with no apparent elution shift of the dimer peak for the mutant, suggesting that the affinity for heparin was not altered (Fig.5.6). There were no significant differences in the monomer to dimer mass ratio as assessed by the areas under the curve or by peak fractions.
Table 5.4. *In vitro* expression of Asn9 variant: LPL activity and mass measured in media from transfected cells.

<table>
<thead>
<tr>
<th>Expt / # dishes per construct</th>
<th>Construct</th>
<th>Activity (mU/ml) [% Asp9-LPL]</th>
<th>Mass (ng/ml) [% Asp9-LPL]</th>
<th>Spec Act (mU/µg) [% Asp9-LPL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transfection 1 (single dish for each construct)</td>
<td>Asp9-LPL</td>
<td>32.8 [100]</td>
<td>267 [100]</td>
<td>123 [100]</td>
</tr>
<tr>
<td></td>
<td>Asn9-LPL</td>
<td>24.1 [73]</td>
<td>248 [93]</td>
<td>98 [80]</td>
</tr>
<tr>
<td>Transfection 2 (pooled medium from 2 dishes)</td>
<td>Asp9-LPL</td>
<td>28.2 [100]</td>
<td>143 [100]</td>
<td>197 [100]</td>
</tr>
<tr>
<td></td>
<td>Asn9-LPL</td>
<td>20.7 [73]</td>
<td>135 [94]</td>
<td>154 [78]</td>
</tr>
<tr>
<td>Transfection 3 (four separate dishes)</td>
<td>Asp9-LPL</td>
<td>37.5 23.0 30.1 23.6</td>
<td>x=28.5 [100] 261.0 309.0 259.5</td>
<td>x=315</td>
</tr>
<tr>
<td></td>
<td>Asn9-LPL</td>
<td>15.5 15.9 17.6 19.8</td>
<td>x=17.2 [60] 202.5 209.6 207.5 240.5</td>
<td>x=215</td>
</tr>
</tbody>
</table>

*p<0.05  **p<0.019

*2-tailed t-test*
Fig. 5.6 LPL activity (open symbols) and mass (filled symbols) in pooled media from transfected cells following separation by heparin-Sepharose chromatography. Cells were transfected with the LPL-Asn9 construct (ASN9)(lower graph, circles) and the control LPL-Asp9 construct (ASP9)(upper graph, diamonds). One milliunit (mU) corresponds to 1 nmol of fatty acid per min. The arrows show the salt concentration at the elution peak. Note that the activity is very low compared to human plasma activity (see Fig. 6.5) and this is near the detection limit. 182
5.5 Protein secondary structure modelling.

The effect of amino acid substitutions on LPL secondary structure was modelled with the Protein component of the DNAstar software. The data are summarised in Fig. 5.7. All the mutations identified in this study were predicted to alter the hydrophobicity profile of 5-10 residue segments encompassing the mutated sites, as estimated by the Hopp-Woods algorithm or the hydrophobic moment calculation. These profile alterations were particularly marked for the A158T, G188E, S193R, P207L and L303P substitutions. In contrast, the H183Q and D9N hydrophobicity profiles departed only slightly from the native LPL one.

When Chou-Fasman and Garnier-Robson secondary structure predictions were considered, a more striking pattern emerged. For the four mutations where low or intermediate levels of LPL activity have been observed in vitro (D9N, A158T, S193R and N291S, this study) (N291S, Ma et al. 1993), no changes affecting the conformation of surrounding residues were observed using either algorithm. In fact, in these four variants, only the predicted conformation of the T158 residue was altered from a helical to an extended conformation with the remainder of the helix being preserved.

In contrast, either the Chou-Fasman or Robson-Garnier secondary structure predictions were altered for amino acid segments comprising W86G, H183Q, G188E, P207L, M301T and L303P. Helical segments were shortened or interrupted for W86G, H183Q and L303P while predicted turns in the structure were removed or introduced by the G188E or M301T substitutions respectively. Three of these substitutions, G188E, P207L and H183Q, led to completely inactive enzyme when studied in vitro (Emi et al., 1990b; Ma et al., 1991, Tenkanen et al., 1994), while the effects of the remaining three have yet to be tested. However, this analysis would predict that the W86G, M301T and L303P mutations would lead to complete loss of LPL activity.
Fig. 5.7 Effect of mutations on secondary structure predictions. Nucleotide substitutions identified in chapter 3 were used to modify the human LPL sequence in Genbank and the mutated sequences were analysed with the DNAStar software. The tracings show variations in Chou-Fasman structural predictions (CF, left) and hydrophobicity (H, right). Dotted lines represent the mutant sequences and solid lines the wild-type sequence.

Conformations
H: helix  E: extended  T: turn  C: coil
5.6 Summary and brief discussion

Four mutations identified in the LPL gene of type I or CHL subjects were reproduced by *in vitro* mutagenesis. These were A158T, S193R, N291S and D9N. The effect of the mutations on LPL activity was studied using a transient expression system in cultured mammalian cells. Additionally, LPL mass in the culture medium was obtained for the D9N mutation. A control plasmid was not co-transfected with the mutant LPL constructs to standardize the measured enzyme activities for possible differences in transfection efficiency between dishes. In these conditions, the current system is at best a semi-quantitative assay.

The functional significance of two of the novel mutations identified in type I subjects, A158T and S193R, was confirmed by the *in vitro* mutagenesis experiments. Both mutations resulted in very low levels of activity. The fact that no other variants were detected by SSCP analysis in the LPL gene of the individuals carrying the A158T and S193R substitutions supports the interpretation that these mutations cause the phenotype. However, these results must be interpreted with caution since no LPL mass data was available for these two transfection experiments. Therefore, it cannot be ruled out that the transfections failed or were very inefficient due to the low transfectability of the LPL158 and 193 constructs.

Assuming a transfection efficiency similar to that of the wild-type construct, the low activity observed with the LPL158 construct agrees well with the *in vivo* data. LPL specific activity measured in subject SAk, homozygous for A158T, represented 3-4% of normal levels (Table 3.1). Recent modelling data suggests that A158T may alter a segment of the hydrophobic substrate binding pocket (van Tilbeurgh et al., 1994). This might restrict access of the substrate to the active site, which would be expected in turn to decrease the turnover rate and would be measured as lower specific activity.

The situation is more complex for the S193R mutation. Subject LA is a compound heterozygote and carries a second defective allele with a 2kb insertion which
has been reported to abolish correct mRNA splicing and hence LPL synthesis (Langlois et al., 1989). Yet, adipose tissue LPL activity for this individual ranges from 8-17% of levels measured in age-matched subjects (Lithell et al., 1978). This differs by several fold from the values obtained in the two transfection experiments and suggests the latter are an underestimate, possibly due to lower transfection efficiency. Alternatively, it is possible that the effect of this mutation is modulated in a tissue-specific manner such that it is less severe in adipose tissue and is not accurately reflected in COS cells. Brunzell and colleagues (1980) have described patients with adipose tissue-specific LPL deficiency although the molecular basis for these observations remains unclear.

Lower LPL activity and mass were consistently observed in the medium from Asn9 transfected cells compared to cells transfected with the wild type construct, although the decrease in activity was slightly more marked (range 17-40% for activity and 6-32% for mass). There was some variation in the results from the in vitro expression studies. This was not surprising, since for several reasons the transient expression system used was not ideal for testing relatively small differences; namely the absence of a true internal control for the transfection efficiency of the plasmids and the large amount of inactive LPL protein. Although the number of cells per plates used was kept constant, there was no control for transfection efficiency. However, this issue is at least partially addressed by the use of multiple dishes which are randomized to different constructs, by combining data from several plates and by the consistency of the results in repeat experiments.

Secreted LPL-Asn9 appears to have only slightly reduced function. The heparin-Sepharose chromatography profile also demonstrated that the ability to bind heparin is preserved and the affinity appears unchanged. This suggests that the lower mass and activity in the medium is not caused by impaired release from the cell surface. This is not surprising since it is proposed that the main heparin-binding domains are located
between amino acids 270-305 (Hata et al., 1993; van Tilbeurgh et al., 1994), which are predicted to be distant from the N-terminal sequence in the tertiary structure of LPL. The data from these two sets of experiments imply that the Asn9 substitution may impair post-translational processes or secretion from the cell. This might be expected to lead to some accumulation of LPL within cells, which could not be determined accurately in these experiments due to the low sensitivity of the current transfection assay. These issues could be resolved by cell fractionation and metabolic labelling experiments in permanently transfected cell lines.

Finally, the N291S variant is of great interest because it is the first LPL mutation with a major effect on LPL activity which appears to be common both in hyperlipidaemic subjects (type I by this study, type III HLP by Ma et al., 1993c) and in the general population (chapter 4). The lower activity observed with the LPL291 construct (range 31.7 to 50.7%) has confirmed the report of Ma and colleagues. We have shown that this decrease appears to be caused largely by the lability of the mutant enzyme. The loss of activity is probably caused by the rapid dissociation of the active LPL dimer (Peterson et al., 1992). In agreement with this, a high level of LPL monomers has recently been reported by Reymer et al. (1995) in the media from cells transfected with the Ser291 allele. In addition, the denaturation assay provides an explanation for the apparent inability of heparin to effect the release of LPL activity from the surface of LPL291 cells in the first transfection experiment. This result was somewhat unexpected as the mutation did not involve a positively charged amino acid which could bind to a surface heparan-sulphate molecule. In fact, it is likely that the mutant enzyme is released rapidly (and normally) by heparin but is inactivated after 24 hrs in the culture medium. Overall, these results suggest that residue 291 may play a role in the maintenance of a stable LPL dimer conformation.
6. HETEROZYGOUS EXPRESSION OF LPL GENE MUTATIONS - POPULATION STUDIES.

Complete LPL deficiency due to two defective alleles at the LPL gene locus is a rare event with an estimated frequency of 1:1 000 000 and is expressed phenotypically as type I HLP (Brunzell, 1989). Based on this estimate, approximately one individual in 500 is expected to carry a non-functional LPL allele. In contrast, the studies presented in chapter 4 have identified two common, mild mutations (2-5% carrier frequency) in samples from the general population and from hyperlipidaemic subjects. Both the Asn9 and Ser291 variants (section 5.3 and 5.4) cause a decrease in LPL activity when expressed in vitro although this is more marked for the N291S substitution. In this chapter, the impact of these variants in vivo will first be investigated by examining LPL activity and lipid levels in unrelated heterozygous carriers. As a second step, the segregation of several LPL variants will be studied in families with FCHL and type I HLP.

Statistical analysis was carried out by Dr. Jackie Cooper for the Camberley and St.Andrews samples and by Dr. Viviane Nicaud for the ECTIM study.

6.1 Impact of the D9N substitution on Tg and LPL levels in subjects from the UK, Sweden and the Netherlands.

6.1.1 Triglyceride levels.

The possible impact of the D9N substitution on lipoprotein metabolism was investigated by comparing lipid/lipoprotein traits and LPL activity (PHLA) in the carriers of the Asn9 allele identified in section 4.5 and in non-carriers from the same groups. Lipid levels, LPL activity where available, and biometrical data for the 55 Asn9
carriers that were identified in those groups are shown in Tables 6.1a & b.

In the first instance, the data from the two groups of healthy men recruited from the Camberley (England) and St. Andrews (Scotland) general practices were examined for the effect of the Asn9 allele. The 27 individuals with at least one Asn9 allele had significantly higher plasma triglycerides at entry in the study (2.25 vs 1.82, p < 0.02, 24% increase) than non-carriers (Table 6.2). When the individual data for triglycerides were plotted (Fig. 6.1) and compared to the Asp9 group mean, a considerable scatter in the levels of triglycerides emerged. One of the two homozygous individuals for Asn9 had relatively high levels while the other had levels slightly below the Asp9 group mean. The values were nevertheless distributed relatively evenly using the logarithmic scale and the higher Tg seen in carriers was not simply due to a few outliers. There were no other significant differences in age, BMI, total chol, and apoB between the two groups.

For 22 of 27 Asn9 carriers and 631 non-carriers, complete lipid data were available for baseline and three subsequent annual measurements. In the group of non-carriers, there was a small but significant decrease in plasma triglyceride levels over the three years (p = 0.001). A similar trend could be seen in the carriers but it did not reach statistical significance (p = 0.07). However, the higher plasma triglycerides seen at baseline in LPL-Asn9 carriers were maintained throughout the study period with the difference being significant at year 2 and overall (p = 0.005 and p = 0.01 respectively)(Fig.6.2). The magnitude of the increase ranged from 20 to 35% over the three years and averaged 27% overall. The slightly lower cholesterol levels seen in carriers at baseline (Table 6.2) was also maintained over time, but overall the difference was still not significant (5.35 ± 0.18 mmol vs 5.66 ± 0.04, p = 0.1).
Table 6.1 Age, body mass index and lipid levels of individuals with the Asp9 to Asn substitution.

a) Patients

<table>
<thead>
<tr>
<th>Sample</th>
<th>Age</th>
<th>BMI</th>
<th>Chol</th>
<th>Tg</th>
<th>HDL-C</th>
<th>LPL activity (percentile)*</th>
</tr>
</thead>
<tbody>
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<td>-</td>
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<td>6.95</td>
<td>0.82</td>
</tr>
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<td>2</td>
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</tr>
<tr>
<td></td>
<td>3</td>
<td>56</td>
<td>-</td>
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<td>2.00</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>57</td>
<td>-</td>
<td>6.2</td>
<td>2.72</td>
<td>1.38</td>
</tr>
<tr>
<td>F 5</td>
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<td></td>
</tr>
<tr>
<td>F 6</td>
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<td>7</td>
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<td>6.7</td>
<td>7.06</td>
<td>0.63</td>
<td></td>
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<tr>
<td>8</td>
<td>44</td>
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<tr>
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b) Control individuals

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<th>Tg</th>
<th>LPL Activity (Percentile)*</th>
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<td>4.80</td>
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<td>4.45°</td>
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<tr>
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<td>26.1</td>
<td>5.9°</td>
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<tr>
<td>Swedish controls</td>
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<td>26.4</td>
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<td>2.75°</td>
</tr>
<tr>
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<td>6.09°</td>
<td>1.65°</td>
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<td>4.91</td>
<td>1.22°</td>
</tr>
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</tr>
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<tr>
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<td>3</td>
<td>48</td>
<td>20.0</td>
<td>5.79°</td>
<td>1.58°</td>
</tr>
</tbody>
</table>

* The difference in the range of values between the study groups is due to the use of different heparin doses (50 or 100 units/kg body weight) and different LPL activity assays. Activity is expressed as nmoles FFA/ml/min. F = females, * Individuals homozgous for the LPL-Asn9, ° at or above sample mean
Table 6.2 Mean (±SE) plasma lipid levels at base line in healthy LPL-Asp9 and LPL-Asn9 carriers from England and Scotland.

<table>
<thead>
<tr>
<th></th>
<th>No</th>
<th>Age (years)</th>
<th>BMI (kg/m²)</th>
<th>Chol mmol/l</th>
<th>Tg* mmol/l</th>
<th>apoB g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPL-Asp9</td>
<td>746</td>
<td>55.5 (0.18)</td>
<td>26.4 (0.14)</td>
<td>5.77 (0.03)</td>
<td>1.82 (0.07)</td>
<td>0.848 (0.05)</td>
</tr>
<tr>
<td>LPL-Asn9</td>
<td>27</td>
<td>56.2 (0.63)</td>
<td>27.7 (0.66)</td>
<td>5.55 (0.17)</td>
<td>2.25 (0.34)</td>
<td>0.796 (0.24)</td>
</tr>
<tr>
<td>Test for difference</td>
<td></td>
<td>p = 0.75</td>
<td>p = 0.09</td>
<td>p = 0.14</td>
<td>p &lt; 0.02</td>
<td>p = 0.24</td>
</tr>
</tbody>
</table>

*Tg were loge normally distributed. Values presented are antilogged.
Fig. 6.1. Scatter plot of triglyceride data in healthy carriers of the LPL Asn9 mutation from the two UK samples. Also shown is the mean triglyceride levels (± SEM) for the non-carriers from the UK samples. Filled circles represent data from Asn9 homozygous individuals. Some unfilled circles represent more than one individual.
Fig. 6.2. Plot of mean (± SEM) plasma triglyceride levels in Asn9 carriers and non-carriers with time. Mean values at baseline and at subsequent three annual measurements for the 22 carriers individuals and 631 non-carriers with no missing data. Both non-carriers ($p < 0.0001$) and Asn9 carriers ($p = 0.07$) show a negative trend over time. The two groups do not differ in trends ($p = 0.61$).
A similar analysis could not be carried out in the Dutch and Swedish control groups since the total number of carriers was too small. However, 5/6 carrier individuals had triglyceride levels at or above their respective sample mean, 4 of whom also had elevated cholesterol relative to their sample mean (Table 6.1b).

6.1.2 Impact on LPL activity.

Where available, PHLA was examined in Asn9 carriers. Data was available from 12/55 carriers, of which 9 were patients. Figure 6.3 shows LPL activity values from individuals originating from the various studies plotted relative to their own group’s mean. From the patient groups, 6/9 carriers had LPL activity measurements below their group mean and 7/9 had activity below their respective control group mean. The three carriers from the control groups all had LPL activity well below their group mean. Overall, the lower activity in carriers represented a 15-40% decrease in LPL activity relative to their respective sample means.

Without LPL mass data, it was not possible to distinguish whether this lower activity in carriers was due to lower specific activity of the mutant enzyme or overall decreased release or secretion from the cells. To examine this question, experiments were carried out to characterize the Asn9 variant biochemically by analysing PHLA and LPL mass in normolipidaemic carriers. Three Asn9 carriers from the English general practice sample were recalled and samples from three non-carrier normal laboratory controls taken for comparison as shown in Figure 6.4. In the carriers, the total activity was 30% lower (177 vs 251 mU/ml) and mass was 30% lower (985 vs 1538) although these differences did not reach statistical significance (Mann-Whitney, p = 0.15 for mass and activity; t-test, p=0.12 and p=0.16 respectively). Separation
Fig. 6.3. Post-heparin LPL activity in patients and controls from the Charing Cross and Swedish studies. Data from the Swedish NTG and HTG groups are plotted using the scale at the right hand side. The mean (± S.E.M.) for each groups is shown by horizontal bars. One milliunit (mU) corresponds to 1 nmol of fatty acid released per min.
Fig. 6.4. LPL activity and mass in three normolipidaemic carriers of the Asn9 from the Camberley sample and in three healthy non-carriers (laboratory controls). Horizontal bars indicate mean LPL activity or mass. One milliunit (mU) corresponds to 1 nmol of fatty acid released per min.
of plasma by heparin-Sepharose did not reveal any striking differences in lipase activity or mass elution profiles between carriers and non-carriers. In particular, there was no striking variation in the ratio of the two mass peaks or the two areas under the curve, representing the inactive monomeric LPL and the active dimeric enzyme respectively, and both forms eluted in the expected fractions. Profiles from a representative individual from both groups are shown in Fig. 6.5. The first activity peak is due to hepatic lipase which expresses about 70% of full activity in the assay for LPL used here. The semi-purified LPL material from both Asn9 carriers and non-carriers was incubated at 37°C for increasing periods of time to test its stability. Again, no differences were observed between the control and carrier material (Fig. 6.5).

**6.2 Impact of the D9N substitution in carriers from the ECTIM study.**

The relationship between the Asn9 variant and various plasma lipid traits was examined in the cases and controls separately (after adjusting for the effects of age, BMI and centre according to the ECTIM study protocol) and the results are presented in Tables 6.3 and 6.4. Cases who were carriers of the mutation had significantly higher levels of plasma triglycerides (30%), VLDL cholesterol (18.9%), apoE (24.2%), Lp E:B (31.8%), and Lp CIII:B (39.3%), with non-significantly higher levels of total cholesterol and apoB (Table 6.3a). Because of previously reported differences in lipid traits and lifestyles between the Belfast and French participants, the data was re-analysed separately for each group. The effects were consistent in both the Belfast group and the combined French centres, with a larger effect generally on lipid traits in the Belfast group (Table 6.3b). By contrast, no significant or consistent differences were observed for those lipid traits between carriers and non-carriers from the control.
Fig. 6.5. Lipase activity (open symbols) and LPL mass (filled symbols) in plasma fractionated by heparin-Sepharose chromatography. Profiles from two subjects is shown: one individual heterozygous for the Asn9 substitution (lower graph, circles) and one individual homozygous for the (wild-type) Asp9 allele (upper graph, diamonds). One milliunit (mU) corresponds to 1 nmol of fatty acid released per min. Arrows indicate the salt concentration for the fractions at which peak or near peak activity and mass elute. Differences in the activity peaks for human plasma samples and transfection media (0.8M NaCl vs. 0.9M NaCl) may be due to the presence of other ions and the inaccuracy of the mass and activity assays at very low levels.
Table 6.3a. Lipids, apoliproteins and lipoparticles (g/l) adjusted* mean levels and SEM according to LPL-Asn9 mutation in cases. Tests are performed on log-transformed variables.

<table>
<thead>
<tr>
<th></th>
<th>Mutation carriers</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 22</td>
<td>N = 614</td>
</tr>
<tr>
<td>T-Chol</td>
<td>2.402 [0.085]</td>
<td>2.292 [0.017]</td>
</tr>
<tr>
<td>Tg</td>
<td>2.275 [0.239]</td>
<td>1.744 [0.048]</td>
</tr>
<tr>
<td>LDL-C</td>
<td>1.608 [0.074]</td>
<td>1.570 [0.015]</td>
</tr>
<tr>
<td>VLDL-C</td>
<td>0.340 [0.036]</td>
<td>0.286 [0.007]</td>
</tr>
<tr>
<td>HDL-C</td>
<td>0.456 [0.024]</td>
<td>0.435 [0.005]</td>
</tr>
<tr>
<td>ApoE</td>
<td>0.077 [0.007]</td>
<td>0.062 [0.001]</td>
</tr>
<tr>
<td>LpE:B</td>
<td>0.597 [0.059]</td>
<td>0.453 [0.012]</td>
</tr>
<tr>
<td>LpCIII:B</td>
<td>0.234 [0.028]</td>
<td>0.168 [0.006]</td>
</tr>
</tbody>
</table>

* for centre, group, age and BMI.

Table 6.3b. Lipids, apoliproteins and lipoparticles (g/l) adjusted* mean levels and SEM according to LPL-Asn9 mutation in cases of Belfast and France.

<table>
<thead>
<tr>
<th></th>
<th>Mutation carriers</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 5</td>
<td>N = 197</td>
</tr>
<tr>
<td>T-Chol</td>
<td>2.661 [0.182]</td>
<td>2.419 [0.029]</td>
</tr>
<tr>
<td>TG</td>
<td>3.365 [0.623]</td>
<td>2.013 [0.099]</td>
</tr>
<tr>
<td>VLDL</td>
<td>0.452 [0.063]</td>
<td>0.340 [0.010]</td>
</tr>
<tr>
<td>LpE:B</td>
<td>0.722 [0.134]</td>
<td>0.550 [0.021]</td>
</tr>
<tr>
<td>LpCIII:B</td>
<td>0.331 [0.067]</td>
<td>0.228 [0.011]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Mutation carriers</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 17</td>
<td>N = 417</td>
</tr>
<tr>
<td>T-Chol</td>
<td>2.308 [0.097]</td>
<td>2.242 [0.020]</td>
</tr>
<tr>
<td>TG</td>
<td>1.879 [0.235]</td>
<td>1.629 [0.047]</td>
</tr>
<tr>
<td>VLDL</td>
<td>0.302 [0.043]</td>
<td>0.267 [0.009]</td>
</tr>
<tr>
<td>LpE:B</td>
<td>0.555 [0.065]</td>
<td>0.423 [0.013]</td>
</tr>
<tr>
<td>LpCIII:B</td>
<td>0.206 [0.030]</td>
<td>0.154 [0.006]</td>
</tr>
</tbody>
</table>

* for age and BMI.
Table 6.4a Lipids, apolipoproteins and lipoparticles (g/l) adjusted* mean levels and SEM according to LPL-Asn9 mutation in controls. Tests are performed on log-transformed variables.

<table>
<thead>
<tr>
<th>CONTROLS</th>
<th>Mutation carriers N = 22</th>
<th>Others N = 780</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-Chol</td>
<td>2.252 [0.097]</td>
<td>2.321 [0.016]</td>
</tr>
<tr>
<td>Tg</td>
<td>1.615 [0.277]</td>
<td>1.639 [0.047]</td>
</tr>
<tr>
<td>LDL-C</td>
<td>1.488 [0.085]</td>
<td>1.542 [0.014]</td>
</tr>
<tr>
<td>VLDL-C</td>
<td>0.255 [0.035]</td>
<td>0.264 [0.006]</td>
</tr>
<tr>
<td>HDL-C</td>
<td>0.509 [0.029]</td>
<td>0.517 [0.005]</td>
</tr>
<tr>
<td>ApoE</td>
<td>0.062 [0.008]</td>
<td>0.060 [0.001]</td>
</tr>
<tr>
<td>LpE:B</td>
<td>0.445 [0.059]</td>
<td>0.430 [0.010]</td>
</tr>
<tr>
<td>LpCIII:B</td>
<td>0.169 [0.029]</td>
<td>0.163 [0.005]</td>
</tr>
</tbody>
</table>

* for centre, group, age and BMI.

Table 6.4b Lipids, apolipoproteins and lipoparticles [g/l] adjusted * mean levels and SEM according to LPL-Asn9 mutation in controls of Belfast and France.

<table>
<thead>
<tr>
<th>BELFAST</th>
<th>FRANCE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mutation carriers N = 4</td>
</tr>
<tr>
<td>Tot.chol</td>
<td>2.457[0.219]</td>
</tr>
<tr>
<td>TG</td>
<td>1.566[0.449]</td>
</tr>
<tr>
<td>VLDL</td>
<td>0.286[0.078]</td>
</tr>
<tr>
<td>LpE:B</td>
<td>0.373[0.159]</td>
</tr>
<tr>
<td>LpCIII:B</td>
<td>0.202[0.079]</td>
</tr>
</tbody>
</table>

* for age and BMI.
group (Table 6.4a,b). No consistent differences were seen in HDL-chol, Lp Al, Lp Al:AII and apo E for either group. Exclusion of 58 individuals from the analysis who reported the use of lipid-lowering drugs did not influence the results of the analysis.

Two factors were examined to explain the case-control difference in the effect of the Asn9 mutation on plasma lipid and lipoparticle levels. Firstly, the possibility that variation at the apoE locus, known to influence lipid levels, might be responsible for the observed effects was explored. Pooled data from the carriers and non-carriers irrespective of disease status still showed the increase in Tg, VLDL-chol and lipoparticle levels that had been observed for the carriers from the cases group. However, the distribution of apoE genotypes in all carriers of the Asn9 allele was not different from the whole group (F.Cambien, personal communication), with 18% having the genotype apoE2E3, 66% the genotype apoE3E3 and 16% having one or more copy of the apoE4 allele. Moreover, plasma triglycerides were highest in the carriers with the apoE4 allele by 19.6%, but the size of the effect was similar to that seen in the non-carriers with one or more apoE4 allele (20.1%). Therefore, it is unlikely that the apoE polymorphism is the source of the difference between the cases and controls. The more appropriate, separate comparisons between carriers and non-carriers from the cases and controls group could not be attempted for the apoE genotype distribution and apoE4 effect, due to small subgroup size.

The second factor investigated was the effect of BMI on Tg, apoE, Lp E:B and Lp CIII:B in carriers and non-carriers. Subjects were divided according to BMI levels (below or above 26) and carrier status for Asn9, separately for cases and controls. In both groups, the increase in the levels of the four traits associated with higher BMI was greater in carriers of the Asn9 variant than in non-carriers. For the controls, this effect
Table 6.5 Mean level of lipids according to BMI (< or > 26) in carriers and non-carriers of the Asn9 mutation. All values are in g/l.

a) controls

<table>
<thead>
<tr>
<th>Trait</th>
<th>BMI &lt; 26</th>
<th>BMI &gt; 26</th>
<th>% incr.</th>
<th>BMI &lt; 26</th>
<th>BMI &gt; 26</th>
<th>% incr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg</td>
<td>1.313</td>
<td>1.872</td>
<td>43%</td>
<td>1.430</td>
<td>1.835</td>
<td>28%</td>
</tr>
<tr>
<td>ApoE</td>
<td>0.054</td>
<td>0.070</td>
<td>30%</td>
<td>0.055</td>
<td>0.064</td>
<td>16%</td>
</tr>
<tr>
<td>LpE:B</td>
<td>0.339</td>
<td>0.537</td>
<td>58%</td>
<td>0.376</td>
<td>0.480</td>
<td>28%</td>
</tr>
<tr>
<td>LpC3:B</td>
<td>0.131</td>
<td>0.201</td>
<td>53%</td>
<td>0.145</td>
<td>0.180</td>
<td>24%</td>
</tr>
</tbody>
</table>

b) cases

<table>
<thead>
<tr>
<th>Trait</th>
<th>BMI &lt; 26</th>
<th>BMI &gt; 26</th>
<th>% incr.</th>
<th>BMI &lt; 26</th>
<th>BMI &gt; 26</th>
<th>% incr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg</td>
<td>1.789</td>
<td>2.838</td>
<td>60%</td>
<td>1.615</td>
<td>1.843</td>
<td>14%</td>
</tr>
<tr>
<td>ApoE</td>
<td>0.059</td>
<td>0.097</td>
<td>64%</td>
<td>0.059</td>
<td>0.064</td>
<td>8%</td>
</tr>
<tr>
<td>LpE:B</td>
<td>0.387</td>
<td>0.845</td>
<td>118%</td>
<td>0.423</td>
<td>0.476</td>
<td>13%</td>
</tr>
<tr>
<td>LpC3:B</td>
<td>0.142</td>
<td>0.342</td>
<td>241%</td>
<td>0.154</td>
<td>0.180</td>
<td>17%</td>
</tr>
</tbody>
</table>
was particularly strong for Lp E:B, with a 58% increase in carriers of the high BMI class compared to 28% in non-carriers with high BMI (Table 6.5a). In cases, the effect was more striking. The percent increases in trait levels observed in the high BMI group ranged from 60% for Tg to 241% for LpCIII:B in carriers compared to a range of 8% (ApoE concentration) to 17% (LpCIII:E) in non-carriers (Table 6.5b). With the exception of Tg in the carriers from the cases group, measured trait levels were similar or slightly lower in carriers from the low BMI class compared to non-carriers. These results suggested that BMI was mediating the effect of the Asn9 variant on lipid and Lp traits in the ECTIM samples. The interaction of Asn9 carrier status with BMI was tested for the four traits using the combined data from cases and controls. As expected, the increases in trait levels due to BMI were greater in Asn9 carriers and there was significant interaction between BMI and the Asn9 allele for the LpCIII:B trait (p = 0.01). The interaction term did not reach statistical significance for the other traits tested (Fig. 6.6).

6.3 Relationship between lipid levels or LPL activity and carrier haplotype.

From the results of section 4.7, it was concluded that the Asn9 mutation was carried on two major haplotypes as defined by the PvuII and HindIII polymorphisms, P+ H+ and P- H+. A third rarer haplotype was detected unequivocally only in the UK/Swedish/Dutch study. An important question is whether the effect of the Asn9 variant on lipid and LPL activity levels is similar for either of the major haplotypes. The detection of heterogeneity in lipid levels amongst haplotypes would suggest that the Asn9 variant is in linkage disequilibrium with another mutation causing the lipid phenotypes. Unfortunately, this question can only be partially addressed because the
Fig. 6.6. Effect of BMI on selected lipid/lipoprotein traits in Asn9 carriers and non-carriers from the ECTIM population. Data from cases and controls was pooled and subjects were re-classified into low (<26, n=23) or high (>26, n=21) BMI groups for both carriers and non-carriers, and analysis of variance was performed. The significance level for the interaction term is shown on the graph for each BMI-lipid trait pair tested.
number of carriers with unequivocal haplotypes is small and only very limited data is available for LPL activity.

In the UK/Swedish/Dutch study, only three individuals with unambiguous carrier haplotypes had LPL activity data. All three individuals displayed low activity relative to their sample means and two had the P-H+ carrier haplotype while the other one had the P+H+ haplotype. No other test was performed on this small group. In the ECTIM study, no LPL activity data was available. Plasma triglyceride levels were examined in thirteen subjects where the Asn9 carrier haplotype could be determined unequivocally, seven individuals who were homozygous for the P+ H+ Ser haplotype and six homozygotes for the P- H+ Ser haplotype. Mean plasma triglyceride levels were elevated in both groups compared to non-carriers (2.27g/l for P+ H+ and 2.02g/l for P- H+ vs. 1.69g/l). Thus, these data argue against the occurrence of heterogeneity amongst carrier haplotypes.

6.4 Effect of the N291S mutation in normolipidaemic subjects.

The impact of the N291S substitution on cholesterol and triglyceride levels was assessed in the two groups of healthy men from the UK. At entry in the study, the 24 men carrying the N291S mutation had slightly higher cholesterol (6%, 6.10 vs 5.77mmol/l) and higher triglyceride (18%, 2.09 vs 1.77mmol/l) levels than their non-carrier counterparts but this increase was not statistically significant. In 21 of these subjects, data was available from three subsequent cholesterol and Tg measurements. Cholesterol levels were non-significantly higher at all time points and overall in Ser291 carriers (Table 6.6). Tg were significantly higher in Ser291 carriers for the second and third year measurements and overall (p=0.05)(Fig.6.7). The elevation in Tg levels measured in carriers ranged from 12 to 30% with the largest difference seen at year 3.
Table 6.6 Cholesterol levels (mmol/l) in carriers of the N291S substitution and non-carriers from the UK and Scottish healthy men studies. Data from both groups was pooled and mean cholesterol levels were compared at baseline (BL) and during three-year follow-up. None of the differences are significant.

<table>
<thead>
<tr>
<th></th>
<th>BL</th>
<th>Year 1</th>
<th>Year 2</th>
<th>Year 3</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>N291S</td>
<td>6.11</td>
<td>5.75</td>
<td>5.76</td>
<td>5.69</td>
<td>5.83</td>
</tr>
<tr>
<td>controls</td>
<td>5.78</td>
<td>5.63</td>
<td>5.60</td>
<td>5.60</td>
<td>5.65</td>
</tr>
</tbody>
</table>

Fig. 6.7 Plot of mean (± SEM) plasma triglyceride levels in Ser291 carriers and non-carriers with time. Mean values at baseline and at subsequent three annual measurements for the 21 carriers individuals and 600 non-carriers with no missing data. Non-carriers show a negative downward trend over time ($p < 0.0001$).
6.5 Interaction between BMI and LPL mutations in normolipidaemic men.

In light of the effect of obesity on the expression of the Asn9 in the ECTIM study, the data from healthy UK men was re-examined to investigate the relationship between BMI (divided into tertiles), Tg levels and carrier status for the Asn9 and Ser291 variants (Fig. 6.8). As expected, there was a graded increase in plasma Tg across the BMI tertiles in the non-carrier groups. In contrast, both in Asn9 and Ser291 carriers, a much larger increase in Tg levels with was observed in the upper two BMI tertile group compared to the low BMI tertile group, with a significant BMI x genotype interaction for the Ser291 variant (p=0.02). The interaction term for the Asn9 variant approached statistical significance (p=0.07), similar to the ECTIM data presented in section 6.2.

Fig. 6.8. Effect of BMI on Tg levels in Asn9 and Ser291 carriers vs non-carriers from the healthy UK men (Camberley and St. Andrews) population. Data from cases and controls was pooled and subjects were re-classified into low (<25.0), intermediate (25.0 < BMI < 27.7) or high BMI (>27.7) groups and analysis of variance was performed. The probability value for interaction is shown for each mutation.
6.6 Summary and short discussion.

Lipid levels were examined in hyperlipidaemic and normolipidaemic carriers of the D9N and N291S substitutions from the UK, Sweden and the Netherlands. LPL activity and mass was also measured in D9N carriers.

In 10/13 carriers of the LPL-Asn9 variant (UK and Sweden) where data was originally available, LPL activity is below the mean for non-carriers in their respective control groups. The effect is more consistent in the healthy group (3/3) than the patients (7/10). This is not surprising since the positive correlation observed between plasma Tg levels and LPL activity in normolipidaemic individuals is not always present in HTG patients (Huttunen et al., 1976; Taskinen et al., 1987). This indicates that in HTG other factors modify the relationship between Tg and LPL. LPL is synthesised in skeletal muscle and adipose tissue and its expression is controlled in a complex tissue-specific manner by factors such as insulin levels, obesity, diet composition and alcohol intake (Garfinkel and Schotz, 1987; Braun and Severson, 1992). For instance, hyperinsulinaemia, together with obesity, has been shown to decrease muscle LPL activity and increase adipose tissue LPL activity (Brunzell et al., 1983b; Pollare et al., 1991). Weight loss is generally expected to correlate directly with a decrease in post-heparin plasma activity but it has been reported to increase adipose tissue LPL activity in some individuals (Schwartz and Brunzell, 1981; Kasim et al., 1991). Given the substantial contribution of adipose tissue to total LPL production (Cryer, 1987), the two situations outlined above (hyperinsulinaemia/obesity and weight loss) may cause an elevation of post-heparin LPL activity in some subjects and could explain our results. While insulin levels had not been measured in our high LPL/Asn9 carrier individuals, two of them were markedly above their ideal body weight, and one had been on a diet
at the time of sampling.

In the healthy UK men, those carrying the Asn9 variant have mean triglyceride levels elevated 24% above non-carriers while cholesterol levels remain unaffected. A similar increase in Tg is observed in carriers of the Ser291 variant. These effects are observed consistently over the 3-year study period. It can also be seen from the data that 23/33 Asn9 carriers have triglycerides above the group mean. However, there are thus 10 carriers, including one individual homozygous for the Asn9 variant, whose plasma triglyceride levels are not elevated (fig.6.1). This suggests that another factor, either genetic or environmental, must be interacting with the mutation in some carriers to cause the elevation in Tg. Alternatively, the observed scatter in Tg levels may be due to the influence of a Tg-lowering factor in carriers with low/normal Tg levels. The modulation of LPL expression may compensate to some extent for the decrease in activity associated with the Asn9 mutation. Studies using animal and tissue culture models have shown that LPL can be regulated by a variety of stimuli at the synthetic level or post-translationally, through increased transport from a large LPL intracellular pool (Semb and Olivecrona, 1989; Semenkovich et al., 1989b; Vannier and Ailhaud, 1989). Alternatively, on the assumption that the maximal capacity of the lipolytic system is lower in all Asn9 and Ser291 carriers, possible environmental factors include those such as diet or obesity which are known to affect the production of triglyceride-rich lipoproteins. I have presented evidence for an interaction between obesity, as measured by BMI, and both the Asn9 and Ser291 mutations in the determination of plasma Tg concentrations in healthy UK men (section 6.5). Additionally, the Asn9 homozygote with low Tg levels (subject 643, GP practice SE England, table 6.1b) has a low BMI (21.9 vs sample mean of 26.6 kg/m²), possibly reflecting a low-fat, low carbohydrate diet, as well as normal
LPL activity (Mailly et al., unpublished results). Further studies will be required to properly assess the relative importance of mechanisms involved in the determination of Tg and LPL levels in carrier individuals for these mutations.

In the ECTIM study, there were no differences in Tg or lipoproteins levels between carriers and non-carriers of the Asn9 variant in the control group. This is in contrast to the results obtained in the UK control groups and may be due to differences in genetic background or subject selection. In particular, the age range of the study subjects was much wider in the ECTIM group and included men as young as 25 years old. Wilson et al. (1990) have shown that age modulates the expression of hyperlipidaemia in heterozygotes for LPL deficiency in whom the disorder does not usually appear before age 40. It is therefore possible that the Tg-raising effect of the Asn9 allele had not yet become manifest in the younger ECTIM carriers. However, when individuals were divided according to their BMI (below or above 26), the increase in Tg, Lp CIII:B and apoE associated with high BMI was greater in carriers of the Asn9 variant compared to non-carriers. This combined effect of the Asn9 allele and BMI was even more pronounced in the ECTIM cases group suggesting that a large increase in the number of Tg-rich lipoproteins may put individuals at greater risk for CAD. The fact that no significant Asn9 frequency differences were seen between the cases and control groups may reflect the heterogeneity of the ECTIM cases. A smaller frequency difference between cases and controls was also observed in the other study where case selection was based on the occurrence of a myocardial infarction rather than hyperlipidaemia (YMI Swedish study).

In summary, it is clear that both the Asn9 and Ser291 variants are associated with an elevation of Tg concentration which is mediated at least in part by BMI levels and
the results from both the ECTIM and UK healthy men studies are in general agreement. However, fasting Tg levels give only partial indications on the dynamics of the metabolic process and are influenced by a wide range of factors. Useful insight into the effects of partial LPL deficiency may be gained through post-prandial studies involving larger number of carriers where the accumulation of specific lipoprotein particles such as Lp CIII:E or Lp B:E could be measured.
7. CONCLUSIONS & GENERAL DISCUSSION.

Hyperlipidaemia and atherosclerosis are polygenic diseases influenced to a significant extent by environmental factors. Even monogenic disorders of lipid metabolism such as lipoprotein lipase deficiency and familial hypercholesterolaemia display considerable biochemical and clinical heterogeneity, due to differences in genetic background and environment. Many individuals who develop premature heart disease report a family history of CHD although the pattern of disease seen may not be compatible with the inheritance of a single major gene. In most families, several different genes are likely to be contributing to the disease, their effects being modulated by dietary and biometrical factors, with each family having a different constellation of causative genetic and environmental factors. In this model, a mutation altering the function of a given gene would confer susceptibility on carriers but would have insufficient impact to cause disease on its own. Such a mutation would therefore be present in healthy individuals in the general population but should be found more frequently in patients with hyperlipidaemia.

7. 1 Short summary.

In this thesis, I describe the identification and characterisation of rare mutations as well as common variants in the LPL gene of healthy and hyperlipidaemic subjects and show the impact of these variants on LPL function and plasma lipid levels.

Chapter 3 reports the identification by SSCP and characterisation of mutations in the LPL gene of subjects with type I HLP from the UK and Northern Europe. SSCP proved a rapid and efficient first-line screening method for the detection of genetic variation. Eleven potentially functional mutations were identified in subjects with type
I HLP, of which six had not been reported elsewhere. Together with the gene defect (2kb deletion/insertion) detected by Southern blotting, the mutations identified in the type I cohort described here accounted for 75% (30/40) of the defective alleles. The molecular heterogeneity of LPL mutations underlying type I HLP in subjects of North European origin was highlighted by the presence of four different mutant alleles (S193R, P207L, 2kb insertion and one as yet unidentified) in the two patients from Sweden (LA and MVA). Additionally, both the heterogeneity and the clustering of mutations within exons 5 and 6 observed in our studies concurs with previous reports from North America (Santamarina-Fojo, 1992; Hayden and Ma, 1992; Reina et al., 1992).

Chapter 4 describes the screening by SSCP of the LPL gene of CHL patients with low plasma LPL activity and the identification of the common D9N variant. This variant detected originally in two subjects with low LPL and CHL was found to be common in healthy subjects but twice as frequent in matched hyperlipidaemics and MI sufferers from the UK, Holland and Sweden (section 4.5). The higher frequency seen in the patient groups suggested that the Asn9 variant might confer susceptibility to hyperlipidaemia and CHD. The overall frequency difference between MI cases and controls was not as marked in the ECTIM study (3.8 vs 3.1%) although two of the four populations studied (Lille and Toulouse) showed larger differences (section 4.6). The N291S substitution, originally identified in a type I subject (WG - chapter 3), was present in the CX CHL group but was also found to be common in the general population (section 4.9). None of the LPL mutations detected by SSCP in type I HLP patients were present in the CX CHL group. Moreover, the G188E substitution (the commonest mutation causing LPL deficiency) could not be detected in over 2300 normolipidaemic individuals and in over 800 MI sufferers from the
Camberley/St. Andrews, ECTIM and Swedish YMI studies.

Chapter 5 presents data from *in vitro* expression studies. Two of the mutations appeared to markedly decrease LPL activity (A158T, S193R) and one mutation (N291S) resulted in a protein with decreased stability (chapter 5). The D9N mutation was associated with a moderate decrease in LPL activity when expressed *in vitro*. The effect on LPL mass was slightly weaker in these experiments (section 5.4).

Finally, lipid levels or LPL activity measures were examined in normolipidaemic and hyperlipidaemic carriers of the D9N and N291S substitutions from the UK, Sweden and the Netherlands. These data are presented in chapter 6. A majority of normolipidaemic D9N carriers (10/13) had LPL activity levels below their sample mean. Normolipidaemic UK carriers of either variant had slightly (but significantly) elevated mean triglycerides compared to non-carriers while cholesterol levels were unaffected. These higher triglyceride levels were maintained through three subsequent annual measurements (section 6.1). Moreover, an interaction between BMI and LPL genotype with regards to Tg levels was apparent for both variants though this did not quite reach statistical significance for the Asn9 mutation. In the ECTIM case-control study, elevated Tg and apo B-containing lipoproteins were present in the Asn9 carriers from the case group compared to non-carriers while no effects were detected in the control group (section 6.2). When Asn9 carriers from both groups were pooled, significant interaction between BMI and the mutation was detectable for LpCIII:B particles (representing remnant particles), with a clear trend for Tg. Thus, these results clearly suggest that the metabolic alterations which underlie an increase in BMI can potentiate the impact of the Asn9 and Ser291 mutations on Tg or Tg-rich particles. Given their well-known links with obesity and hyperlipidaemia, insulin levels or other parameters of insulin resistance
represent prime candidates for explaining the observations reported in chapter 6.

7.2 Conclusions.

1) Extensive genetic heterogeneity underlies LPL deficiency in type I HLP patients from the UK and Northern Europe.

2) LPL gene mutations which cause type I HLP are not common in patients with CHL and/or low LPL activity and thus do not appear to cause FCHL.

3) There is no evidence yet for a separate set of LPL mutations causing FCHL but not type I HLP although some regions of the LPL gene have not been investigated, and the relationship between LPL mutations (in particular those with little or no effect on activity) and the LRP remains unexplored.

4) Common LPL gene variants are present in hyperlipidaemic and MI patients as well as in general population controls.

5) The higher frequency observed in the patient groups suggests that the variants confer susceptibility to hyperlipidaemia and CHD.

6) These variants are functionally significant as they decrease LPL activity (Asn9 and Ser291) and mass (Asn9) when expressed in vitro and they may therefore directly contribute to hyperlipidaemia, including FCHL.

7) SSCP is an efficient screening method for the detection of new, functionally important mutations.

8) PH LPL activity measurements may not always be sufficient for the identification of carrier individuals for mutations in the LPL gene as some subjects will have normal LPL activity.
7.3 Discussion.

7.3.1 Nature and functional significance of LPL variation in FCHL.

The SSCP screening of the CX CHL/low LPL group (chapter 4) suggests that LPL gene mutations which abolish LPL activity (and cause LPL deficiency and type I HLP in homozygous individuals) are unlikely to be responsible for a major proportion of FCHL cases. The two variants found in the CX CHL group do not cause FCHL on their own as they are commonly detected in the general population.

It is still possible that the results may have been influenced by the relative heterogeneity of the study group. However, my findings are in general agreement with two recent, independent reports where a similar approach was taken. In the first study, the D9N and S447stop substitutions were the only changes affecting the LPL amino acid sequence detected in 20 FCHL subjects with low plasma LPL activity (Nevin et al., 1994). In addition to these, Gagné et al. (1994) identified three other mutations resulting in amino acid substitutions (D21V, H44Y, G188E) in 31 French-Canadian FCHL patients. While the Asn9 mutation was shown by these workers to decrease LPL mass slightly in vitro, the G188E substitution, present in a single individual, was the only mutation that markedly decreased LPL activity (Emi et al., 1990b). It therefore appears that while sequence changes are quite common in the LPL gene of FCHL patients, few of these markedly affect LPL activity or mass. Thus, the lower LPL activity and mass seen in a subset of FCHL subjects (Babirak et al., 1992; this study) remains unexplained. Nevertheless, both the in vivo and in vitro evidence supports the view that the mutations with moderate effects on activity, such as the Asn9 and Ser291 substitutions, alter LPL function. They may thus contribute to the development of hyperlipidaemia, thereby conferring increased susceptibility to atherosclerosis in carrier
individuals. For the Asn9 variant, the small effect on activity and mass observed in the in vitro experiments is consistent with the in vivo data: in the three UK normolipidaemic carriers that were recalled, mean LPL activity and mass were reduced by 25-30%. For both mutations, higher Tg levels were found in normolipidaemic carriers and a clear interaction with BMI was observed. A recent study of NIDDM pedigrees has suggested that the Asn9 variant may contribute to the hypertriglyceridaemia in some families (Elbein et al., 1994).

Two additional elements support the contention that the Asn9 substitution is functional. Firstly, both common haplotypes observed with the Asn9 variant are associated with diminished LPL activity or increased triglyceride levels, making it less likely that the Asn9 variant is in linkage disequilibrium with an unidentified functional mutation elsewhere in the coding region or in the upstream promoter region of the LPL gene. It cannot be ruled out however that recombination events upstream of the PvuII or HindIII polymorphic sites may have given rise to the three haplotypes, and further studies with additional markers might clarify this point. Secondly, the Asp9 residue is completely conserved across species from guinea pig to man for LPL and is in close proximity to a $\beta$-strand motif, highly conserved amongst lipases suggesting its importance in the maintenance of enzyme structure (Bensadoun 1991; Derewenda and Cambillau, 1991). For instance, this segment may be crucial for the correct folding of newly synthesized LPL, ensuring normal cellular processing and effective secretion.

7.3.2 Functional LPL mutations may not always result in low LPL activity in carriers.

While the data that suggest that LPL gene mutations represent an unlikely cause
of FCHL, it is possible that some mutations may have been missed (also discussed in section 3.8) by the selection of individuals for molecular analysis based on a single low LPL activity measurement. Three of the Asn9 carriers identified in the present study had LPL activity levels at or above the sample group mean (figure 6.5). In the Swedish YMI study, two of the N291S carriers had amongst the highest LPL levels measured in that group (Fisher et al., submitted) and the two additional carriers identified in the CX CHL group had not been selected for SSCP screening as their LPL activity levels were not low. Moreover, Tenkanen and colleagues (1994) have described two individuals heterozygous for the Gln183 mutation (which abolishes activity) who have plasma LPL activity levels very close to the reported population mean. This implies either that higher LPL synthetic rate occurs naturally in these individuals or that more effective compensatory up-regulation of the functional protein can be induced through a feedback loop. Given the known complexity of LPL regulation, several site(s) of action and mechanisms are possible for both these processes. Depending on the tissues examined (muscle or adipose tissue), numerous factors including insulin levels, diet or exercise have been shown to affect LPL levels at the transcriptional, post-transcriptional or post-translational stages (Braun and Severson, 1992).

7.3.3 Possible mechanisms linking partial LPL deficiency to FCHL.

It is still not known whether partial LPL deficiency contributes to the hepatic VLDL apo B overproduction which is the hallmark of FCHL. However, mounting evidence suggests that fatty acid metabolism plays a crucial role in the development of this common hyperlipidaemia. An elegant series of studies by Sniderman and colleagues, combining tissue culture modelling of peripheral triglyceride synthesis
together with observations made in hyperapoB individuals (which are indistinguishable from FCHL subjects according to these workers), led to the proposal that the failure of peripheral tissues to take up FFA results in their increased uptake by the liver (Cianflone et al., 1989 & 1990a; Sniderman et al., 1992a). This is thought to be due to the inability of peripheral cells from these patients to increase triglyceride synthesis in response to the presence of ASP/BP I. This in turn is expected to increase VLDL synthesis and apo B secretion, as the influx of FFA appears to be the rate-limiting factor in the latter process (Boren et al., 1993). The insulin-resistant state characteristic of NIDDM, which often accompanies obesity and syndromes related to FCHL such as syndrome X and FDH, appears to further increase the availability of FFA for VLDL synthesis, probably through its stimulation of adipose-tissue lipolysis (Castro-Cabezas et al., 1993). In this context, an increase in plasma LPL activity rather than the decrease seen in LPL heterozygotes might be expected to promote the development of hyperlipidaemia.

An alternative role for LPL in the pathogenesis of FCHL should therefore be considered. Studies by Williams et al. (1991), using cultured hepatoma cells, have shown that incubation in the presence of LPL results in a reduction of the net secretion of new VLDL from the cells, because of rapid re-uptake of VLDL, possibly through interaction with the LRP and/or surface heparan sulphate proteoglycans (Williams KJ et al, 1992; Beisiegel et al., 1991). It is not yet known whether such a process actually takes place in vivo but LPL is present in the space of Disse in the liver. These data suggest that a futile cycle for VLDL secretion may exist and that fewer VLDL particles may be re-absorbed in the space of Disse in individuals with partial genetic or acquired deficiency of LPL. Thus, in combination with defective free fatty acid uptake in peripheral tissues, a decrease in LPL protein mass or in the ability to bind to the LRP
might result in apparent VLDL over-production and higher lipid levels in the plasma. Additionally, it has been shown that cellular uptake of normal VLDL as well as chylomicron remnants can be mediated through an interaction involving the LRP, LPL and/or HSPG (Mulder et al., 1992; Ji et al., 1993; Chappell et al., 1994). All classes of circulating plasma lipoproteins have been shown to contain LPL (Vilella et al., 1993). Although they are largely enzymatically inactive, these LPL molecules may still be able to interact with cell-surface HSPG which have been proposed to act as 'concentrators' of lipoproteins on the cell surface (Beisiegel et al., 1994). The relative importance of the LRP/HSPG pathway in the clearance of LPL-containing lipoproteins is not yet known but a decrease in LPL molecules could conceivably contribute to the impaired clearance of chylomicron remnants seen in FCHL patients (Cabezas et al., 1993).

Taken together, these studies suggest that the LPL protein’s ability to interact with the LRP, rather than LPL activity, would be the critical factors in the presumptive LPL-mediated hepatic uptake of lipoproteins, since the LPL-LRP interaction can occur independently of lipolysis (Williams et al., 1991; Beisiegel et al., 1991). An impaired interaction between Asn9-LPL and the LRP could be envisaged to explain the similar impact of the Asn9 and Ser291 variants on lipid traits, in spite of a more moderate effect on LPL activity and mass of the former. The proposed site of interaction between the LRP or HSPG and LPL, recently located to the C-terminal domain of the molecule (Nykjaer et al., 1994; Williams et al., 1994), argues against this hypothesis but if the head-to-tail model of the LPL dimer is correct (Wong et al., 1994), mutations in the extreme N-terminus might affect the C-terminus conformation. It cannot be excluded that the Asn9 substitution exerts its effect primarily through a reduction of LPL mass due to decreased secretion and that the more pronounced decrease in LPL mass seen with the
Ser291 mutation is counterbalanced by another factor in the subjects studied.

7.3.4 Relative contribution of heterodimers to LPL activity and mass in plasma.

One important question which cannot be readily assessed with current techniques is the relative proportion of LPL present as the Asn9 or Ser291 variant proteins in the plasma of carriers. As LPL is secreted as a dimer, heterodimers should theoretically account for approximately half of the heparin-releasable LPL mass in individuals heterozygous for LPL mutations, with the other half divided equally between the two homodimers. However, this distribution only holds true if the synthesis and stability of both proteins is comparable and if the variant monomer retains a comparable ability to form homodimers and heterodimers as the wild-type monomer. Based on the evidence presented in chapter 5 and on recent reports (Ma et al., 1993c; Reymer et al., 1995), it is expected that due to its lability, the Ser291 mutant will be under-represented in plasma relative to the wild-type protein. The \textit{in vitro} studies in section 5.4 also suggests that Asn9 homodimers are not produced as efficiently as their Asp9 counterparts. For both mutations, it is possible that heterodimer production may be even less efficient or unstable and if this is the case, the current tissue culture model would underestimate the impact of these variants. This could explain the similar decrease (20-30\%) in mass and activity observed in both the homozygous (\textit{in vitro}) and heterozygous (carrier individuals) settings for the Asn9 mutation.

7.4 Future studies.

The limitations inherent to the transient transfection technique when studying protein variants which only moderately decrease function have been pointed out earlier.
This is especially true for dimer proteins like LPL. The development of permanently transfected cell lines expressing high levels of LPL would therefore be of great usefulness, particularly if both the normal and mutant alleles could be transfected on a single vector and simultaneously expressed. This might be achieved with Chinese Hamster Ovary (CHO) cells transformed with the neomycin resistance gene (Cockett et al., 1990) or with insect cells infected with a recombinant baculovirus vector. Stable LPL exhibiting normal affinity for heparin and activation by apo CII has already been obtained using the latter system (T. Bruin, Ph.D. thesis 1994). Both systems might provide large amounts of mutant and wild-type LPL for the cell fractionation or pulse-chase type experiments needed to investigate the rate of secretion and dimerisation behaviour of interesting LPL variants such as Asn9 and Ser291.

However, the latter set of experiment will have to await the development of a method enabling the direct detection of heterodimers such as the Asn9/Asp9 species. One interesting possibility would be the separation of LPL homodimers and heterodimers with different charges, through isoelectric focusing in polyacrylamide gels. The Immobiline system, which allows the production of stable and narrow pH gradients, might be well suited for this task (Bjellqvist et al., 1982). In contrast to the traditional ampholyte species, the Immobiline amphoteric compounds that determine the pH gradient are acrylamide derivatives and thus are incorporated into the gel matrix. This characteristic could prevent the precipitation of LPL observed with traditional ampholyte mixtures. In addition, several investigators have reported the resolution of protein variants with polar amino acid substitutions in these gels (Bjellqvist et al., 1983; Gorg et al., 1985). The separation of Asn/Ser 291 homodimers and heterodimers might thus be achieved in this...
Complex segregation analysis of large pedigrees was used in two recent reports which present fresh evidence in favour of genetic heterogeneity in FCHL. The data suggest that up to three loci with large effects on apo B levels, triglyceride levels and the presence of LDL pattern B respectively may be involved (possibly acting additively) in determining the FCHL lipid phenotype, with contribution from minor loci (Jarvik et al., 1994; Cullen et al., 1994). Meanwhile, studies in FCHL patients have failed to identify common mutations in the coding segment of the LPL gene which lead to markedly decreased LPL activity and mass (Nevin et al., 1994; Gagné et al., 1994, this study). Additionally, neither the Asn9 or Ser291 substitutions appear to cause FCHL on their own in co-segregation studies (Elbein et al., 1994; de Bruin et al., unpublished results). Consequently, LPL protein polymorphisms are unlikely to represent a major cause of FCHL although they may well play a contributory role in the etiology of the disorder. Two other possibilities for LPL involvement should nevertheless be explored. Firstly, in view of the ability of LPL to bind the LRP and thus mediate the hepatic uptake of lipoproteins independently of its lipolytic function (Beisiegel et al., 1991; Williams et al., 1991; Chappell et al., 1994), it would be interesting to study the impact of recently reported mutations on the interaction with the LRP. Secondly, no investigations have been reported to date regarding genetic variation within the promoter of the LPL gene, which contains a number of regulatory elements, or the 2kb 3' untranslated region where alternative polyadenylation sites have been identified (Wion et al., 1987; Previato et al., 1991; Tanuma et al., 1995). Mutations in these regions could potentially reduce LPL synthesis through decreased transcription rate or lower mRNA stability and might provide an explanation for the low activity and mass seen in
a proportion of FCHL patients. Alternatively, alterations in tissue-specific control elements leading to an elevation in LPL production may either confer protection against hyperlipidaemia (muscle and adipose tissue LPL) or susceptibility to CHD due to increased lipid deposition in the atherosclerotic plaque (macrophage LPL).

Even if these studies finally exclude LPL as a major player in FCHL, they are likely to identify additional functional variation at this locus. A number of studies have demonstrated that genetic variation in the LPL gene contributes to the determination of lipid levels (Chamberlain et al., 1991; Stocks et al., 1992; Peacock et al., 1992; Ahn et al., 1993). LPL variants have also been examined in the context of atherosclerotic disease and the HindIII polymorphism was found to be associated with the severity of atherosclerosis in a Welsh population (Mattu et al., 1994) and the progression of the disease in the Swedish YMI study (Peacock et al., 1992). However, these polymorphisms are located in introns 6 (PvuII) and 8 (HindIII) and are unlikely to affect LPL function directly (Kirchgessner et al., 1989a). Recently discovered common LPL variants which alter the amino acid sequence such as the Ser447/Stop (Hata et al., 1990b), Asn291/Ser (Mailly et al., 1993; Ma et al., 1993c) and Asp9/Asn substitutions (Lohse et al., 1991; Mailly et al., 1995), do not appear to explain the associations detected between lipid traits and the LPL gene variants (Peacock et al., 1992). This strongly suggests that there are as yet unknown functional genetic variants which are in linkage disequilibrium with the HindIII and PvuII polymorphisms and can directly influence lipoprotein metabolism.

The results presented in this thesis provide strong additional evidence for a direct
role of variation in the coding region of the LPL gene in conferring susceptibility to hyperlipidaemia and ultimately increasing the risk of CHD, though a major role in FCHL is less likely. Further studies are warranted in hyperlipidaemic carriers of the Asn9 and Ser291 variants, since these individuals will have inherited other predisposing genetic factors, or adopted a high-risk lifestyle. It will be of interest to study the post-prandial response to fat or carbohydrate loads in such individuals as the effect of the mutation in this situation is physiologically more relevant and its magnitude may be greater. In particular, the nature and plasma half-life of potentially atherogenic lipoproteins should be monitored and related to insulin levels and other parameters of insulin sensitivity. Miesenbock and colleagues (1993) have demonstrated that in normolipidaemic carriers of the G188E mutation, an abnormal lipoprotein pattern is apparent in the post-prandial state, with increased residence time of atherogenic particles in plasma. Such experiments are labour-intensive however and require that a sufficient number of subjects be recruited for meaningful results to be obtained. Fortunately, it is now feasible to screen large numbers of subjects rapidly to identify carriers of specific mutations. This will undoubtedly facilitate the initiation of studies that are expected to shed light on the complex gene-gene and gene-environment interactions involving LPL, a fascinating and multi-faceted enzyme with an expanding role in metabolism.
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Estimation of the Frequency of Isoform–Genotype Discrepancies at the Apolipoprotein E Locus in Heterozygotes for the Isoforms


University College and Middlesex School of Medicine, Department of Medicine, The Rayne Institute, London, UK (F.M., S.E.H.), Department of Epidemiology, University of Michigan, Ann Arbor, Michigan (P.M.), Mayo Clinic and Foundation, Rochester, Minnesota (B.A.K.), and Department of Human Genetics, University of Pittsburgh Graduate School of Public Health, Pittsburgh, Pennsylvania (M.I.K., R.E.F.)

Estimates of the impact of apolipoprotein E (apo E) alleles coding for the three common isoforms on plasma lipid levels assume genetic homogeneity among the genotype classes. To test this assumption, we have determined the apo E genotype at the two common polymorphic sites (amino acids 112 and 158) by DNA amplification and hybridisation with allele-specific oligoprobes, in 195 unrelated Caucasian participants of the Rochester Family Heart Study previously classified as heterozygotes by isoelectric focusing (IEF). Fourteen discordant samples were initially detected. Repeat typing of these samples by both methods resolved nine discrepancies and analysis of additional blood samples from the remaining five individuals eliminated a further four discrepancies. The only truly discordant allele was found in a female subject who had an E3 isoform with the common E2 (Cys112, Cys158) genotype. Transmission of this allele from the mother was demonstrated. From these results, we estimate the frequency of discrepancies between isoforms and common genotypes to be 0.25% in this population. Allele misclassification was caused by poor amplification of the DNA in six samples and superimposition of glycosylated and nonglycosylated apo E isoforms on isoelectric focusing gels in five samples. We conclude that the assumption of genetic homogeneity among genotype classes is valid and that misclassification due to technical difficulties is more frequent than true discrepancies. © 1992 Wiley-Liss, Inc.
INTRODUCTION

Polymorphic allelic variation at the human apolipoprotein E (apo E) locus is known to have a significant role in determining interindividual variation in plasma levels of apolipoprotein E, apolipoprotein B, total cholesterol, and LDL-cholesterol in the general population [Sing and Davignon, 1985; Davignon et al., 1988; Kaprio et al., 1991; Xhignesse et al., 1991]. These effects have been replicated in a variety of human populations of diverse genetic background exposed to a variety of cultural and dietary environments [Boerwinkle et al., 1987; Sepehnia et al., 1989a]. Apolipoprotein E genetic variation can be detected at the protein level using a variety of electrophoretic techniques [Utermann et al., 1977; Zannis et al., 1981; Bouthillier et al., 1983; Kamboh et al., 1988]. Detection at the DNA level can be achieved by amplification of the DNA segment containing E2/E3 and E3/E4 allelic substitution sites using the polymerase chain reaction (PCR) followed by hybridisation to allele specific oligonucleotides [Funke et al., 1986; Emi et al., 1988; Houlston et al., 1989 Smeets et al., 1988] or digestion with the restriction endonuclease Hhal to yield allele specific cleavage patterns [Hixson and Vernier, 1990]. Genetic heterogeneity in the common E2, E3, and E4 phenotypes has been recognized in allele products which give a similar or identical isoprotein pattern as for one of the common alleles. These involve distinct, rare, amino acid substitutions and are frequently associated with familial hyperlipoproteinemia.

The measured genotype approach used in the estimation of allelic effects associated with the apo E polymorphism assumes allelic homogeneity within phenotype classes. With the exception of the study of Emi et al. [1988], there have been no population-based studies to estimate the frequency of discrepancies between the isoforms determined by IEF and the genotypes at the apo E locus. In this context, discrepancies refer to alleles giving rise to the protein phenotype of one of the common apo E alleles, but which contain nucleotide substitutions different than those causing the common polymorphism. We have used a combination of protein typing by IEF-immunoblotting and genotyping using the PCR technique followed by hybridization to allele specific oligonucleotides (ASO) to estimate directly the frequency of discrepant alleles occurring in the E2, E3, and E4 isoform classes in a sample of individuals heterozygous for the protein polymorphisms and selected from the general population of Rochester, MN. In the course of comparing genotype assignments using these two methods, we have identified common sources of error associated with phenotyping and genotyping. The results are discussed in the context of application of the measured genotype approach to the estimation of allelic effects in population samples.

MATERIALS AND METHODS

Sample

All samples for this study were drawn from the Rochester Family Heart Study described by Moll et al. [1989] and Turner et al. [1989]. The Rochester Family Heart Study was designed to evaluate the effects of environment and inherited traits on lipid transport and hypertension. In January 1984, we arranged for officials of the school system in Rochester, MN, to send letters to 5,270 households having two or more children enrolled in the city's public and parochial schools. In the letter, we first described
the purpose of the study and then requested each household willing to consider participation in the study to return an enclosed questionnaire providing information about the household (number of children in the household, projected future length of residence in Rochester, number of grandparents living in the Rochester area), phone number, and address. The questionnaire did not contain any queries regarding disease status or family history of disease. From a total of 1,812 questionnaires that were returned (response rate of 34.4%), 159 households were judged unsuitable for sampling either because they (1) did not want to be contacted further \( n = 105 \), (2) reported they planned to move from Rochester within 12 months \( n = 12 \), (3) reported there were only adopted children in the household \( n = 17 \), or (4) gave various other reasons \( n = 25 \) that participation was unlikely (e.g., unwillingness to visit the clinic or have blood drawn). Between December 1, 1984 and January 1, 1988 we contacted 436 of the 1,653 households eligible for sampling, and 2,004 individuals identified by 300 households agreed to participate and completed a clinic visit. These 2,004 individuals were distributed among 276 pedigrees ranging in size from 1 to 24 (mean 7.3). The study reported here is based on 567 unrelated spouses from these pedigrees who were parents of the index school children.

Among the 567 individuals eligible for the present study, 565 were typed for Apo E. Three were E2/2, 65 were E3/2, 329 were E3/3, 10 were E4/2, 149 were E4/3, and 9 were E4/4. Of the 224 heterozygotes, 195 (58 E3/2, 10 E4/2, and 127 E4/3) had both plasma and DNA samples available for the present study and were used to estimate the frequency of discrepancies.

**LABORATORY METHODS**

Plasma cholesterol, triglyceride, HDL-cholesterol (HDL-C), and apolipoproteins AI, AII, B, CII, CIll, and E levels were measured as described by Kotkke et al. [1991].

Apolipoprotein E phenotypes were determined from frozen EDTA plasma by the isoelectric focusing-immunoblot method of Kamboh et al. [1988] on samples transported on dry-ice. DNA was extracted from peripheral white blood cells by the Triton X-100 lysis method [Kunkel et al. 1977] and sent to London on dry-ice for analysis.

Apo E genotypes were determined by direct amplification of a 330 bp region spanning both the E2/E3 and E3/E4 allelic sites (amino acids 94–203), and detection by hybridisation to end-labelled allele-specific oligonucleotides, following transfer to Hybond-N filters (Amersham), essentially as described by Houlston et al. [1989]. The allele-specific oligoprobes (ASO) were however shortened as follows: Arg112, 3’-CCGGCCGGTGAGCACT-5’; Cys112, 3’-CCTGACACGCCAGG-5’; Cys158, 3’-GGTCGCGAGCAGAC-5’; Arg158, 3’-CGTCTTACCGGACGG-5’. Hybridisation was carried out at 42°C and filters were washed briefly at 49–50°C. In later experiments, a second Arg112 probe, 3’-CCGGTGGTCGAGG-5’, containing an additional mismatch at the 6th nucleotide from the 3’ end (T for C), was used. The discriminant washing temperature for this probe was 53–54°C in 3× SSPE (1× SSPE = 0.15 M NaCl, 0.01 M NaH₂PO₄, 0.001 M EDTA, pH 7.4).

Genotyping was also carried out by amplification of a 216 bp region, again spanning both polymorphic sites (amino acids 104–177), with internal primers (5’-CTGGGCGGACATGGAGGAAGCT-3’ and 5’-GATGGCCGTAGACGCGCCTG-3’), followed by digestion with the restriction enzyme HhaI. Separation of the frag-
ments was achieved on 8% polyacrylamide gels (acrylamide:bis, 19:1), run for 3 hr at 35 mA (constant current) in Tris-borate buffer (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA).

Statistical Methods

Statistical tests that gave $P < 0.05$ were considered significant unless otherwise noted. Analysis of variance was used to compare the 195 heterozygotes included in this study with the 29 heterozygotes that were not. Lipids, lipoproteins, and apolipoproteins levels were compared between individuals with discrepant alleles and all other heterozygotes from the Rochester Family Heart Study with the same isoforms (or phenotype). Gender- and phenotype-specific multiple regression was used to estimate the effects of concomitants (date of assay, age, age$^2$, height, weight, and smoking) in those without the discrepant alleles. The levels of the traits were then adjusted for variability in concomitants for all of the individuals with the same gender and phenotype (both with and without the discrepant alleles) using these regression equations. Percentiles were calculated for the individuals with the discrepant alleles.

RESULTS

The distribution of age, measures of body size, lipids, lipoproteins, and apolipoproteins for the heterozygous individuals used in this study are described in Table I for the 91 males and 104 females. When the 195 heterozygotes included in this study were compared to the 29 heterozygotes not included, there were no differences in the mean levels of any traits considered or in the relative frequency of males (data not shown).

Biochemical and molecular analysis of 195 unrelated individuals heterozygous for apo E phenotypes by isoprotein typing revealed a number of inconsistent phenotypes, as shown in Table II. Among the 14 inconsistent phenotypes, five were resolved by repeat isoprotein typing (two on the redrawn sample), six were resolved by repeat ASO analysis, two were found to be due to sample mislabelling and were resolved by resampling of the individuals involved, and one was found to represent a true discrepancy. Thus, this analysis detected one discrepant allele among the 195 individuals tested.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Females (n = 104)</th>
<th>Males (n = 91)</th>
</tr>
</thead>
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<tr>
<td>Age (years)</td>
<td>41.2 (6.3)</td>
<td>43.5 (6.9)</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>164.8 (6.1)</td>
<td>178.2 (6.1)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>68.9 (13.7)</td>
<td>85.4 (12.8)</td>
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<tr>
<td>Chol (mg/dl)</td>
<td>179.7 (36.8)</td>
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</tr>
<tr>
<td>Trigs (mg/dl)</td>
<td>111.1 (68.9)</td>
<td>163.0 (108.9)</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>50.0 (13.0)</td>
<td>39.6 (10.2)</td>
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<tr>
<td>Apo AI (mg/dl)</td>
<td>140.1 (18.7)</td>
<td>130.4 (15.8)</td>
</tr>
<tr>
<td>Apo AII (mg/dl)</td>
<td>34.2 (4.9)</td>
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<td>Apo B (mg/dl)</td>
<td>75.6 (15.3)</td>
<td>80.0 (14.4)</td>
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<tr>
<td>Apo CII (mg/dl)</td>
<td>2.0 (0.8)</td>
<td>2.7 (1.0)</td>
</tr>
<tr>
<td>Apo E (mg/dl)</td>
<td>4.4 (1.9)</td>
<td>5.5 (4.3)</td>
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TABLE II. Apo E Genotype-Phenotype Discrepancies and Partial Resolution Following Retyping of Original Samples and Typing of Redraw Samples

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<th>Sample</th>
<th>First protein typing</th>
<th>First DNA typing — original samples</th>
<th>Second protein typing — original samples</th>
<th>Second DNA typing — original samples</th>
<th>Third protein typing — redraw samples</th>
<th>Third DNA typing — redraw samples</th>
<th>Status</th>
<th>Probable cause of discrepancy</th>
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<td>288</td>
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<td>3/3</td>
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<td>4/3</td>
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<td></td>
<td>Resolved</td>
<td>PA*</td>
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<tr>
<td>296</td>
<td>3/2</td>
<td>3/3</td>
<td>3/2</td>
<td>3/2</td>
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<td></td>
<td>Resolved</td>
<td>PA</td>
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<tr>
<td>330</td>
<td>4/3</td>
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<td>4/3</td>
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<td></td>
<td>Resolved</td>
<td>PA</td>
</tr>
<tr>
<td>575</td>
<td>4/3</td>
<td>4/4</td>
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<td>4/4</td>
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<td>Resolved</td>
<td>?</td>
</tr>
<tr>
<td>1068</td>
<td>3/2</td>
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<td>4/3</td>
<td>4/3</td>
<td></td>
<td></td>
<td>Resolved</td>
<td>SGI</td>
</tr>
<tr>
<td>1651</td>
<td>3/2</td>
<td>4/2</td>
<td>3/2</td>
<td>3/2</td>
<td></td>
<td></td>
<td>Resolved</td>
<td>PA</td>
</tr>
<tr>
<td>1843</td>
<td>3/2</td>
<td>4/2</td>
<td>3/2</td>
<td>3/2</td>
<td></td>
<td></td>
<td>Resolved</td>
<td>PA</td>
</tr>
<tr>
<td>499</td>
<td>4/2</td>
<td>3/3</td>
<td>4/2</td>
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<tr>
<td>1084</td>
<td>3/2</td>
<td>2/2</td>
<td>—</td>
<td>2/2</td>
<td>2/2</td>
<td>2/2</td>
<td>Resolved</td>
<td>Sample mix</td>
</tr>
<tr>
<td>1612</td>
<td>3/2</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
<td>Resolved</td>
<td>SGI</td>
</tr>
<tr>
<td>631</td>
<td>3/2</td>
<td>2/2</td>
<td>3/2</td>
<td>2/2</td>
<td>3/2</td>
<td>2/2</td>
<td>Unresolved</td>
<td>Mutation</td>
</tr>
</tbody>
</table>

*PA, poor DNA amplification.
*SGL, superimposition of glycosylated isoform.
*Cause of inconsistency unknown.
*Sample not available.

an E3 allele at the protein level which was a common E2 (Cys112, Cys158) at the gene level, for an overall frequency of approximately 0.25%. Subject 631 was heterozygous by isoprotein typing (E3/2) was apparently homozygous by ASO analysis (E2/2). To confirm the genotyping, DNA fragments were amplified spanning the region of the apo E gene coding for amino acids 104–177, the DNA digested with the enzyme HhaI, and the size of the resulting fragments analysed by polyacrylamide gel electrophoresis. The pattern of fragments seen was as expected for their ASO genotype. DNA and plasma samples were available for analysis for family members of the individual carrying a discrepant allele, and the results and pedigree ascertained through household 1749 are shown in Figure 1. The proband’s mother also showed the same discrepancy, having an allele which behaved as an E3 allele on isoelectric focusing but appeared as an E2 allele by ASO typing, but neither of the children of the proband inherited the discrepant allele.

Description of Subjects With Discrepant Alleles

A description of the individual and her mother with a discrepant allele is presented in Table III. Based on the physical examination data and medical record review, subject 631 had fasted, had never smoked, and did not report the use of any medications in the 2 months prior to the study. In 1980, she was diagnosed with mitral prolapse at the Mayo Clinic. Otherwise she was considered not to have any other cardiovascular disease, was normotensive, and had had four normal ECGs. Her trait levels, after adjustment for concomitants, placed her above the 95th percentile for HDL-C and below the 5th percentile for apolipoprotein B compared to all other females in the
DISCUSSION

A useful approach to the study of quantitative traits associated with risk of common disease is to characterize the association between allelic variation at candidate gene or random marker loci and quantitative trait levels [Boerwinkle et al., 1986]. The measured genotype approach has proven to be useful for estimating the impact of specific alleles on quantitative traits in samples from the general population. This approach has demonstrated a significant contribution of allelic variation at the apolipoprotein E locus in determining plasma levels of apo E, apo B, LDL-cholesterol, and total cholesterol in a number of populations [Sing and Davignon, 1985; Boerwinkle
TABLE III. Description of the Proband and One Relative With Discrepant Alleles (Percentile Compared to Other Females of Same Phenotype After Adjustment for Concomitants)

<table>
<thead>
<tr>
<th>Trait</th>
<th>Proband</th>
<th></th>
<th>Mother</th>
</tr>
</thead>
<tbody>
<tr>
<td>Identification No.</td>
<td>631</td>
<td>677</td>
<td></td>
</tr>
<tr>
<td>Household</td>
<td>1749</td>
<td>1749</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>Female</td>
<td>Female</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>35.7</td>
<td>62.4</td>
<td></td>
</tr>
<tr>
<td>Height (cm)</td>
<td>162.4</td>
<td>161.2</td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>55.4</td>
<td>82.5</td>
<td></td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>149.0 (18.3%)</td>
<td>237.0 (74.2%)</td>
<td></td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>82.0 (43.1%)</td>
<td>405.0 (98.8%)</td>
<td></td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>79.0 (96.3%)</td>
<td>49.0 (59.5%)</td>
<td></td>
</tr>
<tr>
<td>Apo AI (mg/dl)</td>
<td>170.0 (89.9%)</td>
<td>163.0 (86.1%)</td>
<td></td>
</tr>
<tr>
<td>Apo AII (mg/dl)</td>
<td>41.1 (79.8%)</td>
<td>40.3 (91.3%)</td>
<td></td>
</tr>
<tr>
<td>Apo B (mg/dl)</td>
<td>51.0 (3.7%)</td>
<td>84.0 (31.1%)</td>
<td></td>
</tr>
<tr>
<td>Apo CII (mg/dl)</td>
<td>2.3 (51.3%)</td>
<td>4.1 (90.8%)</td>
<td></td>
</tr>
<tr>
<td>Apo CIII (mg/dl)</td>
<td>18.0 (79.8%)</td>
<td>25.5 (95.6%)</td>
<td></td>
</tr>
<tr>
<td>Apo E (mg/dl)</td>
<td>8.1 (89.0%)</td>
<td>5.3 (62.7%)</td>
<td></td>
</tr>
</tbody>
</table>

and Sing, 1987; Boerwinkle et al., 1987; Davignon et al., 1988; Kaprio et al., 1991]. Additionally, it has been used to examine the impact of genetic variation at a variety of loci coding for genes that are directly involved in lipid metabolism on quantitative lipoprotein lipid traits [Sepehmia et al., 1989b]. The measured genotype approach assumes allelic homogeneity. If heterogeneity exists, then the interpretation of the estimated allelic effects could be biased. The definition of what constitutes similar alleles would affect the estimate itself. Variability between subgroups with respect to the frequency of and variability in discrepant alleles could impact the interpretation of comparison between presumed genotype classes.

Initially, we identified 14 inconsistencies between phenotype and genotype. Six of these were due to technical difficulties with the ASOs used. Poor amplification of DNA, which occurs with some samples possibly because of contamination with salt during the preparation, resulted in a poor discrimination between the signal seen with the $\text{Cy}_{512}$ probe and the $\text{Arg}_{112}$ probe. This was resolved on a second amplification and by the use of an ASO with an additional mismatched base which enabled the different washing conditions used to be more effective. The major source of discrepancy for protein typing (four samples) appears to be due to the glycosylation of apo E isoforms. The electrophoretic patterns of apolipoprotein E isoforms on IEF gels are a direct result of charge variations dependent on both genetic variation at the apolipoprotein E gene locus, and posttranslational modification effects which cause a shift in isoform mobility [Zannis and Breslow, 1981]. This in turn results in the superimposition of glycosylated and nonglycosylated forms in heterozygotes. Recently, Snowdon et al. [1991] have reported that diabetic subjects, which have a 2- to 10-fold increase in protein glycosylation, show a disparity between the apo E allelic frequencies obtained by protein and DNA typing. Therefore, it is likely that differences in the level of glycosylation, determined in part by the plasma glucose level, explain some of the inconsistencies. In particular, individual 1622, initially E3/2 at the protein level but with an E3/3 genotype, had elevated plasma glucose (97% percentile compared with other E3/2 RFHS individuals after adjustment for concomitants). Alternatively, the amount of protein
loaded on the gels for these individuals might influence the typing. Another possible source of error, not encountered in this study, is that lipoproteins with the apo E4 protein are known to have a faster metabolic rate, and are cleared rapidly from the circulation. This causes a low plasma concentration of the apo E4 isoform which therefore may escape detection.

It is of interest that of the original 14 inconsistencies between phenotype and genotype, only one involved an individual with a truly discrepant allele. Two could only be resolved by obtaining new samples from the participants due to an error in sample labeling. This represents a laboratory misidentification rate of approximately 1% (2 of 194 if excluding the individual with a true discrepant allele). Of the remaining 11 inconsistencies, 6 samples were incorrect for only the first DNA typing, 4 samples were incorrect for only the first protein typing, and one sample was incorrect for both the first typings. We estimate the sample error rates to be approximately 3.6% for the DNA typing (7 of 192) and 2.6% (5 of 192) for the protein typing. Both of these error rates are 2 to 3 times higher than the relative frequency of errors in sample identification and at least 5 times higher than the relative frequency of individuals with discordant alleles in this study. Based on these data, laboratory errors in typing for apo E have the potential to contribute more to bias in measured genotype studies than sample identification errors or discordant alleles for apo E heterozygotes.

Apolipoprotein and lipoprotein levels in the two individuals identified in this study as carrying a discordant allele were not indicative of the lipoprotein profile associated with Type III hyperlipoproteinemia. This disorder, characterized by hypercholesterolemia, hypertriglyceridemia, remnant lipoproteins, and the E2/2 phenotype, has been the basis of identifying variant apo E alleles in a number of studies. However, the discordant allele may be involved in the development of hyperlipidemia. While individual 631 had very low lipid levels, characteristic of normolipidemic individuals with the E2/2 phenotype, her mother was clearly hypertriglyceridemic. Drawing a parallel with type III, it is possible that their apo E genotypes predisposes them to this phenotype but that an additional factor, such as obesity, is needed to cause hyperlipidemia. Although we have not attempted the further molecular characterization of this mutant allele, the presence of E2-Christchurch in these individuals can be ruled out as this mutation would have been revealed by the cleavage pattern with Hhal.

The results presented here support the assumption of near homogeneity within the three heterozygous apo E genotype classes in Caucasian populations, with an estimated frequency of discrepancies of less than 1%. This is very likely to represent an underestimate as neutral amino acid substitutions, which are expected to be more frequent, were not accounted for by the methodology used. However, the methodological problems outlined above may still be proportionately much more important for the misclassification of individuals. We cannot rule out that a discrepant allele could have been missed although this is less likely since the inheritance of apo E alleles was checked in the families of all the subjects involved in this study. The results are nevertheless consistent with the estimate of 2% (two variant alleles among 100 alleles tested) seen by Emi et al. [1988] in the Caucasian population of Utah. Whether this estimate applies to non-Caucasian populations and whether population differences in the frequency of apo E variant alleles contributes to differences in estimated allelic effects in some populations, remains to be determined.
ACKNOWLEDGMENTS

This work was supported by NIH Grant 5 RO1 HL 39107-03, Grants HL24489 and HL30428, by the Charing Cross Sunley Research Trust, and Grants from the British Heart Foundation (RG5). France Mailly is the recipient of a Research Traineeship from the Canadian Heart and Stroke Foundation. We thank Dr. Charles F. Sing for his constructive comments on this manuscript.

REFERENCES


Edited by Aravinda Chakravarti
ABSTRACT. Over the last 10 years, the explosion of molecular biology and molecular genetic techniques have allowed major advances in the diagnosis and management of a wide variety of human disorders. These range from accurate and simple screening for carriers of thalassemia (Old JM, Varawalla NY, Weatherall DJ: Lancet 2:834-837, 1990) to the use of preimplantation diagnosis of embryos at risk for untreated congenital defects (Monk M, Holding C: Lancet 1:985-988, 1990) and the development of gene therapy for treatment of disorders such as adenosine deaminase deficiency (Sharp D: Lancet 1:1277-1278, 1991). These same molecular techniques have also been applied to pediatric lipid disorders with some notable successes, both in their diagnosis and understanding the mechanisms of the resulting pathology, including the recent experiments (Wilson JM, Grossman M, Wu CH, Chowdhury NR, Wu GY, Chowdhury JR: J Biol Chem 267:963-967, 1992) that have led to proposals to treat homozygous familial hypercholesterolemia by gene therapy. The purpose of this review is to detail this molecular genetic progress for two of the disorders that result in disturbed triglyceride metabolism in infants, lipoprotein lipase deficiency and apo CII deficiency, and four disorders that lead to disturbed cholesterol levels in infancy, abetalipoproteinemia, hypobetalipoproteinemia, familial defective apo B, and familial hypercholesterolemia. We will also address the question of how knowledge of the mutation causing the defect in a particular patient could be clinically useful and highlight areas of research for the future. (Pediatr Res 34: 403-415, 1993)

Abbreviations

FH, familial hypercholesterolemia
LPL, lipoprotein lipase
FDB, familial defective apo B100
PCR, polymerase chain reaction
SSCP, single-strand conformational polymorphism
TG, triglyceride
RFPL, restriction fragment length polymorphism
ABL, abetalipoproteinemia
HBL, hypobetalipoproteinemia
LDL-R, LDL-receptor

MOLECULAR TECHNIQUES FOR IDENTIFICATION OF MUTATIONS

The genes for most of the apolipoproteins, enzymes, and receptors that are involved in lipid metabolism have now been cloned, and their DNA sequence and arrangement of introns and exons determined and published (reviewed in 1 and 2). The control of expression of these genes is very complex and must be coordinated in response to a number of environmental challenges, rapidly in the postprandial state and more slowly in adaptation to hormonal changes, for example, at puberty or during pregnancy. Molecular details of these control processes are not yet fully understood, but excellent progress has already been made (3). Taken together, this information provides the framework for the identification of the mutations occurring in different patients with pediatric lipid disorders.

Several methods have been published that allow rapid comparison of the sequence of specific fragments of DNA from different individuals amplified in vitro by PCR (4). The first uses chemical cleavage of mismatched bases in the duplex formed between two heterologous DNA fragments after hybridization (5). This is a slow but robust technique that has been used successfully to look for mutations in the apo B gene (6). Because it is based on chemical methods, the technique is able to detect all mismatched bases irrespective of sequence (7), and individuals can be identified who are heterozygous for any sequence difference compared with the normal "probe" DNA, which is radio-labeled with $^{32}$P deoxyctydine triphosphate. Fragments of DNA of about 500-600 bp give good results, but for longer fragments the yield of amplified DNA is reduced, and cleaved mismatches within 50-60 bp of the ends of the DNA fragments may not be detected (as a size reduction) on gel fragments of over 500-600 bp. This can be overcome by using amplifying oligonucleotides that produce fragments that overlap by 100 bp.

A second approach is the use of denaturing gradient gel electrophoresis (8), which again appears to detect all possible mutations. A recent report has used this method to screen the promoter region of the LDL-R for mutations in FH patients, but none were identified (9). The third method is called SSCP and is based on the fact that single base changes result in conformational changes in single-stranded DNA that can be detected as a different pattern of migration on a polyacrylamide gel (10). DNA fragments are labeled by inclusion of $^{32}$P deoxyctydine triphosphate in the amplification mixture and subsequent detection by x-ray film. The advantage of this method is that it is rapid and does not use toxic chemicals or require a hybridization step, but it is not yet clear whether it detects all single base changes. The frequency of such "false negatives" has been reported to be low in one study (11), but this probably depends on the specific sequence or base composition of the DNA being studied, and the procedure may not be equally efficient for all genes or all exons of the same gene. To attempt to overcome this problem, different SSCP gels are run with varying conditions of tempera-
ture and gel porosity. As with chemical cleavage of mismatched bases, the ability to detect differences in migration patterns is reduced in fragments over 300-400 bp. Over the last year, we have used this method to detect sequence changes in the apo B and LPL gene (12), and an example of this method to detect differences in the migration pattern is shown in Figure 1 (13). DNA samples were available from patients with five different mutations in this part of the gene, including a 3-bp and a 2-bp deletion, and three different single base substitutions. All of these gave a pattern of fragments distinguishable from that seen with normal DNA.

Once a sequence change in a gene has been identified by these methods, direct sequencing of amplified DNA can be used (14) to determine the precise change and its potential effect on the function of the protein. This may be confirmed by expression of the protein in vitro and appropriate assays of function. The next stage would be to develop screening methods to identify relatives of the proband or individuals in other families who are carriers of the mutation. A number of PCR-based methods are available for such screening that do not require radiolabeled probes, but rely instead on fluorescence (15) or chemiluminescence (16). This can be carried out directly where the mutation creates or destroys a restriction enzyme site in the gene by separating the different-sized amplified DNA fragments by gel electrophoresis (17), or by the amplification refractory mutation system method using differential amplification oligonucleotides (18, 19). Alternatively, sequence changes can be detected in a few hours by allele-specific oligonucleotide melting with, for example, biotinylated oligonucleotides and streptavidin conjugated with horseradish peroxidase and an appropriate chromogenic system (20). Reagents for such detection systems are commercially available; the labeled probe is stable for many months and thus is suitable for use in a routine diagnostic laboratory. For mutations that occur with low frequency, a “pooling” strategy may be appropriate. Small volumes of whole blood (or DNA isolated by rapid methods from Guthrie blood spots) can be pooled, for example, in batches of 10, and these batches are then pooled in a cumulative fashion. Using PCR methods, the detection of one variant allele in 100 (i.e. 50 samples pooled) has been reported (21), and in the future new methods may result in greater sensitivity. Such pools of DNA can be efficiently screened for many rare mutations in different genes, and the relevant individuals can be easily identified by rescreening the subpools.

**MUTATIONS AFFECTING PLASMA TRIGLYCERIDE LEVELS**

**LPL.** LPL is a heparin-releasable enzyme, bound to glycosaminoglycan components of the capillary endothelium, with a central role in lipid metabolism (22, 23). LPL is found in a variety of tissues, including muscle, adipose tissue, and macrophages. It is a glycoprotein and is active as a dimer of two identical subunits each of approximate molecular weight 60,000. LPL, which has an essential requirement for an apoprotein cofactor apo CII, hydrolyzes TG in large TG-rich lipoproteins (chylomicrons and VLDL) (reviewed in 22, 23). The three-dimensional structure of human LPL has been deduced by comparison with the x-ray crystallography data obtained from the highly homologous human pancreatic lipase (24). The amino terminal two thirds of the protein contain the catalytic triad in a mostly parallel β-sheet structure, with the catalytic triad residues (Leu^232, His^235, and Ser^237) being buried in a hydrophobic pocket covered by a surface loop comprising residues 238-262, which is rotated away as part of the interaction of the enzyme with the lipid substrate. The carboxy-terminal domain consists mainly of antiparallel β-sheet structures and is thought to have a major function in lipid binding (27). The region that interacts with the apo CII cofactor is thought to be within the N-terminal portion of the protein, although the precise location has yet to be determined (28). There is evidence to suggest that binding to heparin is conferred by residues in the regions 290-300 (29).

The gene for LPL has been located to the short arm of chromosome 8 (30), and cDNA sequence and the gene structure have been elucidated (31–34). The 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. Several RFLP in the LPL gene have been reported including a Pvull RFLP in the intron between exons 6 and 7 (35) and a HindIII RFLP in the intron between exons 8 and 9 (36). Neither of these RFLP alters any amino acids of the enzyme. One polymorphism that does lead to a change in the amino acid sequence is a C to G transversion at nucleotide 1595 of the cDNA sequence (37) (the Ser^417→Stop substitution) causing production of a protein truncated by two amino acids. Current evidence suggests this truncated protein maintains normal enzyme activity (38, 39) but may have altered lipid binding properties and thus substrate specificity (38).

In recent years, over 30 nonsense or missense mutations or deletion/rearrangements have been described in the gene for LPL (40–46, reviewed in 47) in patients with chylomicronemia syndrome (23) (type I hyperlipidemia). This disorder is characterized by extreme plasma levels of TG resulting in “creamy” plasma and pancreatitis and is usually diagnosed in childhood. In the absence of TG hydrolysis, chylomicrons are not converted to remnants, and thus cannot be recognized by specific lipoprotein receptors and consequently accumulate in the plasma. The phenotype is caused occasionally as a defect in the essential cofactor for LPL, but more usually by homozygosity for defects in LPL itself (23). The amino acids of the catalytic triad of the enzyme are encoded in exons 4–6 of the gene and, as shown in Figure 2, many of the reported mutations are clustered in these exons and appear to effect the conformation of the predicted secondary structure or the hydrophobic nature of the pocket surrounding these important residues. Many of the patients reported to date are compound heterozygotes, but this

![Fig. 1. SSCP gel to detect mutations in the 3' half of exon 4 of the LDL-R gene. A different pattern of bands is seen in all patients with known mutations in exon 4, compared with the control.](image-url)
is not the case in those populations where a single mutation occurs frequently because of a "founder" effect, for example, in patients of French-Canadian origin where a Pro<sub>207</sub>Leu change is common (45). Interestingly, one mutation that results in the substitution of Glu<sup>142</sup> with Glu has been found in patients from many different countries, including those from Europe, Asia, and the Indian subcontinent (46). This suggests that this mutation is of ancient origin, possibly predating the divergence of the major ethnic groups. We have been examining a group of 20 patients with type I hyperlipidemia from the United Kingdom and Europe by using the SSCP method to screen exons 2–6 for mutations (12). To date, out of the 40 mutant alleles in this group of patients, a mutation has been identified in 27 alleles, and with the exception of the Gly<sup>142</sup>–Glu mutation, all occurred only in a single family. None of the mutations have been reported previously, and their effect on lipase activity is being determined in expression studies. Overall in this sample, the Gly<sup>142</sup>–Glu mutation has been found in 12 of 40 mutant alleles, but among Caucasian patients only, it explained 12 of 26 mutant alleles, or roughly 50%. It would seem likely that this mutation is therefore the most common mutation in patients in the United Kingdom and may be present in the general population at an appreciable frequency. The prevalence of type I hyperlipidemia is hard to estimate, but it is at least as common as homozygous FH (one per 10 000 000), suggesting a carrier frequency of about 1 in 1000 individuals. If 50% of these defects are caused by the Gly<sup>142</sup>–Glu mutation, it is possible that the frequency of this single defect may be as high as one per 1000 individuals.

Clinical relevance and future research. Although there have been a large number of reports of specific defects in different patients with type I hyperlipidemia, there has been no attempt to date to compare the severity of hyperlipidemia or clinical symptoms in these different individuals. Our preliminary evidence from the sample of 20 patients suggests those with the Gly<sup>142</sup>–Glu mutation have the typical pattern found in this disorder with severe symptoms, often showing signs of pancreatitis shortly after birth and developing xanthomas, with plasma TG levels of 20 mmol/L or greater. They respond well to appropriate dietary treatment, with resolution of all clinical symptoms. Biochemical studies on postheparin plasma from these individuals and in vitro expression studies (46) have shown that this mutation results in LPL dimers that are extremely unstable, with the result that the patient has virtually undetectable levels of LPL mass and activity. By contrast, there are other mutations that have been reported in patients with type I that have a much less severe effect on lipase activity. For example, one of the patients we have examined is homozygous for a novel mutation that changes Ala<sub>159</sub> to Thr, and this individual shows about 10% of normal postheparin lipase activity (12), which is significantly higher than the activity found in the patient with the Gly<sup>142</sup>–Glu mutation. Interestingly, neither of the affected children in the family has ever reported pancreatitis or xanthomas and their hyperlipidemia has been easily resolved by moderate adherence to dietary recommendations. In the future, as more information is known about the relationship between specific mutations and their effect on lipase function, it is likely that more differences will be reported on the effect of different mutations, and this information may be useful in clinical management.

One of the important aspects of LPL deficiency is whether carriers for a mutation in the lipase gene have an elevated risk of developing familial combined hyperlipidemia. This is a disorder first described in the early 1970s with an estimated prevalence in the general population at least two or three times higher than that of carriers for FH and a strong association with increased risk of coronary artery disease (48, 49). It is characterized by elevated cholesterol or TG in a proband and relatives, with the primary mechanism thought to be overproduction of apo B-containing lipoproteins. The incidence in the living population at risk is estimated at 1 in 1000 per 50 000 individuals. The molecular defects underlying this disorder are not understood, although in some patients, mutations in the apo AI-III-AIV gene cluster have been implicated (52, 53). Several lines of evidence suggest that low lipase activity, either genetic or acquired, may be involved in causing the combined hyperlipidemia pattern in at least a subset of individuals. The first comes from investigations of the relatives of probands with type I hyperlipidemia, where it was found that carriers of the Gly<sup>142</sup>–Glu mutation were prone to hypertriglyceridemia, with high plasma levels of apo B and low HDL cholesterol concentrations. The effect of the mutation was modulated by age, with the largest effects being noted in individuals over 40 y (54). Similar results have been reported from the large French-Canadian kindred with the Pro<sub>207</sub>Leu change (45). The second line of evidence comes from studies in patients with familial combined hyperlipidemia, where LPL mass and activity have been measured with accurate assays. Roughly a third to a fifth of these individuals have levels of activity and mass below the 10th percentile for their age and gender (55, 56), suggesting heterozygosity for an allele that codes for a lower percentage of normal LPL activity.
for a defective LPL enzyme. This might predispose an individual to a defective clearance of TG-rich lipoproteins, which could develop into the full pattern of combined hyperlipidemia (23). This other environmental or genetic factors result in VLDL overproduction or LPL down-regulation, which saturates this reduced clearance system. Two recent reports have suggested possible mechanisms to explain how low LPL levels could result in overproduction of apo B-containing lipoprotein from the liver, but the precise mechanism is not yet established (57, 58).

There still remain several unresolved questions about the mechanism of the association between factors that cause an individual to have low levels of LPL activity and the subsequent development of hyperlipidemia. However, the data so far suggest the strong possibility that individuals who are carriers for a mutation in the LPL gene may benefit from an early identification of such a risk, followed by appropriate counseling on lifestyle changes that would help them to reduce their subsequent risk, and more detailed monitoring so that if they should develop hyperlipidemia at a later age they could be given appropriate therapeutic advice. Thus, although being a carrier for a mutation in the LPL gene does not require immediate pediatric treatment, such information may be useful in adulthood for the individuals identified and may be of immediate use for their relatives.

*Apo CII.* Apo CII is a 79–amino acid protein that acts as a cofactor for the activation of LPL (reviewed in 59). The gene spans 32 kb (60) and is part of the apoprotein gene cluster on chromosome 19 together with apo E, apo CI, and the apo C pseudogene (61). Apo CII, apo E, and apo CI show strong sequence homology and a similar intron/exon arrangement reflecting their evolution from a common progenitor (62). Three functional domains of apo CII have been elucidated by studies on synthetic peptides and proteolytic fragments (59) and by studies on the expression of normal and mutated variants of human apo CII in a bacterial system (63). The lipid-binding domain, between residues 14 and 50, consists of three amphipathic α-helices that anchor the molecule to the lipoprotein surface. The LPL-activation domain consists of residues 55–65 at the carboxy-terminal third of the protein, whereas the LPL-binding domain is residues 65–79, on the basis of the predicted amphipathic helix structure of this part of apo CII (59) and including the charged terminal tetrapeptide.

In some individuals with type I hyperlipoproteinemia, LPL is normal and the disorder is caused by a defect in apo CII (24, 64, 65). Apo CII deficiency is extremely rare, with a frequency considerably lower than LPL deficiency, but the clinical expression of the two are very similar, both being characterized by massive fasting chylomicronemia and recurrent pancreatitis. The pattern of inheritance of the clinical phenotype of apo CII deficiency is recessive, with obligatory heterozygous having half normal apo CII levels in the normal lipid levels. To date, 14 apo CII-deficient families have been identified worldwide and, as shown in Table 1, the mutation in eight families has now been elucidated. The defect can be caused either by frameshift mutations due to insertions or deletions, by base substitutions that amount of apo CII in the plasma. To date, no single amino acid substitution leading to loss of apo CII function has been reported, and in all but two families (73) the mutations are unique.

**Clinical relevance and future research.** So far there are no data to suggest that different mutations in apo CII might be associated with different clinical consequences or that the dietary management of type I hyperlipidemia caused by apo CII deficiency should be different from that caused by LPL deficiency, although there is general agreement that type I caused by apo CII deficiency is usually associated with milder symptoms (23). This may be in part because, even in the complete absence of apo CII, LPL still has some residual activity (27, 59), and thus some lipoprotein metabolism occurs. However, the extreme rarity of apo CII deficiency makes any such comparison difficult and any definite conclusion must await further data. There is also little evidence to indicate any strong association between the carrier status for apo CII deficiency and hyperlipidemia. One study reporting recently on a large kindred from Toronto (74) indicated that carriers for apo CII deficiency who are also heterozygous for the apo E4 allele of apo E that is associated with elevated levels of plasma lipids have significantly higher levels of TG and lower HDL cholesterol than relatives with apo CII deficiency and other apo E isoforms. Although this is a very informative example of how genetic variations at two different loci, coding for proteins that are both involved in lipid metabolism, may interact to determine an individual's plasma lipid levels, the low frequency of the apo CII deficiency makes this not of immediate clinical relevance.

**Table 1. Mutations in apo CII gene causing type I hyperlipoproteinemia**

<table>
<thead>
<tr>
<th>Variant</th>
<th>Mutation Effect</th>
<th>Effect on apo CII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo CII*3rd</td>
<td>T deletion — frameshift</td>
<td>96-amino acid protein — last</td>
</tr>
<tr>
<td>Apo CII*26</td>
<td>Leu14 — stop</td>
<td>6 residues abnormal</td>
</tr>
<tr>
<td>Apo CII*26</td>
<td>Single base insertion</td>
<td>6 residues 71–95 abnormal</td>
</tr>
<tr>
<td>Apo CII*18</td>
<td>G — C/intron 2 donor splice defect</td>
<td>No protein synthesized</td>
</tr>
<tr>
<td>Apo CII*18</td>
<td>G deletion — frameshift</td>
<td>No protein synthesized</td>
</tr>
<tr>
<td>Apo CII*18</td>
<td>Val14 — stop</td>
<td>Truncated protein 36</td>
</tr>
<tr>
<td>Apo CII*18</td>
<td>C — A/Tyr57 — stop</td>
<td>Truncated protein 36 amino acids long</td>
</tr>
<tr>
<td>Apo CII*18</td>
<td>C — G/Tyr57 — stop</td>
<td>Truncated protein 36 amino acids long</td>
</tr>
<tr>
<td>Apo CII*18</td>
<td>A — G/Initiation Met22 — Val</td>
<td>No protein synthesized</td>
</tr>
<tr>
<td>Apo CII*18</td>
<td>C — T/Arg19 — stop</td>
<td>No protein synthesized</td>
</tr>
<tr>
<td>Apo CII*18</td>
<td>C deletion codon 2 or 24</td>
<td>No protein detected</td>
</tr>
<tr>
<td>Apo CII*18</td>
<td>C deletion codon 23 or 24</td>
<td>No protein detected</td>
</tr>
</tbody>
</table>

**Apo B.** Apo B is the sole protein component of LDL and therefore the principal cholesterol-transporting protein. The mature apo B100 protein is 4536 amino acids long (75) and is secreted from the liver and found in VLDL, intermediate density lipoproteins, and LDL. Amino acid sequence comparisons with the receptor-binding domain of apo E (76) and from studies with MAbs that inhibit binding of LDL to the LDL-R (77) suggested that the region encompassing amino acids 3359–3780 is responsible for the interaction with the receptor. There is a second, shorter form of apo B (B48) that is associated with chylomicrons and secreted from the intestine. The apo B48–containing chylomicron remnants are rapidly cleared from the circulation, whereas the apo B100 containing particles are partially metabolized to LDL that has a relatively long half-life in the circulation. The receptor binding region of apo B is absent from apo B48, and it has been suggested that this may have evolved to ensure the rapid delivery of the remnant particles of dietary lipid to the liver mediated by apo E binding to the LDL-R or the remnant receptor.

There is a single apo B gene on chromosome 2 (p23–p24) (78). The gene contains 29 exons spanning 43 kb (62), and its intron/exon organization is very different from that of the other apoprotein genes, suggesting a different evolutionary history. The intriguing question of how both the liver and intestinal forms of apo B are synthesized from the same gene has now been elucidated. The intestinal apo B48 form of apo B has been shown to be colinear with the amino terminal half of apo B100 and
terminates at isoleucine123 of apo B100 as the result of the introduction in the mRNA of a C→U transition at nucleotide 6666 that creates a stop codon and results in protein termination (79). This apo B mRNA editing mechanism is under tissue-specific and developmental regulation (80). Because apo B48 lacks the sequence that is required for interaction with the LDL-R, the differential expression of the two forms of apo B is important in directing the metabolism of lipoproteins.

**ABL.** Two primary disorders of lipoprotein metabolism exist, characterized by a complete absence or deficiency in apo B-containing lipoproteins. These are ABL and HBL (75). ABL is inherited as an autosomal recessive disorder, with heterozygous parents having normal lipid levels, whereas affected individuals have trace amounts of apo B-containing lipoproteins in the plasma and this is associated with the clinical symptoms of fat malabsorption, acanthocytes, retinitis pigmentosa, and muscular neuropathies (75). Most of the neuropathies are caused by the deficiency of fat-soluble vitamin A and E transportation by the chylomicrons, and once identified, these individuals can be treated very successfully by appropriate dietary measures and vitamin supplements (81). Mutations in the apo B gene would be an obvious possibility for causing this disorder, but because there is a single apo B molecule per LDL particle, it can be postulated that defects in the apo B gene would be expressed in a codominant fashion, with half the circulating LDL having the normal apo B protein and half having the abnormal variant of apo B. Thus, the recessive inheritance of ABL suggests strongly that the mutation may be in a gene other than that for apo B. In addition, a study carried out on liver biopsies from two patients with ABL showed that apo B mRNA could be easily detected, and that levels were 8-fold enhanced (82) and immunohistologic techniques have shown that apo B accumulates within both hepatocytes and enterocytes in this disorder (83, 84). This suggests that the defect is most likely in a gene involved in posttranslational modification or secretion of apo B. Genetic confirmation of this, ruling out the apo B gene as a candidate, was obtained by cosegregation studies such as those shown in Figure 3. For a recessive disorder caused by a mutation in the apo B gene, all affected children in a family should have the same genotype at the apo B locus, having inherited the same defective allele from each of their parents, as shown in family I. The results obtained from several families each with two affected children showed the pattern demonstrated in family II with no cosegregation of the apo B gene (85, 86), thus confirming that the defects in another gene must be the cause of the disorder. One possibility is that the defect is in a “chaperon” protein that is involved in translocation of the apo B protein from the cytosolic compartment or through the Golgi apparatus, where the lipoprotein particle is assembled. The identification of the gene or genes involved in this process will provide valuable insights into the mechanisms that control secretion of lipoproteins from the liver and intestine, both in this disorder and in individuals in the general population.

**HBL.** In contrast with ABL, HBL is inherited as a codominant disorder with heterozygotes having total cholesterol, LDL cholesterol, and apo B levels below the 5th percentile. Cosegregation studies have supported the hypothesis that the mutation is in the apo B gene (87). All mutations identified to date result in truncated proteins and most have been initially detected at the protein level using PAGE of plasma or LDL to estimate protein size. Further identification and sizing of truncations have been achieved with immunoblots and binding with MAbs with well-defined epitopes. In a recent screening carried out on blood donors in St. Louis, the frequency of familial HBL was estimated to be less than 0.01% (88). In the homozygous form, patients have trace levels of apo B-containing lipoproteins and the clinical characteristics of ABL. More than 15 truncated apo B mutations causing HBL have now been characterized at the molecular level (Table 2). A map of the apo B gene showing the position of some of the mutations that lead to truncated proteins is shown in Figure 4. Except in the case of apo B25, the result of a deletion of the entire exon 21, all the truncated forms reported to date are caused by C→T transitions or base deletions. In addition, all the reported mutations have been unique to the kindreds they have been identified in and no two unrelated families have had the same mutation.

These truncated apo B species have helped define the functional domains of apo B, namely the lipoprotein assembly, lipid binding, and receptor binding regions. Where the mutation creates a protein greatly reduced in size, for example, the truncated apo B species apo B25 (89) and apo B29 (90), no plasma apo B was detected. It has been suggested that these small truncated species lack a domain necessary for lipoprotein association and stability. The amino acid residues in the amino terminus of the protein are hydrophilic and would not bind well to lipid. The truncated species apo B31 (91) and apo B37 (92) are associated with small amounts of TG-rich lipoproteins and occur primarily in the HDL density range; this reflects the small amount of associated lipid and not an HDL-type particle. These conclusions have been confirmed by in vitro expression studies (101, 102). Taken together these results suggest that the hydrophobic amino acids found between apo B31 and apo B39 are necessary for assembly and secretion of apo B-containing lipoproteins. Thus, there appears to be a critical size that the protein must exceed to be secreted, with truncations smaller than apo B31, although detected in the plasma, being very unstable and rapidly degraded after secretion.apo B46 (94), apo B50 (95), and apo B54.8 (88) are found primarily in the LDL density range, although apo B46 is seen in LDL and HDL ranges and apo B54.8 in the LDL density range. The larger apo B species, apo B86 (99), apo B87 (100), and apo B89 (103) are found in a similar density range as full-length apo B100. Thus, the truncated apo B proteins help define the region in the amino terminal end of the protein important in the association with lipids (Table 3, Fig. 4).

The low levels of these truncated forms in the plasma are the result of several different mechanisms. Most of the truncated apo B proteins lack the portion of the protein that binds to the LDL receptor; thus an increase in receptor-mediated catabolism is unlikely to be an explanation for the low concentrations of apo B, and it is probable that the instability of the mutant lipoproteins is the major contribution to their low levels. The larger truncated proteins apo B87 and apo B89, which do encode the primary LDL-R binding domain, provide evidence of secondary binding domains on apo B for the LDL-R. In the case of the apo B89, degradation was increased in cultured fibroblasts compared with apo B100 (103), and in addition there was enhanced clearance of apo B89 in LDL turnover studies performed in rabbits (104). This suggests that apo B89 has increased affinity for the LDL-R, thus explaining the low concentration of apo B89 in the plasma of the original patient (103).
Table 2. Characteristics of truncated apo B species associated with HBL

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Predicted no. amino acids</th>
<th>Density fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo B25</td>
<td>1085</td>
<td>None</td>
</tr>
<tr>
<td>Apo B29</td>
<td>1305</td>
<td>None</td>
</tr>
<tr>
<td>Apo B31</td>
<td>1425</td>
<td>HDL + infranatant</td>
</tr>
<tr>
<td>Apo B37</td>
<td>1728</td>
<td>VLDL, LDL, HDL</td>
</tr>
<tr>
<td>Apo B39</td>
<td>1799</td>
<td>VLDL, LDL</td>
</tr>
<tr>
<td>Apo B40</td>
<td>1829</td>
<td>VLDL, LDL, HDL</td>
</tr>
<tr>
<td>Apo B46</td>
<td>2057</td>
<td>VLDL, LDL, HDL</td>
</tr>
<tr>
<td>Apo B50</td>
<td>2251</td>
<td>VLDL</td>
</tr>
<tr>
<td>Apo B54</td>
<td>2485</td>
<td>VLDL, LDL</td>
</tr>
<tr>
<td>Apo B55</td>
<td>2494</td>
<td>VLDL, LDL</td>
</tr>
<tr>
<td>Apo B61</td>
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</tr>
<tr>
<td>Apo B67</td>
<td>3040</td>
<td>VLDL, LDL</td>
</tr>
<tr>
<td>Apo B86</td>
<td>3408</td>
<td>VLDL, LDL</td>
</tr>
<tr>
<td>Apo B87</td>
<td>3979</td>
<td>VLDL, LDL</td>
</tr>
<tr>
<td>Apo B89</td>
<td>4039</td>
<td>VLDL, LDL</td>
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LIPO PROTEIN ASSEMBLY

<table>
<thead>
<tr>
<th>Type of mutation</th>
<th>No. of unrelated patients</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deletions/rearrangements detected by Southern blotting</td>
<td>9</td>
<td>4/9 same as and 3/9 similar to previously detected mutations</td>
<td>151, 152</td>
</tr>
<tr>
<td>Glu186 → Lys (exon 3)</td>
<td>5</td>
<td>1/9 insertion complementary to previously described deletion</td>
<td>152</td>
</tr>
<tr>
<td>Exon 4 mutations</td>
<td></td>
<td>Probably same allele</td>
<td>150</td>
</tr>
<tr>
<td>Deletion of Gly237</td>
<td>6</td>
<td>Common in Lithuanian Jews</td>
<td>149</td>
</tr>
<tr>
<td>Deletion of 2 bp in codons 206/207</td>
<td>5</td>
<td>Same allele in all; “English” defect</td>
<td>149</td>
</tr>
<tr>
<td>Cys518 → stop</td>
<td>1</td>
<td>Irish/Scottish patient</td>
<td>149</td>
</tr>
<tr>
<td>Asp506 → Glu</td>
<td>3</td>
<td>Same as common Afrikaner</td>
<td>149</td>
</tr>
<tr>
<td>Ser164 → Leu</td>
<td>1</td>
<td>Same as Puerto Rico, but recurrent mutation</td>
<td>154</td>
</tr>
<tr>
<td>Asp506-Gly</td>
<td>1</td>
<td>New: English origin</td>
<td>13</td>
</tr>
<tr>
<td>Pro666 → Leu (exon 14)</td>
<td>5</td>
<td>Recurrent mutation</td>
<td>153</td>
</tr>
<tr>
<td>Total</td>
<td>36</td>
<td>(18% of mutant allele)</td>
<td></td>
</tr>
</tbody>
</table>
where the molecular defect is unknown. These data support the view that a reduced synthetic rate of VLDL occurs in these patients and thus the synthetic rate of LDL containing both the truncated and the normal apo B100 may be reduced. Apo B synthesis is constitutive in liver cells, and overall the output of apo B is controlled by changes in the mRNA translational efficiency, or more likely by changes in degradation of intracellular apo B in response to endogenous factors such as the level of plasma fatty acids or postprandially (108). Thus, it is possible that in the hepatocytes of the hypertriglyceridemic HBL patients, although mRNA coding for both the truncated and normal apo B proteins are translated, the truncated species interferes with the assembly of apo B100 into normal lipoproteins, leading to an overall reduction in secretion of apo B-containing lipoproteins. Further research to explore such mechanisms should lead to a better understanding of the control of secretion of apo B-containing particles from the liver.

**FDB.** It is well known that defects in the LDL-R that destroy function lead to FH. Similarly, hypercholesterolemia could be caused by defects in the apo B gene that would reduce binding to the LDL-R and lead to raised cholesterol levels, and the name FDB has been proposed for such mutations (109). A number of laboratories have carried out systematic searches, using molecular biology techniques, to identify patients who have mutations in the apo B gene that would alter receptor binding. To date, there is only one form of FDB and that is caused by a mutation that substitutes codon Arg3500 with Gln (110), thus it is called the apo B3500 mutation. Originally observed in a patient who showed reduced clearance of autologous LDL compared with clearance of LDL from a normal donor (111), the mutation was identified as a G→A substitution altering codon CGG to CAG (112). This single amino acid change reduces binding of the LDL containing apo B-Gln3500 resulting in the accumulation of such LDL (109), whereas LDL-Arg3500 is cleared with normal efficiency. In some patients, it has proved possible to separate the two species of LDL using binding to MAb (113). Purified LDL-Gln3500 has 5% normal receptor binding affinity and thus accumulates in vivo; in an FDB heterozygote, as much as 70% circulating LDL will be LDL-Gln3500. The mechanism of the effect has been elucidated by studying the differential binding of MAb and using carbon-13 nuclear magnetic resonance (114), which showed that the six lysine residues within the region of amino acid 3500 have altered ionization constants in the presence of Gln3500. Lysine residues are known to be involved in the binding of apo B100 to the LDL-R, and the substitution of Arg3500→Gln, by a large effect on the conformation of the surrounding area of the protein, alters the microenvironment of the receptor binding domain.

To date, only individuals who are heterozygous for the mutation have been identified (115). Individuals with FDB have been identified in the United States, Canada (110), Austria (116), United Kingdom, and Denmark (117), Germany (118), and Italy (119), but not in Finland (120). Haplotyping analysis using apo B gene polymorphisms has shown that in all cases reported to date the mutation is of major clinical interest. All carriers of the mutation in ethnic groups, other than Caucasians, have a common ancestor (121, 122). The occurrence of the mutation in ethnic groups, other than Caucasians, has not yet been reported. Rough estimates of the frequency of the mutation in the general population made on the basis of the frequency found in lipid clinic patients are in agreement, ranging from one per 500 (117) to one per 700, (118), but to date, no systematic study to screen for the mutation in the general population has been carried out. The apo B3500 mutation appears to be the most common single gene defect causing hypercholesterolemia, and although other apo B mutations causing FDB may be identified in the future, it is likely that apo B3500 will be the most common.

The mutation was originally identified in a patient who was moderately hypercholesterolemic (111), but three recent studies have screened patients with a clinical diagnosis of FH and found that roughly 3% of these patients were heterozygous for the apo B3500 mutation (117, 118, 123). Where the patients have been analyzed further, this occurred in individuals who had LDL-R activity within the normal range (124). This mutation can be associated with severe hypercholesterolemia, tendon xanthomas, and a family history of premature coronary artery disease, and it is therefore clinically indistinguishable from FH caused by receptor defects. Furthermore, as with defects in the LDL-R gene, the effect of the apo B3500 mutation on plasma lipid levels can be expressed in children (Fig. 5), with several families (124) now reported with children under the age of 5 y having markedly elevated LDL cholesterol levels.

**Clinical relevance and future research.** The range of expression of the mutation is of major clinical interest. All carriers of the mutation have LDL that binds with reduced affinity to the receptor, but the absolute levels of LDL cholesterol in the blood and thus the future risk of developing coronary artery disease will depend on the interaction between other genetic and environmental factors. In a recent review of all the published biochemical and clinical information on 70 FDB carriers (125), it was clear that the rate of development of coronary artery disease in this disorder was similar to that reported in FH patients, with a faster rise in males than females. Over 85% of carriers have total and LDL cholesterol levels over the 95th percentile for their age and gender, but unlike FH patients, TG and HDL cholesterol levels are within the normal range. This is presumably because the metabolism of the TG-rich lipoprotein, mediated through apo E and the normal LDL-R, is not affected by the apo B mutation. However, the information regarding the range of clinical severity of the disorder is biased, because to date the majority of patients have been identified by screening individuals attending lipid clinics, many of whom had a clinical diagnosis of FH. This raises the possibility that the mutation may not always be associated with such severe clinical consequences, and in support of this, a review of the published data on plasma lipid levels of relatives (125) shows that some have levels within the normal range for their age and gender. Presumably environmental factors, and variation at other gene loci, will contribute to these individual differences and may possibly interact specifically with the apo B mutation. Although no such data on range of expression is yet available for any of the mutations in the LDL-R gene causing FH, *a priori* it is likely that a similar range will be seen. It is therefore important to identify FDB carriers.
from the general population to allow an unbiased estimate of the risk of hyperlipidemia and atherosclerosis associated with this defect. Only then can accurate information be given to carriers and appropriate therapeutic management strategies be started.

It is still not clear what is the best drug therapy to offer FDB patients. Most conventional drug therapies for FH rely on up-regulation of the LDL-R to enhance clearance of LDL, but the LDL-Gln cannot be cleared by this route and may still accumulate. The atherogenic potential of such accumulating defective LDL is not understood, and it is possible that LDL-Gln may be susceptible to oxidation and it thus may accumulate in foam cells and promote atherosclerosis. Studies have been carried out on FDB patients to examine the responsiveness to drugs (126-128), and although plasma levels of LDL do fall in most patients, it is possible that this is caused mainly by a reduction in plasma levels of LDL-Arg. Thus, for FDB patients, treatment with antioxidants or a combination treatment may prove a better therapy, and if this were the case, using genetic tests to distinguish hypercholesterolemia caused by a receptor or a ligand defect would be of major importance.

**LDL-R defects in FH.** FH is a common inherited disease showing an autosomal dominant pattern of inheritance (129). It is characterized clinically by elevation in the concentration of LDL cholesterol in blood, tendon xanthomata, and an increased risk of myocardial infarction. FH is present in 5-10% of individuals under the age of 55 in the United Kingdom and the United States, and it is an important cause of coronary artery disease (130, 131), and is therefore the best understood single-gene cause of hyperlipidemia and thus atherosclerosis risk. Based on the estimated population frequency of carriers of one per 500, there are more than 100 000 FH heterozygous individuals in the United Kingdom, of which probably less than 3000 have been identified to date. Once identified, the hyperlipidemia of these patients is responsive to treatment by diet and drugs (132, 133), and such treatment reduces subsequent morbidity and mortality (134). Children who have inherited two defective alleles of the LDL-R (homozygous FH, but usually compound heterozygous for two different defects) represent one per 1 million of the population. In these children, there is usually little useful lowering of plasma LDL cholesterol levels in response to diet or drugs, and many suffer a major coronary event in the first or second decade of life, but life expectancy can be extended by appropriate treatment (135, 136). Current treatment is usually plasma exchange or LDL apheresis (137), but patients may alternatively be treated by transplantation of a donor liver, possibly in conjunction with a heart transplant (138).

FH results from different genetic defects in a cell surface receptor that normally controls the uptake of plasma LDL (129, 139). Five classes of mutations at the LDL-R locus have been identified on the basis of phenotypic behavior of the mutant proteins (140). Class I mutations fail to produce any immunoprecipitable protein (null alleles). Class II mutations encode proteins that do not fold properly after synthesis and are blocked, either partially or completely, in transport between the endoplasmic reticulum and the Golgi complex (transport defective alleles). Class III mutations encode proteins that are synthesized and transported to the cell surface but fail to bind LDL normally (binding-defective alleles). Class IV mutations encode proteins that bind to LDL and bind LDL normally, but are unable to cluster in clathrin-coated pits and thus do not internalize LDL (internalization-defective alleles). Class V mutations encode receptors that bind and internalize ligand in the coated pits, but fail to discharge the ligand in the endosome and fail to recycle to the cell surface (recycling-defective alleles). These different classes of mutations are caused by defects scattered over the entire LDL-R gene in the case of the first two classes, and mutations in the ligand binding region, the cytoplasmic domain, and in the epidermal growth factor precursor homology domain for classes III-V, respectively.

The cloning of the human LDL-R gene (141) has made it possible to study FH using DNA technology. There have been at least 40 different mutations of the LDL-R gene characterized at the DNA level (140), and they have given valuable insights into the function of the different domains in the LDL-R. Many more mutations are likely to be found, and Hobbs et al. have postulated from the number of homozygous FH patients they have investigated that in the Dallas collection alone there could be as many as 183 mutant alleles, although only the minority have been characterized at the DNA level so far (140). Several studies have been published demonstrating that within a geographically or culturally isolated population, or where a large proportion of people are related by descent because of migration, the frequency of carriers of one per 500, there are more than 100 000 FH heterozygous individuals in the United Kingdom, of which probably less than 3000 have been identified to date. Once identified, the hyperlipidemia of these patients is responsive to treatment by diet and drugs (132, 133), and such treatment reduces subsequent morbidity and mortality (134). Children who have inherited two defective alleles of the LDL-R (homozygous FH, but usually compound heterozygous for two different defects) represent one per 1 million of the population. In these children, there is usually little useful lowering of plasma LDL cholesterol levels in response to diet or drugs, and many suffer a major coronary event in the first or second decade of life, but life expectancy can be extended by appropriate treatment (135, 136). Current treatment is usually plasma exchange or LDL apheresis (137), but patients may alternatively be treated by transplantation of a donor liver, possibly in conjunction with a heart transplant (138).

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DNA tests for identification of FH in relatives. Recently, several overview analyses of the results of published trials of diet and drug therapy to lower lipids in middle-aged high-risk patients, have suggested the possibility of harmful side effects in some individuals (155-157). These side effects include increased frequency of violent deaths and suicides in the treated group, and it has been suggested that precipitous reduction in plasma lipid levels in middle age may alter membrane physiology in the brain, and thus affect behavior (155). However, no study has suggested that lifelong low plasma cholesterol levels are associated with such risk (158), and this strengthens the argument for early identification of children with FH and early commencement of lipid-lowering treatment. Further, it is not yet clear whether atherosclerosis develops in youth, as has been shown recently in a study of postmortem material from young individuals (159). Early identification of children carrying LDL-R gene defects will enable life-style changes, dietary therapy, or, where necessary, drug therapy such as resins to be started, and studies have shown that early commencement of such treatment is associated with better compliance (160). Several studies have shown that measures of total cholesterol or LDL cholesterol alone do not allow unequivocal diagnosis of FH in 10-15% of cases, even in the children of a parent with FH (161, 162). We have also recently shown that some children whose lipid levels are initially within
the normal range for their age and gender show a greater than average rise in lipid levels over time, to a point where it is evident that they have inherited the LDL-R gene mutation (163). Although the frequency of this problem is unknown, there is no doubt that an unequivocal DNA test would be very useful, both to allay fears and to identify children for whom dietary advice and appropriate therapy should be started. When the mutation is known for a patient, DNA tests will give an unequivocal result that can be obtained within 1–2 d.

It has been suggested that a monocyte or lymphocyte assay may be a useful tool for identifying individuals with defective LDL-R function, and there are several reports of Lp(a) levels of Lp(a) (181, 182), it cannot be ruled out that different mutations in the LDL-R gene may be the cause. A recent report from South Africa suggested that the Val392Met mutation is associated with more severe clinical symptoms than the other common mutations in this population, the Asp290Glu and the Asp357Asn mutations (183). If such an association could be established for other mutations, more active therapeutic strategies could be recommended to patients and their relatives who had inherited a mutation associated with a greater risk.

Differences in response to diet or drugs. Several studies have reported that there is a great variation in the fall in total and LDL cholesterol levels when patients with heterozygous FH are treated with drugs such as resins or hydroxymethylglutarate-CoA reductase inhibitors (133, 184). One possibility is that these differences are caused by genetic variation at other loci, and use of these drugs may have been shown to be related to baseline lipid levels or response to therapy in patients with FH (185–187).

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Differences in symptoms. In different FH patients, there is a great deal of variation in the levels of untreated plasma lipids (177) and in the age of onset of coronary artery disease (178). In a recent publication from the UK FH register, strong evidence was obtained for differences in risk of coronary artery disease in different patients, with some individuals developing disease at a very early age, whereas those who had survived past 50 y had a standardized mortality ratio that was only slightly higher than in the general population (179). It has also been shown that the age of onset of coronary artery disease aggregates in families (180), and although it is possible that this may be caused by the presence of environmental or other genetics factors such as genes for higher levels of Lp(a) (181, 182), it cannot be ruled out that different mutations in the LDL-R gene may be the cause. A recent report from South Africa suggested that the Val392Met mutation is associated with more severe clinical symptoms than the other common mutations in this population, the Asp290Glu and the Asp357Asn mutations (183). If such an association could be established for other mutations, more active therapeutic strategies could be recommended to patients and their relatives who had inherited a mutation associated with a greater risk.

SUMMARY

Techniques for molecular biology are extremely powerful and have allowed the precise molecular defect to be identified in a number of pediatric patients with disorders of plasma TG and cholesterol metabolism. In very few situations to date has this information been useful to the pediatrician in decisions about the management of the child, but for disorders such as FH or type I hyperlipidemia caused by LPL deficiency, this may be possible in the future if different mutations are found to be associated with better response to a particular therapy or are associated with a different risk for developing disease in later life. For rare disorders that show a recessive pattern of inheritance, the identification of relatives who are carriers is not of great benefit, because the possibility of these marrying another carrier is too low to be of major concern. However, for "codominant" disorders, where carriers have a greater risk of developing clinical symptoms in adulthood, screening relatives to identify carriers will be of major benefit; this applies mainly to the disorders of FH, FDB, and LPL deficiency. For each of these, carriers may
represent one of 500 in the general population and random or opportunistic screening would be feasible, but a case-finding strategy is likely to be most appropriate to identify individuals who have a specific major genetic predisposition to hyperlipide mia and thus coronary artery disease. However, before such screening can be started, much more information will be required about the relationship between a specific mutation and the resulting plasma lipid levels that occur in a range of environmental situations, such as different diets, smoking, or drug therapy, as well as the possible interaction between the mutation and other ameliorating or exacerbating genetic factors. Only then can appropriate advice be given to an individual about the subsequent long-term risk of developing coronary artery disease. Once such information is available, there will still be ethical dilemmas, and the application of such diagnostic techniques to the relatives of children who will require a sensitive collaborative approach from molecular biologists, molecular geneticists, and particularly from pediatricians.

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Lipoprotein lipase activity in patients with combined hyperlipidaemia

M. Seed1, F. Mailly2, D. Vallance3, E. Doherty1, A. Winder3, P. Talmud2, S.E. Humphries2

1Departments of Medicine and Chemical Pathology, Charing Cross and Westminster Medical School, London W6 8RF, UK
2Centre of Genetics of Cardiovascular Disorders, The Rayne Institute, UCL Medical School, London WC1E, UK
3Department of Chemical Pathology, The Royal Free Medical School, London NW3, UK

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Abstract. The aetiology of familial combined hyperlipidaemia remains obscure, with both genetic and environmental factors contributing to the phenotype, which is frequently associated with premature coronary heart disease. We have studied lipoprotein lipase (LPL) activity and hepatic lipase (HL) activity in patients with coronary heart disease to determine whether variation in lipase activities contributes to this phenotype. Forty-one patients (mean age 50 years; 30 male) were selected on the basis of cholesterol levels above 6.5 mmol/l and triglyceride levels above 2.2 mmol/l, with apo B values over the 90th percentile. There was a family history of premature coronary heart disease in 78% and a personal history in 64%, at mean age 44, the patient group therefore predominantly corresponded to the common definition of familial combined hyperlipidaemia, appropriate in the absence of molecular markers. None of the patients was diabetic; hypertension and smoking were not over represented. Blood samples were taken following intravenous administration of heparin (100 IU/kg body wt), and LPL and HL activities were measured. Mean post-heparin LPL was significantly lower in patients than controls 10 min after heparin administration (2.98 ± 1.04 and 3.86 ± 0.93 μmol ml⁻¹ h⁻¹, respectively, P = 0.001), and 37% patients had values below the 10th percentile of controls. Both male and female patients had significantly higher HL activities than their respective controls at 5, 10, 20 and 30 minutes post-heparin. As expected, both female patients and controls had lower HL activities than males, although this sex difference did not reach statistical significance in the patient group. Mean lipid and lipoprotein results were: cholesterol 8.2 mmol/l; triglycerides 4.2 mmol/l; high-density lipoprotein cholesterol 0.90 mmol/l; apoprotein A1 122 mg/dl; apoprotein B 171 mg/dl; lipoprotein (a) 23 mg/dl (median 10 mg/dl). High-density lipoprotein cholesterol and triglycerides were negatively correlated (r = −0.26, P = 0.05). HL was significantly related to body mass index at all time points whereas the negative correlation between post-heparin LPL and body mass index was significant only 30 min after heparin administration. Post-heparin LPL was only weakly correlated with triglycerides 10 and 20 min after heparin administration. These lipid and lipoprotein results are clearly potentially atherogenic as indicated by the extent of premature coronary heart disease in the group described. A decrease in LPL activity may contribute to this pattern.

Key words: Familial combined hyperlipidaemia – Lipoproteins – Lipoprotein lipase – Hepatic lipase

Abbreviations: FCHL = familial combined hyperlipidaemia; CHD = coronary heart disease; LPL = lipoprotein lipase; HL = hepatic lipase; HDL = high-density lipoprotein; VLDL = very low density lipoprotein; apo = apoprotein; TG = triglyceride; BMI = body mass index

Correspondence to: M. Seed

Familial combined hyperlipidaemia (FCHL) is defined as a variable pattern of hyperlipidaemia in the proband, with relatives having either elevated triglycerides (TG), elevated cholesterol, or both [10]. The disorder was described in the early 1970s with an estimated prevalence in the general population of at least two or three times higher than that of familial hypercholesterolaemia [11] and having a strong association with increased risk of coronary artery disease [7, 10, 26]. In the Seattle study of survivors of myocardial infarction under the age of 60 years, 11% of patients fell into this category [10]. In these individuals there was no evidence of a catabolic defect, such as is found in familial hypercholesterolaemia, the primary mechanism being overproduction of apoprotein (apo) B containing lipoproteins from the liver [30]. Patients have a de-
crease in the very low density lipoprotein (VLDL) TG/apoB ratio, with an increase in particle number, and small, denser VLDL and low-density lipoprotein (LDL), and low levels of high-density lipoprotein (HDL) [6, 8]. Such changes are potentially atherogenic [4, 9], and the hypertriglyceridaemia is also associated with increased thrombotic risk [24].

To date the molecular defects underlying this disorder are not understood, although mutations in the apoAI-CIII-AIV gene cluster have been implicated [13, 35]. However, several lines of evidence suggest that low activity of the enzyme lipoprotein lipase (LPL), either genetic or acquired, may be involved in causing the combined hyperlipidaemia pattern in at least a subset of individuals. The first line of evidence comes from studies of patients with FCHL, among whom roughly a third to a fifth have levels of activity and mass below the 10th percentile for their age and gender [34]. The second comes from investigations in the relatives of probands with type I hyperlipidaemia caused by the defects in LPL. Individuals who are heterozygous for a mutation in the LPL gene have been found to be hypertriglyceridaemic and also to have high plasma levels of apoB and low HDL cholesterol concentrations [1, 2]. It would thus appear that heterozygosity for an allele that codes for a defective LPL enzyme predisposes an individual to a defective clearance of TG-rich lipoproteins. This may develop into the full pattern of combined hyperlipidaemia when other environmental, metabolic or genetic factors lead to increased free fatty acid uptake by the liver, resulting in VLDL overproduction [29]. Recent in vitro studies of apoB production [33] show that the addition of LPL to HepG2 cultures decreases the output of apoB-containing lipoproteins, due to lipolysis and increased repack. This suggests that partial LPL deficiency could be manifest by an apparent increase in apoB production [33]. We have studied LPL activity in a group of patients with combined hyperlipidaemia and a family or personal history of premature coronary heart disease (CHD) in order to investigate the prevalence of low LPL activity.

Materials and methods

Subjects

Informed consent in compliance with the Declaration of Helsinki was obtained from patients attending the lipid clinics at Charing Cross Hospital, London, and King Edward VII Hospital, Sussex, and approval was obtained from the local Ethics Committees. Patients were recruited if they had a variable mixed hyperlipidaemia, i.e. total cholesterol above 6.5 mmol/l, TG above 2.2 mmol/l, and apoB above the 90th percentile, and a personal or family history of premature CHD. All but three patients had a family history of hyperlipidaemia; two of the three were adopted and one was an immigrant. Patients with familial hypertriglyceridaemia were excluded, (i.e. fasting triglyceride value above 8 mmol/l). Patients were screened for secondary causes of hyperlipidaemia, in particular thyroid disease, non-insulin dependent or insulin-dependent diabetes mellitus, renal disease or excessive alcohol intake. Patients were therefore excluded on the basis of abnormal glucose, liver function (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, bilirubin, gamma-glutamyltranspeptidase, albumin globulin), urea, creatinine, thyroid-stimulating hormone, and thyroxine. Patients had not taken lipid-lowering medication for at least 4 weeks prior to the investigation. Eight patients were taking β-blockers (atenolol 50 mg daily), six prophylactically post-infarct and two together with a calcium antagonist for the treatment of hypertension. The clinical characteristics of the subject group are represented in Table 1. The age at onset of CHD was not significantly different between males and females. There was no significant difference in body mass index (BMI) between males and females (26.6 female, 26.9 male). Twenty-three normolipidaemic, healthy controls (11 males, 12 females) were recruited from local Departments of Medicine and Chemical Pathology. Lipid characteristics of both groups are shown in Table 2.

Laboratory methods

Lipids and lipoproteins. Serum lipids and lipoproteins were measured at the time of initial diagnosis (Tables 2, 4). However, apoAI and apoB were measured subsequently, at the time of the investig-
Table 2. Lipid and lipoprotein results among patients and normolipidaemic controls

<table>
<thead>
<tr>
<th></th>
<th>Cholesterol (mmol/l)</th>
<th>TG (mmol/l)</th>
<th>High-density lipoprotein</th>
<th>Lp(a) (mg/dl)</th>
<th>ApoB (mg/dl)</th>
<th>ApoA1 (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Overall (mmol/l)</td>
<td>Males (mmol/l)</td>
<td>Females (mmol/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients (n=41)</td>
<td>8.20 ± 1.12</td>
<td>0.90 ± 0.23</td>
<td>0.88 ± 0.25</td>
<td>10.00*</td>
<td>171 ± 29.8</td>
<td>122 ± 19.8</td>
</tr>
<tr>
<td>Controls (n=23)</td>
<td>4.90 ± 0.90</td>
<td>1.30 ± 0.24</td>
<td>1.16 ± 0.22</td>
<td>5.20*</td>
<td>96 ± 29.8</td>
<td>127 ± 42.7</td>
</tr>
</tbody>
</table>

* Median

Serum was isolated by centrifugation, and total cholesterol and TG levels were analysed enzymatically on a BM/Hitachi 717 analyser using reagent kits purchased from Boehringer Mannheim (Lewes, UK; BM cholesterol C system, BM TG GPO-PAP). HDL was assayed after precipitation of other lipoproteins with dextran sulphate/magnesium chloride [32]. The interassay coefficient of variation (CV) for this method in our laboratory is 8%. Samples of serum were frozen and stored at −70°C for the assay of lipoprotein (a) [Lp(a)] concentration using an enzyme-linked immunosorbent assay (laboratory CV 6%; Biopool, Umeå, Sweden) [19]. ApoAI and apoB concentrations were assayed by rate nephelometry using the Beckman Array protein system and manufacturer's reagents (laboratory CV 5%).

Lipoprotein lipase and hepatic lipase assays. Venous blood was taken after an overnight fast. A 100 IU/kg heparin bolus was given, and samples were taken into EDTA tubes at 5, 10, 20 and 30 min for post-heparin analysis of lipoprotein lipase (LPL) and hepatic lipase (HL). Reference ranges and the time response following heparin administration were calculated using 23 normolipidaemic adults (11 males, 12 females). Post-heparin LPL and HL activities were measured by a previously described method [27] with some modifications. Briefly, LPL activity was measured in duplicate using lysophosphatidyl choline stabilized glycerol tri [9,10(n)-3H]oleate in the presence of heat-inactivated serum as LPL activator, after inhibition of HL with sodium dodecyl sulphate treated post-heparin plasma, which inhibits HL, was assayed for each of the patient's samples. Under these conditions, residual lipase activity was less than 0.2 μmol ml⁻¹ h⁻¹. Linearity of some of the assays was checked by incubating larger volumes of post-heparin plasma and substrate and removing 0.5 ml reaction mixture at 15, 30, 60 and 75 min.

Apolipoprotein E genotyping. The absence of floating beta lipoprotein (chylomicron remnant) was confirmed by the electrophoresis of washed VLDL isolated by flotation. Apolipoprotein E genotypes were determined using the method of Hixson and Vernier [14], with minor modifications. Briefly, DNA was extracted from packed blood cells [25], and a 216-bp fragment was amplified by the polymerase chain reaction as previously described [21] and digested with the enzyme Hhal. The resulting fragments were resolved on a 10% polyacrylamide gel.

Statistical analysis. Correlations and comparisons of group means were performed using the SPSS/PC + program. Apolipoprotein E allele frequencies were determined by the gene-counting method and compared to those obtained in 150 healthy subjects in the United Kingdom using contingency tables. Statistical significance was at the P < 0.05 level.

Results

The characteristics of the group of patients are presented in Table 1. The group was predominately
Table 3. Lipolytic activities (μmol ml⁻¹ h⁻¹) among controls and patients

<table>
<thead>
<tr>
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<th>5 min</th>
<th>10 min</th>
<th>20 min</th>
<th>30 min</th>
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</thead>
<tbody>
<tr>
<td>Hepatic lipase</td>
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</tr>
<tr>
<td>Controls</td>
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<tr>
<td>Males (n = 11)</td>
<td>9.06 ± 0.60</td>
<td>9.40 ± 0.52</td>
<td>9.50 ± 0.45</td>
<td>9.17 ± 0.57</td>
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<tr>
<td>Females (n = 12)</td>
<td>7.62 ± 0.51</td>
<td>7.17 ± 0.47  *</td>
<td>7.13 ± 0.51  *</td>
<td>6.93 ± 0.53  *</td>
</tr>
<tr>
<td>Patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males (n = 30)</td>
<td>12.55 ± 0.64 *</td>
<td>12.87 ± 1.08 *</td>
<td>13.52 ± 0.74 *</td>
<td>12.76 ± 0.71 *</td>
</tr>
<tr>
<td>Females (n = 11)</td>
<td>10.89 ± 0.62 *</td>
<td>11.80 ± 0.97 *</td>
<td>10.92 ± 0.90 *</td>
<td>11.00 ± 0.93 *</td>
</tr>
<tr>
<td>Lipoprotein lipase</td>
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<tr>
<td>Controls (n = 23)</td>
<td>2.64 ± 0.17</td>
<td>3.86 ± 0.20</td>
<td>4.28 ± 0.18</td>
<td>4.48 ± 0.18</td>
</tr>
<tr>
<td>Patients (n = 41)</td>
<td>2.08 ± 0.15 **</td>
<td>2.98 ± 0.16 ***</td>
<td>3.59 ± 0.22 **</td>
<td>3.92 ± 0.27</td>
</tr>
</tbody>
</table>

* Significantly different from (same-sex) controls, P < 0.01; ** significantly different from controls, P < 0.02; *** significantly different from controls, P < 0.01

male, with premature CHD, a family history of CHD and a family history of hyperlipidaemia. The number of present smokers was similar to the national average (28%); hypertension was not over-represented in this group (13%). The lipid and lipoprotein results illustrate the mixed nature of the hyperlipidaemia with significant elevation in total cholesterol, TG and apoB and a reduction in HDL. ApoAI levels were not reduced in the patient group (Table 2). Among the patients 30% had Lp(a) concentrations greater than 25 mg/dl, a higher proportion than that in a normal population [28]. The results of assays for HL and LPL are summarized in Table 3. There was very little variation in HL activities over the 30-min time course in either controls or subjects. HL activity was significantly increased at all time points in patients of both sexes compared to control individuals. HL activity was higher (23%) in control males than females, and there was a similar trend in the patient group although this did not reach statistical significance. LPL activity was significantly lower in patients for the first three time points after heparin administration (P < 0.02 at 5 and 20 min, P < 0.01 at 10 min). There was no statistically significant sex difference in LPL activity in the control or patient group (not shown). The maximum activity of LPL occurred 20–30 min after heparin administration. LPL activity was significantly lower at 5 min than at 10 min (P < 0.01). Although LPL activities continued to rise from 10 to 30 min, there was no significant difference in activities between the 10-, 20- and 30-min samples, and the value of the 10-min sample was therefore used for further comparison between the two groups. Overall, 15 of 41 individuals (36.6%) in the patient group had a 10-min LPL activity below 2.7 μmol ml⁻¹ h⁻¹, the 10th percentile value among controls.

The pairwise relationships between age, BMI, LPL activity and lipid traits was expressed in the patient group with and without the females. HDL cholesterol and TG measured on the day of the LPL assay were negatively correlated, with the relationship strongest when only men were included (r = -0.26, P = 0.05 versus r = -0.37, P = 0.025). LPL activities at the various time points were strongly correlated. A weak positive correlation was detected between LPL activity and BMI although this reached statistical significance only using the value for LPL activity measured 30 min after heparin administration (r = 0.33, P < 0.02). LPL activity was not significantly correlated with any of the lipid traits on the day of the lipase assay although a weak inverse relationship was apparent with TG at 10 and 20 min post-heparin (r = 0.16 and -0.21, respectively). There was no evidence of a positive relationship between LPL activity and HDL cholesterol in the whole group or looking at males separately. None of the lipid traits correlated with BMI except presentation cholesterol levels in the whole group (r = 0.47, P = 0.001). HL activity was significantly related to BMI at all time points, with the strongest correlation at 20 min post-heparin (r = 0.36, P = 0.01).

The results from apolipoproteinE genotyping are presented in Table 4. In the group as a whole the frequency of the E4 allele was marginally higher than that reported for healthy control in the United Kingdom (frequency E4 = 0.195 versus 0.137, P < 0.11) [15] with a corresponding decrease in the
Table 4. Individual lipid, lipoprotein results and lipoprotein and hepatic lipase activities (at 10 min post-heparin) in the patient group

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Cholesterol (mmol/l)</th>
<th>TG (mmol/l)</th>
<th>HDL (mmol/l)</th>
<th>ApoB (mg/dl)</th>
<th>ApoA I (mg/dl)</th>
<th>Lp(a) (mg/dl)</th>
<th>LPL (μmol ml⁻¹ h⁻¹)</th>
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E3 allele (0.707 in patients versus 0.773 in controls). These differences did not reach the level of significance ($P = 0.07$).

**Discussion**

The patients examined here showed combined hyperlipidaemia, elevated apoB and with a high incidence of personal and family history of premature CHD. The group therefore conform to the commonly accepted definitions of FCHL. The interest in examining such a group, who are heterogeneous at the biochemical level and therefore probably at the genetic level, lies in the attempt to delineate a subgroup in whom the genetic defect can be determined as the basis of the lipid abnormality [16]. In FCHL patients there is good evidence for involvement of LPL. LPL activity was reduced in our patients, 37% of the patients having LPL activity below the 10th percentile of control values. This was in agreement with recent observations by Babirak and Brunzell [2], who have shown that low LPL activity is common in such patients.

HL may also contribute to the metabolic abnormalities, and we found an increased HL activity in our patients. High HL activity results in small, dense LDL [18, 29], and it is of interest that HL levels are lower in normal women, who on average...
have larger, more buoyant LDL [22]. Among the patients HL activity was elevated in both males and females compared with values reported for healthy men and women, but the sex difference was preserved. Insulin resistance leads to an increase in HL activity which contributes to the FCHL phenotype. Although the patients had normal fasting plasma glucose, hyperinsulinaemia was not excluded, and indeed we found a positive correlation between BMI and HL. The patient group as a whole was overweight, with a quarter of the individuals being obese (BMI > 30), and therefore more likely to have raised insulin levels. Additionally, hypertriglyceridaemia per se is known to be associated with an elevation in HL activity [17]. In our group the patients also had low HDL levels and therefore were at increased risk of CHD [23]. HDL levels were reduced in both male and female patients, but apoAI concentrations were not reduced, suggesting that HDL were relatively protein rich and lipid depleted, consistent with elevated HL activity [15].

The mixed hyperlipidaemia described in this high-risk group is relatively unresponsive to drug treatment [31], nearly always requiring two, sometimes three, types of lipid-lowering therapy. A reduction in low density lipoprotein cholesterol is achieved with 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors [12] or resins — although the latter tend to increase triglycerides [20]. The other metabolic abnormalities, raised TG and low HDL are likely to respond to the addition of a fibrate or nicotinic acid to therapy. Our patient group was treated chiefly with a combination of fibrate (bezalip mono) and statin (simvastatin); even so the lipoprotein abnormality of raised TG and decreased HDL was not fully reversed.

The extent of CHD in patients with mixed hyperlipidaemia is high and is likely to be due to a combination of elevated levels of atherogenic lipoproteins interacting with additional factors such as insulin resistance and fibrinogen. The hypertriglyceridaemia is likely to result in an increase in atherogenic remnant particles due to reduced LPL activity, leading to increased lipid uptake by the scavenger pathway. Plasma levels of certain proteins in the coagulation cascade may also be raised, given the effect of hypertriglyceridaemia in increasing factor VII [24]. In the group of patients studied here Lp(a) was somewhat elevated but not sufficiently to provide an explanation for the high incidence of CHD. Although there is over-production of apoB in this group, there is no apparent accompanying increase in Lp(a) synthesis.

There still remain several unresolved questions about the mechanism of the association between factors which cause an individual to have low levels of LPL activity and the subsequent development of hyperlipidaemia. It is most likely that FCHL requires the combination of a number of different genetic or environmental factors, only some of which are present in each individual patient. Some of these factors are relatively common, such as central obesity or insulin resistance, possibly associated with disturbance in HL. FCHL is thus a set of disorders, with the phenotype being produced by combinations, in different patients, of both genetic and environmental factors. Any information with regard to an abnormality of the gene for lipoprotein lipase may be helpful in the long term to target specific areas for therapy in this high-risk group of patients. Molecular genetic studies to identify such mutations are currently in progress.

Acknowledgements. This work was supported by the British Heart Foundation (grant RG16) and NIH grant 5 RO1 HL 39107-03.

References
A Common Variant in the Gene for Lipoprotein Lipase (Asp9→Asn)

Functional Implications and Prevalence in Normal and Hyperlipidemic Subjects

France Mailly, Yesim Tugrul, Paul W.A. Reymer, Taco Bruin, Mary Seed, Björn F. Groenemeyer, Anette Asplund-Carlson, David Vallance, Anthony F. Winder, George J. Miller, John J.P. Kastelein, Anders Hamsten, Gunilla Olvecrona, Steve E. Humphries, Philippa J. Talmud

Abstract Subjects with combined hyperlipidemia (CHL) were screened for mutations in the lipoprotein lipase (LPL) gene by single-strand conformational polymorphism, and a previously reported G→A DNA sequence change in exon 2, causing substitution of Asp by Asn at position 9, was identified in 2 individuals. Because this substitution destroys a recognition site for Taq I, pooling of DNA samples, amplification, and digest with Taq I allowed the rapid screening of 1563 healthy individuals and patients of Dutch, Swedish, English, and Scottish origin. In the general populations of all four countries, healthy carriers of the mutation were detected at a frequency of 1.6% to 4.4% (mean, 3.0%; 95% confidence interval, 2.0% to 4.0%). The frequency of carriers was roughly twice as high (range, 4.0% to 9.8%) in selected patients with CHL or type IV hyperlipoproteinemia or in subjects with angiographically assessed atherosclerosis; the frequency was consistently higher in each patient group compared with its matched control group. In 773 healthy men from two general practices in the United Kingdom, 25 carriers and 2 homozygotes for the mutation were identified. In these 27, plasma triglyceride but not plasma cholesterol levels were significantly higher than in noncarriers (2.25 versus 1.82 mmol/L, P<.02), and this difference was maintained in three subsequent annual measurements. Postheparin LPL activity data were available for some carriers and for 7 of 9 individuals from the patient groups, and 6 of 6 individuals from the control groups had LPL activity that was lower than the respective group mean. In vitro mutagenesis and transient expression in COS cells showed that compared with the LPL-Asp9 construct, LPL-Asn9 activity and mass were reduced by 20% to 30% in the culture media. Overall however, LPL-Asn9 had only slightly reduced specific activity (by 18%). Thus, although the precise mechanism of the effect is unclear, the data strongly suggest that the LPL-Asn9 variant is associated with and may play a direct role in predisposing carriers to develop hypertriglyceridemia. (Arterioscler Thromb Vasc Biol. 1995;15:468-478.)

Key Words • familial combined hyperlipidemia • genetic predisposition • lipoprotein lipase

A growing body of evidence supports the hypothesis that elevated levels of plasma triglycerides may increase the risk of coronary artery disease (CAD).1,2 A common disorder that is associated with elevated triglyceride levels and increased risk of CAD is familial combined hyperlipidemia (FCHL). This disorder is defined by elevated plasma levels of cholesterol, triglycerides, or both in the proband and in at least one relative,3,6 but often at a relatively late age of onset (third to fourth decade of life).

The common metabolic defect in FCHL appears to be overproduction of triglyceride-rich, apoB-containing particles from the liver;5 which may be accompanied by an increased number of small, dense LDL particles in the blood,8,9 which are believed to be more atherogenic than larger, buoyant LDL and may be more susceptible to oxidative damage.13 However, a number of environmental and genetic factors are also thought to contribute to the development of FCHL, which is now widely believed to be a genetically heterogeneous condition (reviewed in Reference 13). DNA polymorphism studies have shown associations between variation in the apoAI-CIII-AIV gene cluster and FCHL,14,15 and in a proportion of FCHL families, cosegregation with this gene cluster and hyperlipidemia has been reported;16 although this finding has not been reproduced by other workers.17,18 Of the three genes in the cluster, overproduction of apoCIII appears to be the most likely cause of hypertriglyceridemia, as apoCIII is known to inhibit lipoprotein lipase (LPL) and hepatic lipase and to interfere with clearance of remnant lipoproteins.19

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From the Division of Cardiovascular Genetics, Department of Medicine, UCL Medical School, Rayne Institute, London, UK (F.M., S.E.H., P.J.T.); the Department of Medical Biochemistry and Biophysics, University of Umea, Umea, Sweden (Y.T., G.O.); the Lipid Research Group, Department of Vascular Medicine, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands (P.W.A.R., T.B., B.F.G., J.J.P.K.); the Department of Medicine, Charing Cross Hospital, London, UK (M.S.); the Atherosclerosis Research Unit, King Gustaf V Research Institute, Stockholm, Sweden (A.A.-C., A.H.); the Department of Chemical Pathology and Human Metabolism, Royal Free Hospital School of Medicine, London, UK (D.V., A.F.W.); and the MRC Epidemiology and Medical Care Unit, Wolfson Institute of Preventive Medicine, The Medical College of St Barth's Hospital, London, UK (G.J.M.).

Correspondence to Dr Philippa Talmud, Department of Medicine, Division of Cardiovascular Genetics, University College London Medical School, The Rayne Institute, University St, London, WC1E 6JJ England.

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One key factor that determines the metabolism of triglyceride-rich lipoproteins is the activity of LPL. Patients who are homozygous for a mutation in the LPL gene that causes LPL deficiency occur at a frequency of roughly 1 per million and have type I hyperlipoproteinemia with fasting chylomicronemia; thus, carriers for such mutations may be as frequent as 1 in 500. Study of a large kindred with type I hyperlipoproteinemia has shown that some (but not all) relatives who are heterozygous for LPL deficiency have high plasma cholesterol and/or triglyceride concentrations and that this sign is most evident in individuals over 40 years. Other reports have noted the presence of hyperlipoproteinemia in obligate heterozygotes for mutations in the LPL gene, with multiple lipoprotein phenotypes reminiscent of FCHL. Recently, studies from the United States have demonstrated that a proportion (one fifth to one third) of FCHL patients have levels of postheparin LPL activity and mass below the 10th percentile for the general population. In confirmation of these results, a study of UK patients with combined hyperlipidemia (CHL) and a family history of hyperlipidemia or CAD has recently reported that 37% have LPL levels below the 10th percentile of control values. These data suggest that partial LPL deficiency, either genetic or acquired, may underlie the phenotype of FCHL in some patients.

We investigated the role of LPL mutations in the development of CHL and identified a common variant in the LPL gene that is associated with low LPL activity and hypertriglyceridemia. We now report on the frequency of this mutation, its effect on LPL activity in patients and healthy carriers, and on the findings of in vitro expression studies.

**Methods**

**Study Subjects**

Most groups of individuals examined in this study have been described in detail elsewhere. The initial search for mutations by single-strand conformational polymorphism (SSCP) screening was performed for 15 individuals with CHL who had been recruited at Charing Cross Hospital, London, as part of a larger study, and 10 Swedish individuals who were participating in a case-control study of myocardial infarction (MI) at a young age. LPL activity in the lower third of the each sample distribution was used as the selection criterion for this screening.

Specific screening for the Asp9→Asn substitution was undertaken in DNA samples obtained through five separate studies: (1) 93 control subjects and 100 patients who were taking part in a Swedish study of MI before age 45; (2) 61 normotriglyceridemic individuals from Sweden who were matched for age with 65 hypertriglyceridemic individuals from a community-based survey in Stockholm county; (3) 240 Dutch subjects with CHL who were followed up at the Lipid Research Clinic of the University of Amsterdam and 190 normolipidemic healthy control subjects of Dutch origin who were recruited from a risk factor study and matched with the CHL group for age and sex (individuals with plasma lipid levels above the 95th percentile for age and sex were excluded); (4) 300 male subjects aged 40 to 64 from a general practice in southern England and 413 male subjects from a general practice in Scotland, all of whom had been recruited as part of the Northwick Park Heart Study II project and were free of CAD at the time of entry into the study, as assessed by questionnaire and electrocardiography; and (5) 41 consecutive patients with CHL from the Lipid Clinic of Charing Cross Hospital, 93% of whom had a family history of hyperlipidemia.

**Biochemical Analysis**

Cholesterol and triglycerides determined by standard colorimetric methods and biometric data previously obtained for each study were used for comparison of carriers with noncarriers. Approval was obtained from the appropriate institutional ethics review boards for measurement of LPL activity. Blood samples were collected in the fasting state 15 minutes after an intravenous heparin injection (50 or 100 U/kg body weight) was given, and separated plasma was snap-frozen in a dry ice/ethanol bath and kept at −70°C until analysis. Postheparin LPL activity in plasma was determined by one of two methods, using a stabilized, tritated triolein emulsion as the substrate. For samples from the Charing Cross study and the Swedish young MI study control group, LPL activity was measured as described by Nilsson-Ehle and Ekman, whereas the method of Karpe et al was used for all other samples. Six subjects were recalled for reanalysis of plasma LPL by chromatography on heparin-Sepharose columns as described. Briefly, a blood sample was obtained before and after injection of heparin, 10 mL plasma was injected in the column, and 1-mL fractions were eluted with increasing salt concentration. Total lipase activity was measured in individual fractions, as previously described, whereas LPL mass was determined by an enzyme-linked immunosorbent assay with a chicken polyclonal antibody as the capture antibody and monoclonal antibody 5D2 as the detection antibody. The stability (resistance to denaturation) of LPL in carriers and noncarriers of FCHL was studied by preincubating samples of enzyme fractions from heparin-Sepharose columns at 37°C before performing the activity assay.

**DNA Analysis**

Blood was collected in 10-mL Na-EDTA tubes and kept frozen at −20°C. DNA was extracted by the salting-out method or as previously described. Polymerase chain reaction (PCR) amplification of LPL exon 2, yielding a 237-bp product, was achieved on a Cambio machine with a "touchdown" program in the plate mode, using primers on either side of exon 2 (sequence from Oka et al). The sequence of these oligonucleotides (ABC Biotechnology) is as follows: left hand, 5' CTC CAG TTA ACC TCA TAT CC 3'; right hand, 5' CAC CAC CCC AAT CCA CTC 3'.

Following denaturation at 98°C (1 minute, except for 5 minutes at 97°C during the first cycle), the annealing temperature was decreased from 70°C to 55°C in five steps (70°C, 65°C, 60°C, 58°C, and 55°C) over six cycles while the extension conditions were kept constant at 72°C for 1.5 minutes. The reactions were carried out in standard buffer supplied by Gibco-BRL (10× buffer is 500 mM L KCl; 100 mM L Tris-HCl, pH 8.3, 2 mM dNTP, and 0.01% gelatin) with 100 ng of each primer, 5% W l detergent, and 0.5 U Tag polymerase (GIBCO-BRL) per reaction at a final MgCl2 concentration of 1.7 mM/L. For SSCP analysis, amplification was performed as described above, except that 0.2 µL [α-32P]dCTP at 10 µCi/µL (3000 mCi/mmol, Amersham) was added to each sample. An aliquot of the PCR product was then diluted fivefold in 0.1% SDS-10 mM Na-EDTA and kept frozen until needed. DNA was separated into single strands by the method of Orita et al with minor modifications. Samples were denatured by boiling and were separated by gel electrophoresis for 18 hours on a 10% glycerol/7.5% polyacrylamide gel (40 cm, 0.4 mm thick, with a 3:1 ratio of acrylamide:bisacrylamide) at a constant 15-mA current. Direct sequencing of variants detected by SSCP was carried out by using the same primers as in the amplification reaction. The PCR product was purified using the Geneclean II kit (Bio101) and then sequenced by the dideoxy method of Green et al using modified 17 polymerase (Sequenase, US Biochemical Corp).
The G→A substitution destroys a site for Taq I, and for rapid screening of this substitution, DNA samples were pooled in groups of five and amplified as described above. Pooled samples were then digested with Taq I according to the manufacturer's instructions and analyzed on 10% acrylamide gels (80×70×1 mm) using a Hoefer “Mighty Small” vertical electrophoresis apparatus. Samples were run for 1 hour at 30 mA. Detection of fragments was achieved by silver staining according to the method of Merrill et al,29 but with a shorter (1 minute) nitric acid immersion time and brief (2×<20 seconds) distilled water rinses to compensate for the thinness of the gels. The bands were seen at 179 and 52 bp (G→Taq I cutting→Asp9) or 179 and 58 bp (A→Taq I not cutting→Asp9). Samples from pooled DNA showing the 58-bp band were individually reamplified and processed as described above for final identification of Asp9 carriers. Genotypes for Pvu II and HindIII were determined by PCR using the same primers and amplification conditions as described previously.30

Site-Directed Mutagenesis and Transient Expression Studies

In vitro site-directed mutagenesis was used to synthesize the Asp9 mutant allele (Altered Sites mutagenesis system, Promega). A 2.4-kb fragment of the LPL cDNA containing the entire coding sequence was inserted in the antisense orientation in the pSelect plasmid vector with a defective ampicillin resistance gene. Single-stranded vector containing the full-length LPL-Asp9 cDNA was produced by coinfection of JM109 bacteria with phage R408 and isolated by precipitation with 0.25 vol 20% polyethylene glycol-3.75 mol/L ammonium acetate. The purified material was then annealed to the mutagenic oligonucleotide (5'-ACCTTGATGGATGATGCTAATATG-3') and to the ampicillin repair primer, and second-strand synthesis was performed according to the manufacturer's recommendations. Following two rounds of transformation with ampicillin selection, phagemid DNA from resistant colonies was isolated by standard methods and checked for the presence of the mutation by direct sequencing. After reamplification, a positive LPL insert was excised from pAlter by digest with Xba I/Psa I, purified by electrophoresis and elution on a Spin-Bind column (Bozyn), and ligated into the linearized pcDNA expression vector (Invitrogen Corp) in the sense orientation. The resulting pcDNA-LPL-Asp9 construct had the LPL cDNA under control of the cytomegalovirus promoter and expressed the Tyr tRNA suppressor gene (synthetic SupF gene). When used to transform mc1061 bacteria harboring the mutant p3 plasmid (Invitrogen Corp), this construct conferred ampicillin and tetracycline resistance to mc1061/p3 cells. Large-scale purification of pcDNA-LPL-Asp9 and pcDNA-LPL plasmids for transfection was achieved with the Circle Prep kit (Bio101). The purity of plasmid DNA preparations was confirmed spectroscopically and electrophoretically to confirm the absence of contaminating bacterial DNA and by sequencing of the plasmid to confirm its identity and ensure that no other sequence changes had been introduced during manipulation.

The DEAE-dextran method31 was used for transfection of COS-B cells. Briefly, the DNA to be transfected was diluted in 2.5 mL Dulbecco's modified Eagle's medium with 51.6 µg/mL chloroquine and then mixed 1:1 (vol/vol) with a 0.6-mg DEAE-dextran solution. Plasmid DNA (5 µg) was used for each 60-mm dish (50% to 80% confluent). The cells were washed once with medium, and the transfection mixture was added for 3.5 hours. The cells were washed briefly (1 minute) with DMSO, washed once with PBS, and then allowed to recover with medium containing 10% fetal calf serum. Samples of medium were collected and cells harvested 3 days after transfection, with heparin (20 U/mL medium) being added to half of the dishes 2 hours prior to collection. All material was snap-frozen and kept at −70°C until assayed as described previously.26,27

Statistical Analysis

The gene-counting method with a χ2 test and Yates' correction was used to compare the frequencies of the Asp9 variant allele between the different groups. The estimate of relative risk (RR) of containing a carrier was calculated by standard techniques. To compare RRs between samples, the estimates of logRR were weighted by the reciprocal of the sampling variance. The estimates are combined by calculating a weighted mean and are tested for heterogeneity by a χ2 index (Woolf's method).32 All other tests and transformations were performed with the SPSS statistical package. The Mann-Whitney nonparametric test and t test were used to compare levels of lipid traits, LPL activity, and LPL mass between carriers and noncarriers of the Asp9 variant in healthy men from the two UK studies. To test differences in triglyceride levels, values were logarithmically transformed prior to statistical analysis. Statistical significance was considered at P < .05.

Results

Screening of LPL exons 2 through 6, 8, and 9 for mutations was undertaken using SSCP in a group of 25 hyperlipidemic subjects who were selected on the basis of low LPL activity. A number of SSCPs were detected but data for only one of these SSCPs will be presented here. This variant pattern in exon 2 (Fig 1a) was detected in 3 individuals, 2 from the CHL low-LPL group from Charing Cross Hospital and 1 from the
low-LPL patient group from the Swedish young MI study. No pattern differences in any other exons were detected for these 3 individuals. Direct sequencing of exon 2 revealed a G→A transition at position 280 (sequence numbering according to Wion et al) (Fig 1b), resulting in the substitution of Asn for Asp at amino acid position 9. This mutation was previously reported in a patient with type I hyperlipoproteinemia, who, in addition to being homozygous for the Asn9 variant, was homozygous for the Tyr262→His substitution. This second base change was not present in the carrier subjects studied here.

This G→A transition was predicted to abolish a Taq I restriction site, resulting in the production of a 58-bp fragment, larger than the wild-type 52-bp fragment, upon digest of the exon 2 PCR product with this enzyme (Fig 1c). To estimate the frequency of this base change in hyperlipidemic individuals and to determine whether it was present in apparently healthy individuals, a rapid screening strategy was developed on the basis of pooling the DNA samples prior to PCR amplification. Four to six DNA samples were pooled, amplified, and digested with Taq I. Individual samples from pools found to be positive were then reamplified and redigested for final identification of carriers. Mixing experiments (not shown) demonstrated that this method had the sensitivity to identify a carrier from a pool of as many as 10 samples (1 mutant allele from a total of 20), but given the frequency of this variant, pools of four to six individual samples were optimal.

Patients from England, two groups from Sweden, and one group from the Netherlands who had been selected on the basis of either being hyperlipidemic or having suffered an MI before the age of 45 were screened. Each patient group had a comparison group of control subjects from each general population; the general characteristics, lipid levels, and LPL activities (where available) of these groups are summarized in Table 1. A total of 37 Asn9 carriers were identified. As shown in Table 2, in the four control groups the frequency of carriers was between 1.6% and 2.5% (mean, 2.2%; 95% confidence interval, 1.1% to 3.2%) with no significant evidence for heterogeneity in frequency between groups ($\chi^2 = 0.34$, $P = .89$). One individual in the general practice control group from England was homozygous for the Asn9 mutation. In each group of patients the frequency of carriers was roughly twice as high as in the matched control group. The frequency was fourfold higher (9.8% versus 2.5%) when the CHL sample from Charing Cross Hospital was compared with control subjects in the English general population. The two study groups from Sweden had similar frequency differences, with a prevalence almost twofold higher in young MI patients compared with their healthy counterparts (4.0% versus 2.2%) and more than threefold higher in hypertriglyceridemic compared with normotriglyceridemic individuals (5.0% versus 1.6%). In the Dutch CHL group the frequency was threefold higher compared with Dutch control subjects (4.9% versus 1.6%). There was no significant difference ($P = .91$) between studies in the RR of being a carrier in patients compared with control subjects.

To examine the effect of the mutation on plasma lipid levels, an additional 413 healthy men from a general practice in Scotland were screened. Their characteristics were similar to the English control group (Table 1), and 18 carriers (including one homozygote) were identified. The frequency of carriers in this group was 4.4%, which is higher (but not significantly, $\chi^2 = 1.74$ by gene counting, $P > .2$) than the frequency in the English group. As shown in Table 3, after data from the two groups of

### Table 1. General Description of Population Samples Screened for the Asp9→Asn Substitution

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<th>Sample</th>
<th>n</th>
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<th>BMI, kg/m² Mean±SEM</th>
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<td>2.62±0.18</td>
<td>5.76±0.05</td>
<td>1.96±0.09</td>
<td>. .</td>
</tr>
<tr>
<td>Combined hyperlipidemias, Charing Cross</td>
<td>41</td>
<td>59</td>
<td>51.2±1.59</td>
<td>29.2±0.62</td>
<td>8.05±0.27</td>
<td>4.15±0.21</td>
<td>49.7±2.7</td>
</tr>
<tr>
<td>Hospital</td>
<td>360</td>
<td>86</td>
<td>50.4±0.40</td>
<td>26.4±0.26</td>
<td>6.09±0.12</td>
<td>1.48±0.14</td>
<td>92.1±3.6</td>
</tr>
<tr>
<td>Swedish young MI study control subjects</td>
<td>93</td>
<td>100</td>
<td>40.4±0.38</td>
<td>26.4±0.35</td>
<td>7.24±0.14</td>
<td>2.61±0.19</td>
<td>72.3±4.1</td>
</tr>
<tr>
<td>Swedish young MI patients</td>
<td>100</td>
<td>76</td>
<td>45.4±0.40</td>
<td>24.7±0.35</td>
<td>5.63±0.10</td>
<td>1.22±0.06</td>
<td>286.1±11.1</td>
</tr>
<tr>
<td>Swedish normolipidemic subjects</td>
<td>61</td>
<td>100</td>
<td>44.9±0.47</td>
<td>27.8±0.47</td>
<td>6.80±0.15</td>
<td>4.54±0.20</td>
<td>277.7±10.1</td>
</tr>
<tr>
<td>Swedish hypertriglyceridemic subjects</td>
<td>60</td>
<td>100</td>
<td>46.0±0.65</td>
<td>25.0±0.22</td>
<td>5.55±0.06</td>
<td>1.26±0.03</td>
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</tr>
<tr>
<td>Dutch combined hyperlipidemias</td>
<td>190</td>
<td>100</td>
<td>47.2±0.85</td>
<td>26.2±0.18</td>
<td>8.14±0.08</td>
<td>2.96±0.09</td>
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<tr>
<td>Dutch combined hyperlipidemias</td>
<td>240</td>
<td>62</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

BMI indicates body mass index; Chol, cholesterol; TG, triglycerides; LPL, lipoprotein lipase; and MI, myocardial infarction.

2. LPL activity is presented in milliunits (mU)/mL, which corresponds to 1 nmol fatty acid released per minute per milliliter.

From Reference 22, LPL activity in healthy control subjects was 64.3±3.1 mU/mL.

### Table 2. Frequency of Asn9 Carriers in Patients With Hyperlipidemia or Premature Myocardial Infarction and Control Subjects of Dutch, English, Swedish Origin and the Relative Risk of Being a Carrier

<table>
<thead>
<tr>
<th>Sample</th>
<th>Carrier%</th>
<th>Companion Control Group</th>
<th>Relative Risk of Being a Carrier</th>
</tr>
</thead>
<tbody>
<tr>
<td>UK CHL</td>
<td>3.9</td>
<td>2.5</td>
<td>4.22</td>
</tr>
<tr>
<td>Swedish young MI</td>
<td>4.0</td>
<td>2.2</td>
<td>1.94</td>
</tr>
<tr>
<td>Swedish HTG</td>
<td>5.0</td>
<td>1.6</td>
<td>3.16</td>
</tr>
<tr>
<td>Dutch CHL</td>
<td>4.9</td>
<td>1.6</td>
<td>2.99</td>
</tr>
</tbody>
</table>

UK indicates United Kingdom; CHL, combined hyperlipidemias; MI, myocardial infarction; and HTG, hypertriglyceridemia.

2. Percentage of carriers in patients does not differ between studies: 4 of 41 versus 4 of 100 versus 3 of 60 versus 11 of 240. $P = .46$.

3. Percentage of carriers in control groups does not differ between studies: 9 of 360 versus 2 of 93 versus 1 of 61 versus 3 of 90. $P = .89$.

4. Test for heterogeneity of RR $\chi^2 = 0.53$. $P = .89$.

5. One individual was homozygous.

6. Number of Asp9 homozygous individuals (wild-type allele) compared with the number of carrier individuals for the Asn9 variant.

7. Weighted relative risk.
TABLE 3. Mean±SEM Plasma Lipid Levels at Baseline in Healthy Lipoprotein Lipase (LPL)-Asp9 and LPL-Asn9 Carriers From England and Scotland

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Age, y</th>
<th>BMI, kg/m²</th>
<th>Chol, mmol/L</th>
<th>TG, mmol/L</th>
<th>ApoB, g/L</th>
<th>ApoAI, g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPL-Asp9</td>
<td>746</td>
<td>55.5±0.18</td>
<td>26.4±0.14</td>
<td>5.77±0.03</td>
<td>1.82±0.07</td>
<td>1.73±0.01</td>
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</tr>
<tr>
<td>LPL-Asn9</td>
<td>27</td>
<td>56.2±0.63</td>
<td>27.7±0.66</td>
<td>5.55±0.17</td>
<td>2.25±0.34</td>
<td>1.61±0.08</td>
<td></td>
</tr>
</tbody>
</table>

Test for difference P = .75 P = .09 P=.04 P < .02 P=.24 P = .09

*BMI indicates body mass index; Chol, cholesterol; and TG, triglycerides.

Healthy men from England and Scotland were combined, those with at least one allele for LPL-Asn9 had significantly higher (24%) plasma triglyceride levels than did noncarriers, with no other significant differences in measured traits between groups. The individual data for triglycerides and cholesterol are shown in Fig 2a, which reveals considerable scatter in triglyceride levels, with one homozygote having a relatively low and the other a relatively high level. The individual triglyceride and cholesterol levels for the Dutch and Swedish control groups are also shown in Fig 2a, and 5 of 6 individuals have triglyceride values above their respective sample mean.

In 22 male Asn9 carriers from the United Kingdom, complete lipid data were available for baseline and three subsequent annual measurements, and these are shown in Fig 2b. In the group of noncarriers (n=631) there was a small (but significant, P=.001) decrease in plasma triglyceride level over the 3 years, but the significantly higher plasma triglyceride level in LPL-Asn9 carriers was maintained (P=.01 overall). The slightly lower cholesterol levels in carriers at baseline were also maintained over time, but overall, this difference was still not significant (5.35±0.18 mmol versus 5.66±0.18, P=.1).

To investigate the potential role of obesity in the development of elevated plasma triglyceride levels in carriers, lipid levels were determined in those men with a body mass index (BMI) in the lowest tertile (<25.0 kg/m²) and in the upper two tertiles combined. In noncarriers from the two UK general practice samples, plasma triglyceride values were, as expected, higher in those with a BMI in the upper tertiles (1.54 versus 1.94 mmol/L, 26% higher), but in carriers the effect associated with BMI was larger (1.35 versus 2.93 mmol/L, 117% higher), although this difference did not reach conventional levels of significance (BMI×genotype interaction, P=.07).

Where available, LPL activity data were examined for the Asn9 carriers. Fig 3 shows individual LPL activity values from the various studies plotted against each
of the Asn9 variant by analyzing postheparin LPL activity and mass in normal lipidemic carriers. Three Asn9 carriers from the English general practice sample were recalled, and samples from three noncarrier normal laboratory control subjects were taken for comparison. In the carriers, mean total LPL activity was 30% lower than in the medium. Overall, there was no clear evidence for LPL activity or mass within cells being threefold to fourfold higher than in the medium before and after addition of heparin. However, because both activity and mass were decreased, the overall reduction of approximately 20% to 30% (range, 12% to 22%) for activity and 6% to 32% for mass), with specific activity being a 15% to 40% decrease in LPL activity or overall decreased release or secretion of LPL from the cells. To examine this question, experiments were carried out to characterize biochemically the Asn9 variant by analyzing postheparin LPL activity and mass in normal lipidemic carriers. Three Asn9 carriers from the English general practice sample were recalled, and samples from three noncarrier normal laboratory control subjects were taken for comparison. In the carriers, mean total LPL activity was 30% lower (177 versus 251 mU/mL) and mass 30% lower (985 versus 1538), although these differences did not reach significance (t test, P = .12 for mass and P = .16 for activity). Separation of plasma by heparin-Sepharose did not reveal any striking differences in LPL activity or mass elution profiles between carriers and noncarriers. In particular, there was no clear variation in the ratio of the two mass peaks or the two areas under the curve, representing inactive monomeric LPL and active dimeric LPL, respectively; both forms eluted in the expected fractions (not shown). The semipurified LPL from both Asn9 carriers and noncarriers had a similar stability incubation at 37°C (not shown). Activity was then measured for 22 hours. No striking difference in substrate affinity was observed between lipase preparations from carriers and noncarriers (not shown).

To determine whether the mutation causing the Asn9 substitution may have occurred on more than one occasion, genotypes for the Pvu II (intron 6) and HindIII (intron 8) polymorphic sites were determined for all identified Asn9 carriers except those in the Scottish sample. In 28 individuals for whom unambiguous haplotypes could be determined, two distinct Asn9 haplotypes that differed at the Pvu II site were identified. Seven individuals were P-P/H+H+ homozygotes (ie, noncutting for Pvu II but cutting for HindIII) and thus carried the Asn9 variant on a P-H+ haplotype; 4 individuals were homozygous for the P+H+ haplotype; and in 12 individuals who were heterozygous for the Pvu II restriction site, either haplotype could carry the Asn9 variant. Of the remaining 5 subjects for whom data on both polymorphisms were available, 1 was homozygous and 4 were heterozygous for a third, rarer carrier haplotype, P-H-. Among the healthy men from the United Kingdom, those with different Pvu II/HindIII genotypes had similar levels of plasma triglycerides (not shown), and of those with low LPL activity, 2 had the commonest P-H+ carrier haplotype and 1 had the P+H+ haplotype, but the haplotype could not be determined unambiguously in the remaining 6 individuals (P+P-/H+H-).

To confirm that these effects on LPL activity and plasma triglycerides were due to a direct effect of the Asp9→Asn substitution, the Asn9 variant was constructed by site-directed mutagenesis and expressed in vitro. Following transfection of Asn9 and Asp9 constructs in COS cells, cells and media were collected and tested for LPL activity and mass, and data from three separate experiments are summarized in Table 4. In all experiments, LPL activity and mass were measured in the medium in separate plates, both before and after addition of heparin to release LPL from the cell surface. In cells transfected with the Asn9 construct, both activity and mass in the medium were lower than for the Asp9 construct, and this was seen in all experiments and in all nine replicates from three experiments. There was an overall reduction of approximately 20% to 30% (range, 18% to 40% for activity and 6% to 32% for mass), with similar results for mass and activity measurement in the medium before and after addition of heparin. However, because both activity and mass were decreased, the reduction in LPL specific activity was smaller and not statistically significant (range, 12% to 22%). Levels of activity and mass within cells were measured in all plates, and these values showed a large scatter, with mass measurements being threefold to fourfold higher than in the medium. Overall, there was no clear evidence of intracellular accumulation of LPL-Asn9 (data not shown). Preheparin media from several plates were pooled in the fourth transfection experiment and passed through a heparin-Sepharose affinity column to separate
the excess monomer LPL protein associated with these in vitro assays. The resulting profile still showed decreased dimer mass and activity for the Asn9 construct, with no apparent elution shift of the dimer peak for the mutant, suggesting that the affinity for heparin was not altered (Fig 4). There were no significant differences in the monomer to dimer mass ratio, as assessed by the areas under the curve or by peak fractions. The stabilities of LPL-Asp and LPL-Asn were compared by measuring activity in the medium from cells incubated at 37°C for 2 hours. LPL activity declined slowly over this time at a similar rate for both constructs (not shown).

**Discussion**

Many individuals who develop hyperlipidemia report a family history of hyperlipidemia and/or CAD, although the observed disease pattern may be incompatible with the inheritance of a single major gene. In most families, several different genes are likely to contribute, with their effects being modified by environmental factors, so that each family has a different constellation of causative genetic and environmental factors. In this model, a mutation that alters the function of a given gene would confer susceptibility but have insufficient impact on its own to cause disease. Such a mutation would therefore be present in healthy individuals in the general population but would be found more frequently in patients with hyperlipidemia, and the data we report here suggest that the common Asp9→Asn variant in the LPL gene is such a mutation. The frequency of carriers of the mutation in the healthy populations of southern England, the Netherlands, and Sweden ranged between 1.6% and 2.5%, with a slightly higher frequency observed in healthy men from Scotland. The presence of the mutation in individuals from four different European countries suggests that it may be present in other Caucasian groups, but further studies are needed for a more accurate estimate of gene frequency. The frequency of carriers of the mutation was consistently higher in Swedish MI patients and Swedish, English, and Dutch hyperlipidemic cases than in their corresponding general population control groups. Although combining data from the different patient groups is invalid because the selection criteria are different, the consistently higher frequency in the patient groups strongly suggests that the LPL-Asn9 variant is associated with and contributes to the development of hyperlipidemia.

The hypothesis that the Asn9 substitution has a direct effect on LPL function is supported by data from in vivo studies. In 34 of the carriers, three distinct two-restriction fragment length polymorphism Asn9 haplotypes were identified, suggesting that the mutation has occurred more than once, a likely possibility, considering that a mutable CpG dinucleotide is involved. All three
could account for this observation. LPL is synthesized in both skeletal muscle and adipose tissue, and LPL expression is controlled in a complex tissue-specific manner.

was HTG. The second had a BMI that was 18% lower than the Charing Cross CHL group and 1 from the Swedish HTG group. Several explanations are possible, and in the healthy group (3 of 3) than in the normolipidemic group mean. Thus, 10 carriers, including 1 homozygote, had plasma triglyceride levels that are not elevated, and this raises the possibility that in some circumstances there may be a compensatory up-regulation of the functional allele.

In the in vitro experiments, although there was some variation in results, lower activity and mass were consistently observed in the medium from transfected COS cells. The observed variability is not surprising, because the transient expression systems (for several reasons) are not ideal for testing relatively small differences. Although the number of cells per plate was kept constant within each experiment, there was no control for transfection efficiency. However, this issue was at least partially addressed by using multiple dishes, randomized to the two constructs; by combining data from several plates; and by the consistency of results in repeated experiments. The data for nine replicates of three experiments show that cells transfected with the LPL-Asn9 construct consistently had lower levels of LPL activity (by 18% to 40%) and mass (by 6% to 32%) than did plates of cells with the wild-type construct. This small effect on activity and mass is consistent with the in vivo data: in the three normolipidemic carriers who were recalled, mean LPL activity and mass were reduced by 25% to 30%. However, both in vivo and in vitro, specific activity was within the range for the wild-type LPL-Asp9 construct.

Our in vitro experiments suggest that LPL-Asn9 has a specific activity within the normal range, as originally reported. The stability of the LPL-Asn9 secreted by COS cells did not differ from that of LPL-Asp9, and binding affinity and the ability to bind heparin appear unchanged, suggesting that the lower mass and activity in the medium were not caused by impaired LPL release from the cell surface. This is not surprising, since the main heparin-binding domain has been proposed to lie between amino acids 270 and 305, which are predicted to be distant from the N-terminal sequence in the tertiary structure of LPL. The data from these two sets of experiments raise the possibility that the Asn9 substitution may impair secretion, which might lead to intracellular accumulation of LPL. This could not be accurately determined in these experiments due to the difficulty in measuring low levels of active LPL enzyme and in distinguishing inactive monomer from active dimer forms in the cell lysates. These issues could be resolved by cell fractionation and metabolic labeling experiments in permanently transfected cell lines.

One question still to be addressed is the relative proportion of LPL present as the Asn9 variant protein in the plasma of carriers and the ability of the Asn9 monomer to associate intracellularly with the Asp9 monomer. Theoretically, roughly half of plasma LPL activity should be due to Asp9-Asn9 heterodimers, with the other half divided equally between the two homodimers, although this cannot be readily assessed with...
current techniques. Evidence from the in vitro studies suggests that Asn9 homodimers are not produced as efficiently as their Asp9 counterparts, and it is possible that heterodimer production may be even less efficient. If this is the case, then tissue culture models would underestimate the impact of the Asn9 variant and could explain the similar decrease in mass and activity observed in both homozygous (in vitro) and heterozygous (carrier individuals) settings.

After completion of the work reported here, two small studies reported identification of the LPL-Asn9 variant in 20 patients from the United States and 31 patients from Canada with FCHL. A carrier frequency of 2% to 5% was observed in FCHL patients, but a similar frequency was also reported in 49 healthy individuals. However, neither study was large enough to have the power to detect small differences in carrier frequency, and in addition, case-control frequency comparisons are easily confounded by ethnic heterogeneity, such as is found in the United States. Further larger studies are required to more precisely estimate the frequency of the LPL-Asn9 variant in groups of patients with different hyperlipidemic phenotypes. However, neither previous study would have had the sensitivity to detect the 20% to 30% lower activity and mass that we consistently observed in the more extensive transfection experiments reported here.

The 24% higher plasma triglyceride levels observed in normal LPL-Asn9 carriers support the contention that the moderate decrease in LPL activity associated with the Asn9 substitution can directly affect lipid metabolism and that this factor, when compounded by other factors, may lead to full hyperlipidemia. These data suggest that a search for other common variants in the LPL gene that affect enzyme function or expression will be fruitful.

Table 5. Age, Body Mass Index, and Lipid Levels of Individuals With the Asp9→Asn Substitution

<table>
<thead>
<tr>
<th>Sample/No.</th>
<th>Age, y</th>
<th>BMI, kg/m²</th>
<th>Chol, mmol/L</th>
<th>TG, mmol/L</th>
<th>HDL, mmol/L</th>
<th>LPL Activity, (Percentile)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Dutch CHLs</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>58</td>
<td>8.0</td>
<td>8.05</td>
<td>0.82</td>
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</tr>
<tr>
<td>2</td>
<td>81</td>
<td>8.3</td>
<td>3.06</td>
<td>0.83</td>
<td></td>
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</tr>
<tr>
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<tr>
<td>4</td>
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<tr>
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<td>8</td>
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<tr>
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<td>6.10</td>
<td>0.79</td>
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<td>35†</td>
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<tr>
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<td>43</td>
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</tr>
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<td>5.8§</td>
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<td>28.22</td>
<td>6.3§</td>
<td>4.45§</td>
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### Acknowledgments

This work was supported by the British Heart Foundation (RG16) (Dr Humphries and Talmud), the Swedish Medical Research Council, and the Bank of Sweden Tercentenary Foundation (Dr Olivecrona). The excellent technical assistance of Ann-Sofie Jakobsson and Karla Peters is gratefully acknowledged, and we thank Jackie Cooper for assistance with the statistical analysis.

### References


### Table 5 continued

<table>
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<tr>
<th>Sample/No.</th>
<th>Age, y</th>
<th>BMI, kg/m²</th>
<th>Chol, mmol/L</th>
<th>TG, mmol/L</th>
<th>HDL, mmol/L</th>
<th>LPL Activity (Percentile)*</th>
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<td>4054</td>
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<td>26.4</td>
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<td>2.75$^*$</td>
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<td>45</td>
<td>23.3</td>
<td>6.09$^*$</td>
<td>1.65$^*$</td>
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<td>63 (17)</td>
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<tr>
<td>Dutch</td>
<td>112</td>
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<td>238 (30)</td>
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<td>1.51$^*$</td>
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<td>20.0</td>
<td>5.79$^*$</td>
<td>1.58$^*$</td>
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</table>

(BMI indicates body mass index; Chol, cholesterol; TG, triglycerides; LPL, lipoprotein lipase; FFA, free fatty acids; CHLs, combined hyperlipidemias; MI, myocardial infarction; HTGs, hypertriglyceridemias; GP, general practice; and NTGs, normotrigrayseridemias. *The difference in the range of values between study groups originates from the use of different amounts of heparin (50 or 100 U) and differences in LPL activity assays. †Female subject. ‡Individuals homozygous for the LPL-Asn9 variant. §Value is at or above the mean for the sample.)
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