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Low density lipoprotein (LDL) heterogeneity

Implications for cardiovascular disease and genetic influence

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Abstract

The low density lipoprotein (LDL) particle population is heterogeneous with regard to several structural and functional properties that may influence its atherogenic potential. A preponderance of small, dense LDL is associated with an increased risk of coronary heart disease (CHD), and also predicts future CHD events. However, the metabolic pathways for the formation of the small, dense LDL are not yet fully understood. There is strong evidence that the LDL particle size distribution is under genetic influence, although the major regulating genes remain unknown. The present studies were conducted to further elucidate the role of LDL heterogeneity in cardiovascular disease, and to evaluate the metabolic and genetic determinants of LDL particle size distribution.

In **study I** the influence of LDL particle size distribution on intima-media thickness (IMT) of the common carotid artery (CCA) was investigated in healthy 50-year-old men. A high-resolution nondenaturing polyacrylamide gradient (3-7.5%) gel electrophoresis (GGE) procedure was developed to measure LDL peak particle size (nm), relative distribution (%) and plasma concentration (mg/L) of four LDL subfractions. The IMT of the CCA was measured by B-mode ultrasound. The results of this study show that the plasma concentration of small, dense LDL (LDL-III) is a strong and independent indicator of early atherosclerosis in healthy, middle-aged men.

In **study II** the effects of artificial and exhaustive lipolysis on potentially atherogenic properties of LDL were investigated in healthy normotriglyceridaemic men. After *in vitro* and *in vivo* lipolysis of serum and plasma triglycerides, respectively, the LDL particle population was characterised with regard to size, composition, and susceptibility to oxidative modification. The results of this study demonstrate that an exaggerated or efficient lipolysis of plasma triglycerides results in the generation of new LDL particles with an increased content of α-tocopherol and increased resistance to oxidative modification.

In **studies III-V** the influence of common functional variants in candidate genes (encoding proteins or enzymes with important roles in lipoprotein metabolism) on LDL heterogeneity was investigated.
In study III, polymorphisms in the cholesteryl ester transfer protein (CETP), lipoprotein lipase (LPL), hepatic lipase (HL), and apolipoprotein E (apoE) genes were studied in relation to LDL particle size in 377 healthy, middle-aged men. The results of this study show that the investigated polymorphisms are associated with moderate effects on the LDL particle size, consistent with respect to protein function and proposed association with CHD risk. In study IV, the isolated and combined effects of the apolipoprotein B (apoB) and the apoE polymorphisms on LDL particle size and risk of CHD were investigated in 405 survivors of a first myocardial infarction before the age of 60, and 769 healthy individuals. The results of this study demonstrate that a gene-gene interaction between the apoB and apoE polymorphisms is associated with a markedly elevated concentration of small, dense LDL, which is further conveyed to an increased risk of myocardial infarction. In study V, the influence of a common variant in the MTP gene promoter on the secretion pattern of apoB-containing lipoproteins and plasma LDL heterogeneity was investigated. A total of 12 healthy men were recruited by genotype to participate in apoB stable isotope turnover studies, and kinetic parameters were calculated by multicompartmental modelling. LDL particle size was measured in 377 healthy, middle-aged men. The results of this study show that the MTP promoter polymorphism is associated with a reduced direct production of IDL+LDL particles, which appears to be directly related to lower plasma concentrations of large LDL particles.

Conclusions: The studies presented here demonstrate that the atherogenic properties of plasma LDL goes far beyond the routinely measured LDL cholesterol concentration. LDL heterogeneity appears to be regulated by complex metabolic pathways, which are further modulated by common genetic variability.

Key words: low density lipoprotein, gradient gel electrophoresis, intima-media thickness, atherosclerosis, myocardial infarction, low density lipoprotein oxidation, cholesteryl ester transfer protein, lipoprotein lipase, hepatic lipase, apolipoprotein E, apolipoprotein B, microsomal triglyceride transfer protein, genetics, lipoproteins, metabolism

List of original articles

This thesis is based on the following original articles, which will be referred to by their Roman numerals.

I Skoglund-Andersson C, Tang R, Bond MG, de Faire U, Hamsten A, Karpe F
LDL particle size distribution is associated with carotid intima-media thickness in healthy 50-year-old men
Arteriosclerosis, Thrombosis and Vascular Biology 1999;19:2422-2430

II Skoglund-Andersson C, Karpe F, Hellénius ML, Regnström J, Hamsten A, Tornvall P
In vitro and in vivo lipolysis of plasma triglycerides increases the resistance to oxidative modification of low-density lipoproteins
European Journal of Clinical Investigation 2003;33:51-57

III Skoglund-Andersson C, Ehrenborg E, Fisher RM, Olivecrona G, Hamsten A, Karpe F
Influence of common variants in the CETP, LPL, HL and ApoE genes on LDL heterogeneity in healthy, middle-aged men
Atherosclerosis 2003, In press

IV Skoglund-Andersson C, Samnegård A, Ehrenborg E, van 't Hooft F, Krapivner S, Boquist S, Hamsten A, Karpe F
Interaction between the ApoE and ApoB genes affects plasma concentration of small, dense LDL and risk of coronary heart disease
Manuscript

V Lundahl B, Skoglund-Andersson C, Caslake M, Bedford D, Stewart P, Hamsten A, Packard CJ, Karpe F
Reduced direct production of LDL and IDL apoB in subjects homozygous for the microsomal triglyceride transfer protein -493T gene variant is related to fewer large LDL particles in plasma
Manuscript

Contents

Abbreviations

aa	amino acid
ALP	atherogenic lipoprotein phenotype
apo	apolipoprotein
BMI	body mass index
bp	base pair
CAD	coronary artery disease
CCA	common carotid artery
CE	cholesteryl ester
CETP	cholesteryl ester transfer protein
CHD	coronary heart disease
d	density
DGUC	density gradient ultracentrifugation
ER	endoplasmic reticulum
FC	free cholesterol
FCH	familial combined hyperlipidaemia
FFA	free fatty acid
FH	familial hypercholesterolaemia
GGE	gradient gel electrophoresis
HDL	high density lipoprotein
HL	hepatic lipase
HPLC	high performance liquid chomatography
HSPG	heparan sulfate proteoglycans
IDL	intermediate density lipoprotein
IMT	intima-media thickness
kb	kilo base pair
LDL	low density lipoprotein
LDLR	low density lipoprotein receptor
Lp (a)	lipoprotein (a)
LPL	lipoprotein lipase
LRP	low density lipoprotein receptor-related protein
MI	myocardial infarction
MTP	microsomal triglyceride transfer protein
NEFA	non-esterified fatty acid
NMR	nuclear magnetic resonance
PA	polyacrylamide
PL	phospholipid
RFLP	restriction fragment length polymorphism
TG	triglyceride or triacylglycerol
TRL	triglyceride-rich lipoprotein
Sf	Svedberg flotation rate
VLDL	very low density lipoprotein

Introduction

Cardiovascular disease is a major cause of morbidity and mortality in the industrialised world. Coronary heart disease (CHD) is a prevalent manifestation of cardiovascular disease, and the predominant underlying cause is atherosclerosis of the coronary arteries. The incidence of CHD is related to multiple genetic and environmental risk factors. Well-established risk factors for CHD are age, male sex, family history of atherosclerotic disease, smoking, hypertension, hypercholesterolaemia and diabetes mellitus. However, traditional risk factors for coronary artery disease (CAD) predict only about 50% of the overall cardiovascular risk. Among the emerging risk factors for CAD are elevated levels of homocysteine, prothrombotic factors, markers of inflammation such as C-reactive protein (CRP), and small, dense low density lipoprotein (LDL) particles.

Lipoproteins

Lipoproteins are soluble aggregates of lipids and specific proteins (apolipoproteins) that transport hydrophobic lipids (triacylglycerols, cholesterol and cholesteryl esters) in plasma. All lipoprotein particles have a common structure of a neutral lipid core surrounded by a surface monolayer of phospholipids, free cholesterol and apolipoproteins. The major plasma lipoproteins are classified according to density (Table 1).

The apolipoproteins are essential structural components of the lipoprotein particles, but also serve important functions as cofactors for enzymatic reactions and ligands for selective catabolism of plasma lipoproteins by cell-surface receptors (Table 2).

Table 1. Size, density and composition of the major plasma lipoproteins

	Chylomicrons	VLDL	IDL	LDL	HDL
Density (g/mL)	<0.95	0.95-1.006	1.006-1.019	1.019-1.063	1.063-1.210
Diameter (nm)	80-1200	30-80	25-35	18-25	5-15
Composition (% dry wt)					
Protein	1-2	10	18	25	33
Triglyceride	83	50	31	9	8
Free cholesterol + Cholesterol ester	8	22	29	45	30
Phospholipid	7	18	22	21	29
Apolipoproteins	A-I, -II				A-I, -II
	B-48	B-100	B-100	B-100	
	C-I, -II, -III	C-I, -II, -III			C-I, -II, -III
	E	E	E		E

Table 2. Major apolipoproteins

Apolipoprotein	Function
ApoA-I	Structural protein of HDL. Activates LCAT. Promotes cholesterol efflux from peripheral tissues.
ApoA-II	Structural protein of HDL. Stabilizes the HDL particle? Affects HDL metabolism?
ApoB-100	Structural protein of VLDL, IDL and LDL. Ligand for the LDLR.
ApoB-48	Structural protein of chylomicrons. Truncated form of apoB-100, lacks the LDLR-binding region.
ApoC-I	Modulates the interaction between apoE and VLDL remnants? Inhibits binding of VLDL remnants to LRP?
ApoC-II	Activator of LPL.
ApoC-III	Inhibitor of LPL. Inhibits hepatic uptake of TRL and TRL remnants?
ApoE	Ligand for the LDLR, LRP and other members of the LDLR family.

Low density lipoprotein

LDL is the principal carrier of cholesterol in normal human plasma. The surface of the LDL particle consists of amphipathic molecules; a single copy of apolipoprotein B-100 (apoB-100), phospholipids (mainly phosphatidylcholine and sphingomyelin), and unesterified (free) cholesterol. The hydrophobic core contains mainly cholesteryl esters (CE), and smaller amounts of triglycerides (TG) and free cholesterol (FC). Elevated plasma concentration of LDL cholesterol (hypercholesterolaemia) is an important risk factor for CHD, and clinical intervention studies have consistently demonstrated the therapeutic value of correcting hypercholesterolaemia both for primary and secondary prevention of CHD (Steinberg and Gotto 1999). The plasma concentration of LDL is largely determined by the rate of LDL uptake through the hepatic low density lipoprotein receptor (LDLR) pathway, as evidenced by the fact that lack of functional LDLR is responsible for the massive accumulation of LDL in patients with familial hypercholesterolemia (FH) (Brown and Goldstein 1986).

LDL in atherogenesis

The epidemiological association between elevated plasma LDL cholesterol and CHD strongly suggests a causative role of LDL in the development of atherosclerosis. However, the evidence for a specific pathogenic pathway is mostly circumstantial.

According to the 'response to retention' hypothesis of atherogenesis, a key step in early atherosclerosis is the entrapment of cholesterol-rich lipoproteins in the arterial wall, which is proposed to occur via interactions between segments of the apoB protein and negatively charged glycosaminoglycans of proteoglycans. Retained lipoprotein particles in the intimal extracellular matrix undergo structural changes of the lipid and protein moieties which increase their susceptibility to enzymatic and nonenzymatic modifications (Schwenke and Carew 1989; Williams and Tabas 1998; Borén et al. 2000). Several lines of evidence suggest that oxidative modification of the LDL particle is a key event in the initiation of atherosclerosis development (Navab et al. 1996; Steinberg and Witztum 1999). The development of the macrophage 'foam cells' is thought to be mediated primarily by uptake of oxidatively modified LDL via so-called scavenger receptors (Yamada et al. 1998). Progressive accumulation of foam cells leads to the development of early atherosclerotic lesions, i.e. fatty streaks.

The transition of the fatty streaks to more complex atherosclerotic lesions is characterised by the migration of smooth muscle cells from the medial layer of the arterial wall into the intima, or the subendothelial space. Intimal smooth muscle cells convert from contractile to synthetic phenotype, begin to proliferate and take up modified lipoproteins, and synthesize extracellular matrix proteins that lead to

the development of the fibrous cap of the lesion. This phase of lesion development is influenced by a broad range of cellular and humoral inflammatory responses orchestrated by local cytokine secretions. The antigens responsible for immune activation are not known with certainty. However, substantial evidence supports an important role for epitopes of oxidized LDL as immunogens (Hörkkö et al. 2000).

Oxidized LDL

In vitro oxidized LDL has been described to mediate several potentially proatherogenic events (Steinberg and Witztum 1999). However, the evidence for the presence of oxidized LDL *in vivo* is scarce (Witztum and Steinberg 2001).

Clinical studies have demonstrated that LDL obtained from subjects with CAD showed an increased susceptibility to *ex vivo* oxidative modification compared with LDL from healthy subjects (Regnström et al. 1992; Cominacini et al. 1993; Chiu et al. 1994). A substantial amount of research has been aimed at elucidating the specific properties of the LDL particle that determine its resistance to oxidative modification, and how potential antioxidative mechanisms can be enhanced.

The LDL particle is protected from oxidation by its endogenous content of antioxidants, mainly lipid-soluble vitamins (e.g. α-tocopherol, ubiquinol-10, and carotenoids), as well as by antioxidant defence mechanisms in plasma. The variability in oxidative susceptibility of LDL has been attributed to differences in size and/or density (de Graaf et al. 1991; Tribble et al. 1992; Chait et al. 1993; Dejager et al. 1993), antioxidant concentrations (Stocker et al. 1991), fatty acid composition (Kontush et al. 1994; Thomas et al. 1994; Tribble 1995; Maziere et al. 1998), and lipid composition (Tribble et al. 1992; Frei and Gaziano 1993).

A number of intervention trials with supplementation of synthetic or naturally occurring antioxidants have been performed in animals and humans. In the majority of animal studies, antioxidant treatment has inhibited lesion development. However, in general, data from human studies have been disappointing (Chisolm and Steinberg 2000).

Metabolism of apoB-containing lipoproteins

ApoB is an essential component of chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL) and LDL. Chylomicrons are the largest and most lipid-rich particles, containing principally TG of dietary origin. They are secreted by the intestine, and are abundant in plasma in the postprandial state. TG-rich VLDL particles are secreted mainly by the liver. IDL and LDL are metabolic products of VLDL, and are enriched in CE. Severe disruption in the metabolism of apoB-containing lipoproteins results in hyperlipidaemia, and is also associated with increased risk of developing CHD.

Assembly and secretion

The production of VLDL particles is a complex process, which is regulated at different levels by a variety of factors. Currently the assembly of the VLDL particle is considered to involve two steps (Fig. 1). First, an apoB-containing VLDL 'precursor' is formed in the rough endoplasmic reticulum (ER). This step requires the presence of the microsomal triglyceride transfer protein (MTP), which mediates the transfer of hydrophobic lipids from the ER membrane into the nascent VLDL particle. In the second step, the apoB-containing pre-VLDL particle fuses with a large TG-rich lipid droplet, to form

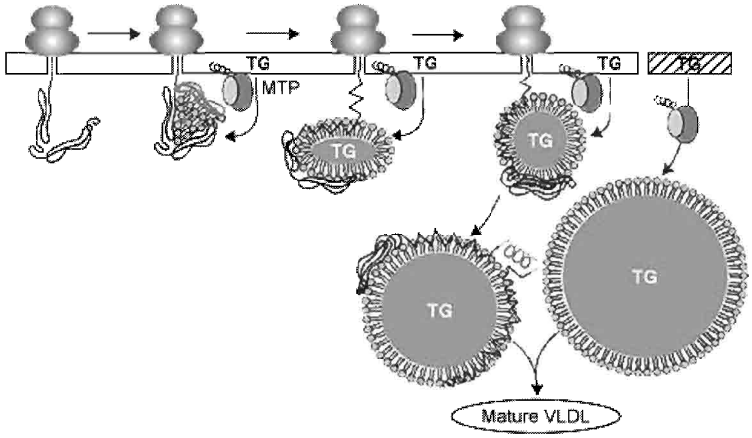

Figure 1. Model of VLDL assembly (adapted from Shelness et al. 2001).

mature VLDL. At present, very little is known about the intracellular mechanisms responsible for the formation of the lipid droplet, which transfers the bulk of the lipids into the VLDL particle.

Important regulatory mechanisms for the assembly and secretion of apoB-containing lipoproteins are the rate of formation and degradation of apoB, and the intracellular content of lipids. The production of large VLDL is promoted by an increase in the flux of non-esterified fatty acids (NEFA) to the liver and also in situations with an increase in the hepatic synthesis of endogenous fatty acids, such as during a period of high carbohydrate intake. Hepatic uptake of TG-rich remnant lipoproteins may also provide substrate for triglyceride excretion from the liver. Insulin suppresses the production of large VLDL by decreasing the substrate availability of NEFA (Adeli et al. 2001), but a direct inhibitory effect of insulin on the secretion process has also been described (Malmstrom et al. 1997; Gibbons et al. 2002). An impaired inhibitory effect of insulin may be an explanation to the increased production rates of VLDL apoB that are characteristically found in insulin resistant states, such as obesity and type 2 diabetes (de Graaf et al. 2002).

In plasma, two VLDL subfractions have been defined; VLDL1 (Sf 60-400) and VLDL2 (Sf 20-60), of which the former is the larger, lighter, and more TG-rich species. Turnover studies suggest that the production of the two VLDL fractions are regulated separately and independently by the liver (Packard and Shepherd 1997). However, the mechanisms by which the liver assembles the different forms of VLDL, as well as the regulatory pathways for their production, remain unknown. A direct production and secretion of an IDL/LDL fraction by the liver has also been suggested, although this remains a controversial issue (Shames and Havel 1991).

Plasma modulation and clearance

In plasma, TG-rich VLDLs are converted to remnant lipoproteins through the actions of triglyceride lipases, most importantly lipoprotein lipase (LPL) and hepatic lipase (HL). Kinetic studies using stable isotope tracers to monitor the metabolism of apoB-containing lipoproteins suggest that the VLDL1 and VLDL2 fractions have different metabolic fates

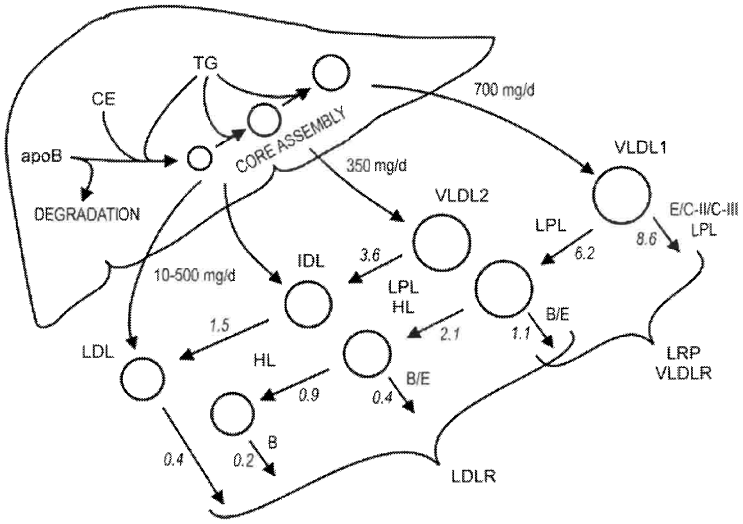

Figure 2. ApoB metabolism (adapted from Packard and Shepherd 1997).

(Fig. 2). Large VLDL (VLDL1) are to a larger extent converted to remnants (VLDL or IDL) which are removed from plasma before undergoing conversion to LDL. However, once formed, LDL derived from VLDL1 has a longer plasma residence time (Demant and Packard 1998). In contrast, VLDL2 particles are rapidly and efficiently converted to LDL (Gaw 1995). The IDL fraction has a short half-life, and its plasma concentration is normally low (about one-tenth of that of LDL). Compared with its TG-rich precursors, the LDL particle has a long plasma residence time, and circulates for days before being cleared by the LDLR in liver and peripheral cells.

A gross approximation shows that about half of the VLDL remnants are directly cleared by the liver, and the rest are converted to LDL. The clearance of TG-rich lipoprotein (TRL) remnants by the liver is primarily mediated by apolipoprotein E (apoE), although interactions between apoE and the triglyceride lipases with heparan sulfate proteoglycans (HSPG), the LDLR, and the low density lipoprotein receptor-related protein (LRP) appear to modulate TRL clearance (Mahley and Ji 1999).

LDL heterogeneity

The LDL particle population has been shown to be heterogeneous with regard to several structural and functional properties, such as size (Shen et al. 1981; Krauss and Burke 1982; Musliner et al. 1986), density (Lindgren et al. 1969), electric charge (Chapman et al. 1981; La Belle et al. 1997; Lund-Katz et al. 1998), and lipid and apolipoprotein composition (Lee and Alaupovic 1970; Chapman et al. 1981; Chapman et al. 1988).

Methodology

During the last 20 years, two principally different methods have commonly been used to characterise LDL heterogeneity; density gradient ultracentrifugation (DGUC) and nondenaturing gradient gel elecrophoresis (GGE).

DGUC separates lipoproteins according to density, and several DGUC procedures have been developed to separate LDL subfractions (Swinkels et al. 1987; Chapman et al. 1988; Griffin et al. 1990).

In principle, a fresh sample of whole plasma, serum, or isolated LDL is applied to a discontinuous salt gradient and subjected to ultracentrifugation. After ultracentrifugation the content of the tube may be recovered by different procedures, e.g. aspiration or upward displacement by infusing a dense, inert solution through a hole in the bottom of the tube. Continuous collection of fractions enables the eluate to be monitored by a UV detector to obtain an absorbance curve. In isolated fractions, the chemical composition of the different LDL subfractions can be measured. When prestained serum is used, the ultracentrifugation tube may be photographed and the photograph scanned by a laser densitometer to obtain a crude LDL particle density distribution profile.

Nondenaturing polyacrylamide (PA) GGE separates LDL according to particle size. In principle, a sample of whole plasma, serum or isolated LDL is subjected to electrophoresis on a gradient gel, which is then stained for protein or lipid. After a densitometric scan, the migration distances of standard particles of known size are used to construct a standard curve, relating particle diameter to migration distance on the gel. GGE has been reported to enable separation of as many as seven discrete LDL subclasses (McNamara et al. 1987). However, many investigators have classified subjects according to a dichotomous LDL subclass phenotype or pattern, where pattern A is defined as a skewing of the LDL particle size distribution towards larger species, and pattern B is used for subjects with a predominance of small LDL particles. If neither pattern is observed, subjects are categorized as 'intermediate' (pattern I).

More recently, a highly efficient method has been described to evaluate the LDL particle size distribution by nuclear magnetic resonance (NMR) spectroscopy (Otvos et al. 1992). The basis for NMR analysis of lipoprotein subclasses is that each lipoprotein particle in plasma within a given diameter range 'broadcasts' a distinctive lipid NMR signal, the intensity of which is proportional to its bulk lipid mass concentration. Thus, the proton NMR spectrum of a plasma specimen is analysed by converting lipid methyl group signal amplitudes to lipoprotein subclass concentrations. Isolated subfractions with chemically measured cholesterol and TG concentrations are used as standards and the particle concentration of the individual suclasses are calculated by dividing their lipid mass concentrations by the known total amount of lipid per particle. These calculations use standard assumptions about the relationship between lipoprotein particle diameter and core volume and the partial specific volumes of the core lipids. Thus, if a subject's LDL particles are 'abnormal' in composition, NMR-derived values will differ from those obtained by chemical methods of lipid measurement.

LDL heterogeneity and CHD

A number of studies have examined the relationship between LDL heterogeneity and risk of CHD (Table 3). In the first population-based case-control study (Austin et al. 1988a), a threefold increased risk of myocardial infarction (MI) was found in subjects with a predominance of small, dense LDL particles (LDL subclass pattern B).

Taken together, the cross-sectional studies consistently suggest an association between small, dense LDL and CHD. In most studies the association has been dependent upon correlated lipid and lipoprotein measures, most importantly plasma TG and high density lipoprotein (HDL) cholesterol concentrations. However, in the most recent case-control

Table 3. Cross-sectional studies of LDL size/buoyancy in relation to CHD

	Gender	Patients/Controls	Relationship
(Crouse et al. 1985)	M	46/47	Lower LDL MW in CAD
(Austin et al. 1988)	M+F	109/121	Subjects with LDL pattern B: OR=3.0 for MI
(Tornvall et al. 1991)	M	36/14	More dense LDL distribution in CAD
(Campos et al. 1992)	M	275/822	Small LDL more prevalent in CAD
(Coresh et al. 1993)	M+F	107/91	Smaller LDL in CAD
(Griffin et al. 1994)	M	86/82	LDL-III mass>100 mg/dL: OR=4.5 for CAD OR=6.9 for MI

M=male, F=female, MW=molecular weight, OR=odds ratio

study (Griffin et al. 1994) a plasma concentration of small, dense LDL (LDL-III) above 100 mg/dL was associated with a 4.5-fold increased risk of CAD (defined as three-vessel disease by angiography or previous MI), after adjustment for the serum TG concentration.

Also, in the majority of prospective nested case-control studies a small LDL particle size has predicted the development of CHD (Table 4).

Few studies have examined the relationship between LDL particle size distribution and CHD risk in women. However, a study in young women (18-44 years) showed an odds ratio of 2.3 for a 1-nm reduction in LDL particle size (Kamigaki et al. 2001), and in a recent prospective nested case-control study of healthy middle-aged women, small LDL particle size (measured by NMR) was a predictor of future cardiovascular events (Blake et al. 2002).

Atherosclerotic measures

The relationship between LDL particle size and degree of CAD as measured by coronary angiography has also been investigated. In two studies by Tornvall et al. (Tornvall et al. 1991; Tornvall et al. 1993), a correlation was found between the concentration of TG in dense LDL (d>1.040 kg/L) and the degree of CAD in young (<45 yrs) postinfarction patients. In contrast, a graded relationship between the LDL peak particle size (determined by 2-16% PA GGE) and the degree of CAD was

Table 4. Prospective studies of LDL particle size and future CAD events

	Gender	Patients/Controls	Follow-up time	
(Stampfer et al. 1996)	M	266/308	7 ys	LDL size –8 Å at baseline: RR=1.4 for CHD
(Gardner et al. 1996)	M+F	124/124	5 ys	LDL size –5 Å in cases at baseline
(Lamarche et al. 1997)	M	114/114	5 ys	LDL size<25.6 nm at baseline: OR=3.6 for CHD
(Mykkanen et al. 1999)	M+F	86/172	3.5 ys	LDL size did not predict CAD events
(Austin et al. 2000)	M	145/296	12 ys	LDL –10 Å at baseline: RR=1.3 for CHD
(Campos et al. 2001)	M+F	416/421	5 ys	LDL size>26.3 at baseline: RR=1.8 for CHD LDL size did not predict CAD events in subjects treated with pravastatin
(Lamarche et al. 2001)	M	108/1949	5ys	LDL size<25.6 nm at baseline: RR=2.2 for CHD

M=male, F=female, RR=relative risk, OR=odds ratio

not found in two studies (Campos et al. 1992; Coresh et al. 1993). In a more recent study, where NMR was used to measure LDL particle size distribution, the level of small LDL was associated with age-adjusted CAD severity (Freedman et al. 1998). However, the association was no longer significant after adjustment for plasma TG and HDL cholesterol concentrations. In contrast, in normotriglyceridaemic men, an LDL score (determined by continuous disc PAGE) was the best discriminator for CAD severity, and accounted for approximately 25% of the variation in the degree of CAD in multivariate analyses (Rajman et al. 1996).

LDL heterogeneity, in particular the plasma concentration of small dense LDL, also appears to be a strong determinant of early cardiovascular changes in an asymptomatic healthy population. In a study of healthy middle-aged men, it was shown that the plasma concentration of small, dense LDL (LDL-III) was significantly correlated with intima-media thickness (IMT) of the common carotid artery (CCA), as measured by ultrasonography (Skoglund-Andersson et al. 1999). A similar relationship has been reported in a large-scale study of healthy men (Hulthe et al. 2000), and recently also in asymptomatic FCH family members (Liu et al. 2002).

Functional cardiovascular studies

Endothelial dysfunction has been proposed to be an early marker of atherosclerosis, and an impaired endothelium-dependent vasodilatation is characteristically found in subjects at high risk of developing CHD (Chowienczyk et al. 1992; Celermajer et al. 1994; Williams et al. 1996), and in atherosclerotic coronary arteries (McLenachan et al. 1991; Zeiher et al. 1994). Whereas several studies have described an impaired endothelium-dependent vasodilatation in hypercholesterolaemic subjects, the relationship between hypertriglyceridaemia and endothelial dysfunction is less well understood, and studies of endothelial function in subjects with isolated hypertriglyceridaemia have given contradictory results (Chowienczyk et al. 1997; Lewis et al. 1999; Schnell et al. 1999). LDL size has been suggested to be a determinant of endothelial function in healthy men (Lupattelli et al. 2000; Vakkilainen et al. 2000), and in subjects with type 2 diabetes (O'Brien et al. 1997; Makimattila et al. 1999). In the study by Vakkilainen et al. (Vakkilainen et al. 2000), it was shown that LDL peak particle size was significantly correlated with the acetylcholine (ACh)-induced endothelium-dependent increase in forearm blood flow. Furthermore, the association between small LDL particles and impaired in vivo endothelial function was shown to be independent of plasma HDL and LDL cholesterol, and TG concentrations.

Atherogenicity of small, dense LDL

Several pathophysiological mechanisms for the increased atherogenicity of small, dense LDL have been suggested. Compared with larger and more buoyant LDL particle species, small dense LDL more readily infiltrate the arterial wall (Bjornheden et al. 1996), bind more tightly to arterial wall proteoglycans (Anber et al. 1996; Anber et al. 1997), and are more susceptible to oxidative modification (Tribble et al. 1992; Chait et al. 1993; Dejager et al. 1993). Furthermore, small dense LDL have a prolonged plasma residence time (Packard et al. 2000b), most probably due to a decreased affinity for the LDLR (Nigon et al. 1991; Galeano et al. 1994), and in effect an extended opportunity to exert atherogenic effects.

Metabolic origin

Although the metabolic origin of small dense LDL is not yet fully understood, the distribution of LDL particle size in human plasma is believed to be the result of the coordinated actions of hepatic and endothelial lipases as well as mediators of neutral lipid exchange. One current hypothesis (Griffin et al. 1994; Tan et al. 1995a) is that the large, light LDL particles are direct products of the action of LPL on the small VLDL species (VLDL2). In contrast, the small dense LDL are believed to arise through the exchange of CE for TG, between LDL and large VLDL (VLDL1), mediated by the cholesteryl ester transfer protein (CETP), producing TG-enriched LDL particles, which are then lipolysed by HL. Such a metabolic relationship could, in part, explain the strong correlation between the plasma TG concentration and the prevalence of small, dense LDL. However, at present, very little is known of the link between the pattern of VLDL production and the heterogeneity of the metabolic end product, i.e. LDL.

A metabolic model for the formation of small, dense LDL has been derived from turnover studies (Packard and Shepherd 1997). This model suggests that there are two metabolically distinct pools of LDL that are derived from different precursors. Pool α has a relatively rapid turnover (plasma residence time 2 days), and is the major source of LDL in subjects with low TG levels. LDL in pool α is primarily derived from small VLDL (VLDL2), secreted directly by the liver. In contrast, a plasma TG concentration above 1.5 mmol/L, as a result of overproduction or insufficient removal of VLDL, favours the production of LDL by delipidation of the larger VLDL (VLDL1). LDL in pool β (derived from VLDL1) has a substantially longer plasma residence time (5 days), which enables its TG-enrichment (by CETP-mediated lipid exchange) and subsequent delipidation by HL to form a small, dense LDL species.

Influence of metabolic and environmental factors

Associated metabolic aberrations – 'partners in crime'

LDL particle size is commonly found to be closely correlated with HDL cholesterol concentrations and strongly inversely related with plasma TG concentrations. Low plasma HDL cholesterol is a recognized independent risk factor for CHD, whereas the role of isolated hypertriglyceridaemia as a CHD risk factor is still controversial (Hokanson 2002). The metabolic mechanisms for the strong inverse relationship between plasma TG and HDL cholesterol are not yet fully elucidated. A current hypothesis connects the dyslipidaemic triad of increased plasma TG, low HDL cholesterol and an increased number of small, dense LDL (Fig. 3).

Several studies have shown an increased catabolism of HDL in hypertriglyceridaemia (Lamarche et al. 1999b), and recent evidence suggests that TG-enriched HDL, produced via an accelerated CETP-mediated heteroexchange of CE and TG between HDL and TRL, is more prone to lipolysis by HL, and subsequently more rapidly cleared from the circulation (Rashid et al. 2002).

The atherogenic lipoprotein phenotype (ALP) is characterised by an essentially normal plasma cholesterol concentration, moderately increased plasma TG, low plasma HDL cholesterol and an LDL particle size distribution dominated by small, dense LDL particles (Austin et al. 1990b). ALP is a common dyslipidaemia in CHD patients (Swinkels et al. 1989; Austin et al. 1990b; Griffin et al. 1994), in FCH (Austin et al. 1990a), and in insulin

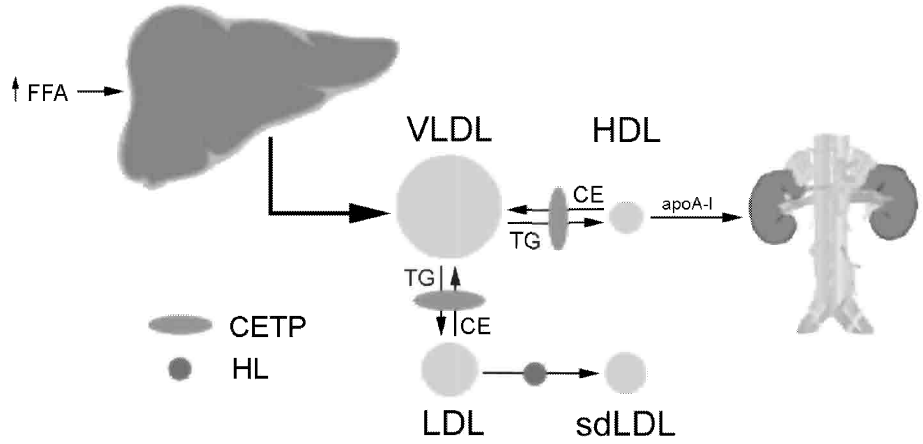

Figure 3. Metabolic mechanisms of ALP (adapted from Ginsberg 2000).

resistant states, such as obesity and type 2 diabetes. A preponderance of small dense LDL particles has been suggested to be a marker for the insulin resistance syndrome (Reaven et al. 1993; Selby et al. 1993), and to confer the association with atherosclerosis. However, the relationship between insulin resistance and LDL particle size is likely to be a function of the elevated plasma TG concentration that is a characteristic feature of insulin resistance (Lahdenperä et al. 1995; Tan et al. 1995b).

Most likely, several aspects of the dyslipidaemia of insulin resistance contribute to an increased risk of cardiovascular disease (Ginsberg 2000). In a recent prospective study (Lamarche et al. 1998), the clustering of an increased fasting plasma insulin concentration, increased plasma apoB levels and small LDL particle size, was shown to confer a remarkable 18-fold increased risk for CHD.

Although plasma TG and HDL cholesterol concentrations appear to be at least in part independent determinants of the LDL particle size distribution, the plasma TG concentration is regarded to be the major determinant of the LDL particle size distribution, and several studies have investigated this relationship. In a study by Tan et al. (Tan et al. 1995a), the correlation between the plasma concentration of small, dense LDL (LDL-III) and plasma TG was observed to be different above a plasma TG level of 115 mg/dL (1.3 mmol/L). A plasma concentration of small, dense LDL >100 mg/dL (1.3 mmol/L) was rarely seen in subjects with a plasma TG concentration <1.3 mmol/L, whereas above this level, the plasma concentration of small, dense LDL increased in proportion to the plasma TG concentration. In the same study, it was noted that women, at a given plasma TG level, have less small, dense LDL compared with men, which illustrates the presence of other, and in this respect gender-specific, regulatory factors.

Gender and lifestyle

A small, dense LDL distribution pattern is more common in men (McNamara et al. 1987; Swinkels et al. 1989), and may be attributed to a higher HL activity (Tikkanen and Nikkila 1987). In parallel, women generally have higher plasma HDL cholesterol and lower TG concentrations (Nikkilä et al. 1996), and after adjustment for these variables, gender is usually no longer associated with LDL

size (Swinkels et al. 1989; Coresh et al. 1993).

Body mass index (BMI) has been associated with the plasma concentration of small, dense LDL (LDL-III) (Griffin et al. 1994), however not independently of plasma TG concentrations. The influence of diet on LDL particle size distribution is not fully understood. In a series of reports by Dreon and colleagues, it was suggested that subjects with an LDL subclass pattern B have a more beneficial response to a low-fat diet in that they exhibit a greater reduction in plasma LDL cholesterol concentration compared with pattern A subjects (Dreon et al. 1994). In contrast, it was noted that a large proportion of the subjects with an LDL subclass pattern A at baseline converted to a pattern B on the low-fat diet. This shift was furthermore shown to be due to a relative decrease in the larger, more buoyant LDL particle species (Krauss and Dreon 1995), and to be related to apoE phenotype (Dreon et al. 1995).

Several studies have suggested that long-term exercise training induces a lowering of the plasma concentration of small, dense LDL (Williams et al. 1986; Williams et al. 1989; Halle et al. 1999). These findings were recently confirmed in a prospective, randomized, controlled clinical study (Kraus et al. 2002), where a six-month exercise intervention was shown to decrease the plasma concentration of small LDL, as well as the total LDL particle concentration, without changing the plasma LDL cholesterol concentration. Furthermore, the beneficial effects of exercise on LDL particle size was shown to be related to the amount of activity rather than to the intensity of exercise or improvement of fitness.

Drugs

Numerous studies have investigated the effect of the most common lipid-lowering agents on LDL heterogeneity. Statins are potent inhibitors of hydroxymethylglutaryl-coenzyme A (HMG CoA) reductase, the rate-limiting enzyme in hepatic cholesterol synthesis, and are currently the drug of choice for the treatment of elevated plasma LDL cholesterol concentrations. Statins have also been reported to lower the plasma TG concentration, and increase plasma HDL cholesterol. Several studies have investigated the effects of statins on LDL particle size distribution (Austin et al. 1994; Lamarche et al. 1999a; Marais 2000; Packard et al. 2000a). However, the results of these studies present a complex picture, which suggests that statin-induced changes in LDL particle size are dependent on the type of substance, and also on the hyperlipidaemic phenotype of the treated subjects. Taken as a whole, statins appear to lower all LDL subspecies simultaneously, and thus not induce any significant shift in the LDL particle size distribution. However, in a recent study by Forster et al. (Forster et al. 2002) the effects of atorvastatin and simvastatin on metabolism of apoB-containing lipoprotein were evaluated in subjects with moderate combined hyperlipidaemia (who often present with an atherogenic lipoprotein phenotype). In this study, it was shown that atorvastatin had a more pronounced effect on the small, dense LDL compared with simvastatin, and thus was able to induce a shift in the overall LDL particle size distribution towards larger, less dense LDL subfractions. In constrast, most studies of the effects of fibrates have reported an overall increase in the LDL particle size. As activators of the peroxisomal proliferation activator receptors (PPAR) (Staels et al. 1998), the fibrates are likely to affect LDL particle size through multiple

pathways, mediated by a reduction in plasma triglycerides. Gemfibrozil has been shown to increase LDL particle size in type 2 diabetics (Lahdenperä et al. 1993) and hypertriglyceridaemic men (Yuan et al. 1994). Ciprofibrate and bezafibrate normalise a small, dense LDL pattern in combined hyperlipidemia (Bruckert et al. 1993; Chapman and Bruckert 1996) and in isolated hypertriglyceridaemia (Eisenberg et al. 1984; Ruotolo et al. 1998), respectively. Thus, to summarize the effects of lipid-lowering drugs on LDL particle size distribution, it appears that at least in part, the effect on LDL particle size may be predicted by the ability of the drug to lower plasma triglyceride levels.

β-blocker medication is associated with smaller LDL (Campos et al. 1992), proposedly due to a relative deficiency in larger, more buoyant LDL (Griffin et al. 1994; Boquist et al. 1998). The effect of β-blocker use was however no longer significant after adjusting for plasma TG concentration (Griffin et al. 1994).

Of note, recent clinical trials have suggested that the improvement in CAD in response to pharmacological therapy may be predicted by a) the baseline LDL particle phenotype (Watts et al. 1993; Mack et al. 1996; Miller et al. 1996), and b) the response in LDL particle size distribution to lipid-lowering treatment (Zambon et al. 1999).

Genetic influence

Studies in healthy families, FCH families and in twins strongly suggest a genetic influence on the distribution of LDL particle size (Austin et al. 1988b; Austin et al. 1990a; de Graaf et al. 1992; Austin et al. 1993; Bredie et al. 1996). However, the specific genetic loci affecting LDL particle size distribution are not yet known. Linkage studies using candidate gene and/or random genomic markers have given partly contradictory results (Austin et al. 1991; La Belle et al. 1991; Nishina et al. 1992; Rotter et al. 1996). Recent studies in women have shown significant linkage between LDL peak particle size and the apoB (Austin et al. 1998) and CETP (Talmud et al. 2000) gene loci. In FCH the CETP gene (Allayee et al. 1998), and recently also the HL gene (Allayee et al. 2000), have been linked to LDL size. In contrast, a study in healthy families did not show any linkage to nine candidate genes (Austin et al. 1999), whereas a genome scan approach showed linkage between the plasma concentration of small LDL and genomic markers on chromosomes 3 and 4 (Rainwater et al. 1999). The discrepancies in these reports probably reflect the complexity of the genetic determination of the phenotype, i.e. the LDL particle size. However, differences in phenotypic characterisation, statistical approach, as well as in the effects of genetic background and environmental factors are likely to contribute to the diversity.

In addition, several association studies have indicated moderate, and sometimes conflicting, effects of common candidate gene variants on LDL particle size distribution in various study populations.

Candidate genes

Recent studies have implied that there exists a genetic correlation between the different dyslipidaemic parameters of ALP and that common genes are likely to influence the different lipoprotein traits by pleiotropic effects (Edwards et al. 1999; Rainwater et al. 2001). Thus, the primary candidates for the regulation of LDL particle size are genes encoding proteins with intricate roles in lipid and lipoprotein metabolism.

Microsomal triglyceride transfer protein

MTP is a heterodimer consisting of protein disulfide isomerase (PDI) and a large, unique 97 kDa (894 aa) protein (M subunit). MTP is found in the ER lumen, primarily of liver and intestine, and plays an essential role in the assembly and secretion of apoB-containing lipoprotein particles. Recently, it was also shown that MTP is expressed in the heart, where it has been suggested to mediate the removal of excess triglyceride from cardiac myocytes (Boren et al. 1998; Nielsen et al. 1998). Absence of MTP due to mutations in the gene encoding the large subunit causes abetalipoproteinaemia (ABL), a rare disorder which is characterised by an inability to secrete apoB-containing lipoproteins and consequently by virtually undetectable levels of apoB in plasma (Berriot-Varoqueaux et al. 2000). However, the specific steps in the assembly process that require MTP have not been identified. MTP associates with membranes and vesicles and mediates the transfer of neutral lipids by a shuttle mechanism. In the ER, MTP binds to the amino-terminal part of apoB, probably already during the translation/translocation of apoB (Olofsson et al. 1999). The binding of MTP to apoB is thought to play two important roles; a) to prevent apoB degradation, and b) to transfer lipids to the nascent apoB (Hussain et al. 2003). Furthermore, recent studies suggest that MTP also plays a role in the accumulation of lipids in the ER lumen, and possibly also in the so called 'second step' of the assembly process; the fusion of the primordial apoB with a lipid droplet (Shelness and Sellers 2001).

The human MTP gene (55 kb, 18 exons) is located on chromosome 4q24. Three promoter polymorphisms (-493G/T, -400A/T, -164T/C), and four missense polymorphisms have been reported in the MTP gene. All polymorphisms in the coding region give rise to an exchange in amino acids and are thus putatively functional; Q95H, I128T, Q244E, and H297Q (Narcisi et al. 1995). The less common allele of the -493G/T polymorphism has been associated with decreased plasma concentration of LDL cholesterol and LDL apoB in healthy men (Karpe et al. 1998; Ledmyr et al. 2002).

Apolipoprotein B

The majority of apoB is produced in liver and intestine, where apoB is a requirement for the assembly and secretion of the TRL (VLDL and chylomicrons). In particles of hepatic origin (VLDL, IDL and LDL) the apoB contains 4536 aa (apoB-100, 550 kDa), whereas a truncated apoB (apoB-48, 260 kDa), containing the amino-terminal 2152 residues (48%) of apoB-100, is found in chylomicrons. ApoB-48 is produced as a result of post-translational editing of the apoB mRNA (Powell et al. 1987), which changes the codon specifying apoB-100 aa 2153 to a premature stop codon. Unlike the other apolipoproteins, apoB does not exchange between lipoproteins. Hence, apoB (B-100) is the principal protein of LDL, and serves as a ligand for the LDLR-mediated uptake of LDL by liver and extrahepatic tissues.

The human apoB gene (43 kb, 29 exons) is located on chromosome 2p24. Mutations in the apoB gene, which impair the assembly of TG-rich lipoproteins, lead to decreased TG secretion from both liver and intestine (familial hypobetalipoproteinemia). Mutations in the LDLR-binding region of apoB result in poor binding of apoB to the LDLR, causing accumulation of CE-rich lipoproteins in plasma (familial ligand-defective apoB-100, FDB) and an increased proneness to CAD. The most 'common' mutation causing FDB is an arginine to glutamine substitution at codon

3500 (R3500Q). In addition to the rare mutations, several restriction fragment length polymorphisms (RFLPs) has been identified in the apoB gene. Some of these have been associated with the plasma concentration of LDL cholesterol or apoB, and also with myocardial infarction. However, results are inconsistent.

A common C to T substitution in the apoB promoter (-516C/T) has been reported to increase transcriptional activity *in vitro*, and to be associated with increased plasma LDL cholesterol in healthy men (van 't Hooft et al. 1999).

Apolipoprotein E

ApoE is a glycoprotein of 299 aa (34 kDa) which is synthesized in many tissues, including brain, kidney, spleen and liver, which is the major source of plasma apoE. ApoE is present on most lipoprotein fractions, and is a high affinity ligand for all members of the LDLR family (e.g. LDLR, LRP and VLDLR). Thus, apoE plays an important role in the turnover and removal of TRL and TRL remnants. In addition to its role in lipoprotein clearance, there is increasing evidence that apoE plays an important role in intracellular lipid metabolism. ApoE has been suggested to regulate VLDL production by hepatocytes (Fazio et al. 2000), and to be necessary for the efflux of cholesterol from macrophages (Zhang et al. 1996b). The role of macrophage-derived apoE as a modulator of atherosclerotic lesion development has been supported by evidence from studies in transgenic mice. However, data are partly conflicting. Whereas macrophage-specific expression of apoE on an apoE-/- background was shown to significantly reduce atherosclerosis (Bellosta et al. 1995), the reverse experiment, i.e. the introduction of apoE -/- macrophages (by bone marrow transplantation) into wild type mice, was reported to increase atherosclerosis by one group (Fazio et al. 1997), but not by another (Boisvert and Curtiss 1999).

ApoE is encoded by a four-exon (3.6 kb) gene located on chromosome 19q13, in close proximity to the genes encoding apoC-I, apoC-II and the insulin receptor. A common polymorphism in the apoE gene gives rise to three major apoE isoforms. In a Caucasian population the apoE e3 (Cys112, Arg158) has an allele frequency of 77% (Davignon 1993), and is considered the wild type. ApoE e2 (Cys112, Cys158) and e4 (Arg112, Arg158) both differ from e3 by one amino acid, and have allele frequencies of 8% and 15%, respectively. The apoE e2 allele encodes a protein with only 1-2% of the normal LDLR-binding activity, and is associated with a decrease in plasma LDL cholesterol. ApoE e2 homozygosity (~1% of the population) is essential, but not sufficient for the development of hyperlipoproteinaemia type III, which is characterised by an accumulation of remnant lipoproteins (chylomicron remnants and IDL) in plasma. Thus, the majority (95-98%) of e2 homozygotes are not hyperlipidaemic. In contrast, heterozygosity for certain defective apoE variants is invariably associated with type III hyperlipoproteinaemia. Compared with the e2 allele, less is known about the functional properties of the apoE E4 isoform. The apoE e4 allele has been associated with an increase in plasma LDL cholesterol concentration, and an increased risk of CHD (Wilson et al. 1996). In mice, germline ablation of both copies of the apoE gene results in the development of advanced atherosclerotic lesions similar to those observed in human CAD (Breslow 1993), and the apoE -/- ('knock-out') mouse is a frequently used model of atherosclerosis.

Cholesteryl ester transfer protein

Human CETP is a 493-aa (55 kDa) hydrophobic glycoprotein, which is synthesized mainly in liver, spleen and adipose tissue (Tall 1993). CETP circulates in plasma mainly bound to HDL particles, and mediates the transfer of neutral lipids between plasma lipoproteins, a process that results in a redistribution and equilibration of CE and TG between lipoprotein fractions. The effect of CETP in normal plasma is to promote the net transfer of CE from HDL to TRL and LDL, and of TG from TRL to LDL and HDL. However, the lipid transfer activity of CETP is modulated by alterations in the substrate lipoproteins particles. In addition, plasma CETP activity has been suggested to be highly influenced by environmental factors, such as obesity (Dullaart et al. 1994b), smoking (Dullaart et al. 1994a), and alcohol consumption (Hannuksela et al. 1992). Also, CETP function and activity has been suggested to be influenced by the metabolic state (Lassel et al. 1999), and in particular, by hypertriglyceridaemia (Mann et al. 1991).

The human CETP gene (25 kb, 16 exons) is located on chromosome 16q21. Several mutations causing CETP deficiency have been identified. CETP-deficient patients have markedly increased plasma HDL cholesterol concentrations, whereas the plasma LDL cholesterol concentration is normal or only slightly elevated (Yamashita et al. 2000). LDL from CETP-deficient patients is poor in CE and enriched with TG, and displays a polydisperse LDL particle size distribution, whereas the HDL particles are enlarged and CE-enriched, compared with normal subjects.

Several polymorphisms have been reported in the human CETP gene. The TaqIB polymorphism in the first intron (Drayna and Lawn 1987) has been extensively studied, but as an intronic variant it is not considered functional. The B2 allele (absence of restriction site) has consistently been associated with increased plasma HDL cholesterol and low plasma levels of CETP activity and mass, and also with a decreased CHD risk (Fumeron et al. 1995; Ordovas et al. 2000). Furthermore, the TaqIB polymorphism has been associated with the progression of coronary atherosclerosis in response to pravastatin treatment (Kuivenhoven et al. 1998). However, in general, the effects of the B2 allele have been suggested to be highly influenced by environmental factors, such as alcohol consumption (Fumeron et al. 1995) and tobacco smoking (Freeman et al. 1994; Hannuksela et al. 1994). In the Framingham population, the B2 allele was associated with increased LDL particle size (Ordovas et al. 2000), whereas several previous studies have reported no association between the CETP TaqIB polymorphism and LDL particle size (Bernard et al. 1998; Carr et al. 2002; Okumura et al. 2002). However, a tetranucleotid repeat in the CETP promoter was recently used to demonstrate linkage between the CETP gene and LDL particle size in a study by Talmud et al. (Talmud et al. 2000). In their study, the distribution of the tetranucleotide repeats was shown to be in strong linkage disequilibrium with the alleles of the TaqIB polymorphic site. Also, the TaqIB polymorphism has been shown to be in almost complete linkage disequilibrium with a common functional polymorphism in the CETP gene promoter (-629C/A). The less common CETP -629A allele has been associated with lower CETP mass, increased plasma HDL cholesterol, and a 25% decreased transcriptional activity *in vitro*, proposedly due to an impaired binding of nuclear factors Sp1 and Sp3 (Dachet et al. 2000).

Lipoprotein lipase

LPL is a 475-aa (~55 kDa) protein that is synthesized mainly in the parenchymal cells of skeletal muscle and adipose tissue. LPL is bound to cell-surface proteoglycans in the capillary endothelium of adipose tissue, skeletal muscle and heart, where it hydrolyses triglycerides in VLDL and chylomicrons. ApoC-II molecules at the lipoprotein particle surface act as cofactors of LPL, whereas apoC-III and possibly also apoE may inhibit LPL-mediated lipolysis (Merkel et al. 2002). LPL also has a nonenzymatic 'bridging' function, mediating the cellular binding and uptake of lipoproteins (Merkel et al. 1998), possibly by acting as a ligand for the LRP (Chappell et al. 1994). Heparin can competitively displace LPL from the endothelium and thereby disrupt the tissue-specific channelling of TRL-derived fatty acids. Small amounts of soluble LPL are found circulating in plasma, which has been proposed to represent a recycle or removal mechanism of the enzyme.

The LPL gene is located on chromosome 8p22 (30 kb, 10 exons). Obligate heterozygotes for LPL deficiency have moderately increased TG, low HDL cholesterol, and small, dense LDL. Furthermore, in these subjects, LDL particle size is linked to the LPL gene (Hokanson et al. 1999). Several common polymorphisms have been described in the LPL gene (Merkel et al. 2002). The LPL S447X polymorphism, which appears to be the most common one, results in a premature stop codon and a truncation of the last two amino acids of the mature LPL protein. The LPL 447X allele has been associated with a beneficial lipid profile (low plasma TG and high HDL cholesterol) and a lower risk of CHD in most but not all studies (Gagne et al. 1999; Gehrisch 1999; Wittrup et al. 1999). The functional characteristics of the LPL S447X polymorphism have been disputed, and the mechanism behind the associations with plasma TG and HDL cholesterol concentrations remains unclear. *In vitro* studies have indicated both similar (Faustinella et al. 1991; Zhang et al. 1996a), decreased (Previato et al. 1994) and increased (Kozaki et al. 1993) catalytic activity of the truncated LPL protein compared with the wild type. *In vivo*, the LPL 447X allele has been associated with increased postheparin LPL activity in CAD patients (Groenemeijer et al. 1997; Henderson et al. 1999). However, several studies also suggest that postheparin LPL activity is not affected by the S447X variant (Wittrup et al. 1999; Garenc et al. 2000). Theoretically, the LPL 447X variant could also affect the endothelial binding or turnover rate of the LPL protein, which would result in more subtle effects on plasma lipids and lipoproteins.

Hepatic lipase

HL is a 476-aa (~65 kDa) glycoprotein which is produced and secreted primarily by the liver, where it is bound via HSPG to hepatocyte and hepatic endothelial surfaces. *In vitro*, HL has a broad substrate specificity. It hydrolyses mono-, di- and triglycerides, cholesteryl esters and phospholipids of plasma lipoproteins. *In vivo* studies of animal models and HL-deficient humans have indicated that HL plays an important role in the conversion of IDL to LDL (Demant et al. 1988) and the conversion of postprandial TG-rich HDL (HDL_2) to post-absorptive TG-poor HDL (HDL_3) (Connelly 1999). Recent *in vitro* and *in vivo* studies also support the hypothesis that HL mediates the uptake of remnant lipoproteins by the HSPG/LRP pathway, and that this function is independent of its catalytic activity (Connelly 1999; Zambon et al. 2000; Dichek et al. 2001). In addition, HL has been suggested to enhance the selective

uptake of HDL-CE via the hepatic scavenger receptor B1 (SR-B1) (Lambert et al. 2000). Like LPL, HL is readily released from its cell-surface binding site by heparin. Post-heparin plasma HL activity is inversely correlated with plasma HDL cholesterol concentrations, in particular the HDL_2 subfraction (Thuren 2000). Several investigators also report an inverse correlation between (post-heparin) plasma HL activity and LDL size (Zambon et al. 1993; Watson et al. 1994; Packard and Shepherd 1997). The role of HL in development of atherosclerosis and CHD remains controversial, and transgenic animal model studies as well as studies in HL-deficient patients have given partially contradictory results, indicating both pro- and antiatherogenic effects of HL action (Jansen et al. 2002).

The human HL gene is located on chromosome 15q15 (35 kb), and consists of nine exons and eight introns. Several mutations in the HL gene have been reported to impair secretion and/or catalytic function of the protein. However, the clinical syndrome of HL deficiency is a very rare lipoprotein disorder, and the variability in biochemical phenotype is considerable among carriers of HL gene mutations. In general, HL-deficient patients are hypercholesterolaemic, hypertriglyceridaemic, and accumulate VLDL remnants. HDL and LDL from these patients are typically enlarged and TG-rich. Four common polymorphisms in the HL promoter are in complete linkage disequilibrium (Guerra et al. 1997), defining a single allele. Several studies have reported a decreased HL activity and elevated plasma HDL cholesterol concentration in association with the less common allele, represented by -514T (also denoted -480T) (Jansen et al. 1997; Murtomaki et al. 1997; Zambon et al. 1998; Jansen et al. 1999; Couture et al. 2000). Recently, the -514T allele was shown to decrease the transcriptional activity of the HL gene *in vitro* (Deeb and Peng 2000). In conflict with the expected anti-atherogenic effect of an increased plasma HDL cholesterol concentration, the -480T allele has been shown to be more common in CAD patients compared with healthy controls (Jansen et al. 1997), and to be associated with a greater extent of CAD, as determined by coronary angiography (Dugi et al. 2001).

Hypothesis

The small, dense LDL phenotype is a genetically regulated risk factor for cardiovascular disease.

Aims

To establish an accurate method to measure plasma concentrations of LDL subfractions.

To further elucidate the relationship between LDL subfraction distribution and cardiovascular disease.

To investigate physiological determinants of the oxidative susceptibility of LDL.

To investigate the influence of common variants in genes encoding physiologically relevant proteins on the metabolism of apoB-containing lipoproteins and plasma LDL subfraction distribution.

To investigate the relationship between genetic regulation of LDL subfraction distribution and cardiovascular disease.

Material and Methods

Study subjects

Studies I, III, and IV

Healthy 50-year-old men living in the county of Stockholm were recruited from a randomised population-based screening program. Exclusion criteria were chronic disease of any kind, a history of CHD or arterial thromboembolic disease, and continuous treatment with anti-hypertensive or lipid-lowering drugs. Only men of North European descent were included. Study participants were interviewed by a nurse who completed a questionnaire regarding smoking habits and alcohol consumption.

For study I additional inclusion criteria were homozygosity for the apoE e3 allele and acquisition of a technically satisfactory carotid ultrasound examination. Furthermore, for this study (I), only subjects with BMI < 32 kg/m^2 were included.

A total of 377 subjects were included in studies III and IV, and 94 of these also participated in study I.

Study II

A total of 21 healthy, normolipidaemic men, aged 57.8±6.1 (mean±SD) years, were recruited from participants in a long-term follow-up of a lifestyle intervention study (Hellenius et al. 1993), and included in the study. Of these 21 men, the 10 subjects with the highest serum TG concentrations were selected for the *in vivo* studies.

Study IV

A total of 405 consecutive survivors of a first MI before the age of 60 were included in study IV. The patients were admitted to the coronary care units of three hospitals in the northern part of Stockholm. Exclusion criteria were type 1 diabetes, renal insufficiency (serum creatinine >200 μmol/L), chronic inflammatory disease, drug or alcohol addiction, psychiatric disease or inability to comply with the protocol. In addition, a total of 41 subjects with type 2 diabetes were excluded, and in 22 patients DNA was not obtained. Thus, analyses were performed on a total of 342 patients. Approximately 34% of the patients were investigated on lipid-lowering medication. A total of 392 healthy, age- and sex-matched controls were recruited from the general population in the same catchment area. Essentially, after inclusion of a postinfarction patient, a matched control subject was identified via the population registry. Control subjects were interviewed to exclude individuals with a history of cardiovascular disease.

Biochemical analyses

Blood sampling

Fasting venous blood samples were drawn into pre-cooled sterile tubes (Vacutainer, Becton Dickinson, Meylan Cedex, France) containing Na_2EDTA (final concentration 4 mM), and plasma was immediately recovered by low-speed

centrifugation (1.750*g*, 20 min, +1°C). Phenylmethyl-sulfonyl fluoride (PMSF) (10 mmol/L, dissolved in isopropanol) and aprotinin (1.4 g/L; Trasylol, Bayer, Leverkusen, Germany) were immediately added to the isolated plasma to final concentrations of 10 µmol/L and 28 µg/ml, respectively.

For preparation of serum (study II), 20 ml of blood was drawn into vacutainer tubes. After blood samples had been allowed to clot for 120 minutes at room temperature, serum was recovered by low-speed centrifugation (1.400*g*, 20 min).

Major plasma lipids and lipoproteins, free fatty acids

Plasma concentrations of cholesterol and TG in VLDL, LDL and HDL were determined by a combination of preparative ultracentrifugation, precipitation of apoB-containing lipoproteins and lipid determinations (Carlson 1973). Cholesterol (free and esterified), TG and phospholipid (PL) concentrations in VLDL and LDL fractions (study II) were determined by enzymatic methods. Free fatty acid (FFA) concentrations in LDL (study II) were determined after extraction (Dole 1956) by chemical methods (Ho 1970).

Lipase activities

Plasma LPL and HL activities were determined (study III) without prior injection of heparin. The lipase assay was performed as described (Karpe et al. 1992), which essentially involved incubation of plasma with a ^{3}H-fatty acid labelled triglyceride emulsion. The two activities were distinguished by inhibiting LPL with high concentration of NaCl (1M) and HL by neutralising antibodies. Lipase activities are expressed in mU, which correspond to 1 nmole of fatty acid released per minute.

Lipid soluble vitamins

The mass content of the lipid soluble vitamins (study II) α-tocopherol, lycopene, α-carotene and β-carotene in isolated LDL was determined by high performance liquid chomatography (HPLC) (Schäfer-Elinder and Walldius 1992). One ml of isolated LDL was mixed with 1.0 ml of ethanol. Heptane (1.5 ml) was added and the upper phase was isolated, evaporated under N_2, and redissolved in 150 µl aceto-nitril:tetrahydrofurane (1:1). A volume of 125 µl was applied to the HPLC system (Beckman System Gold equipped with an Ultrasphere ODS 250 x 2 mm column). Elution was performed with aceto-nitril:tetrahydrofurane:water (74:20:6) at a flow of 0.4 ml/min. The concentration of α-tocopherol was monitored at 292 nm, lycopene at 475 nm and α- and β-carotene at 475 nm. All vitamins were monitored simultaneously on a diode array detector (Beckman System Gold, model 168). Standards (T3251, SL 9879, C0251 and C4582) were purchased from Sigma. For the lipid extraction of co-enzyme Q10, a 100 µl sample was mixed with 100 µl ethanol, 500 µl hexane was added and the upper phase was removed, evaporated and dissolved again in methanol:hexane (90:10). The peak was monitored at 275 nm (Andersson 1992). A standard from Sigma (C 9538) was used.

Subfractionation of apoB-containing lipoproteins

The large and small VLDL (Sf 60-400 and 20-60, respectively), IDL (Sf 12-20) and LDL fractions were isolated by a DGUC procedure (Karpe and Hamsten 1994). LDL for GGE was isolated from plasma by a single spin DGUC procedure according to Redgrave (Redgrave and Carlson 1979) with modifications. After a 16 hour spin at 40,000 rpm and 15°C (Beckman SW40), the top 0.5 ml layer was

aspirated (VLDL). The tube was then sliced 57 mm from the top to harvest the fraction (*d* 1.006-1.061 kg/L) containing both IDL and LDL. Aliquots of isolated LDL were then stored at -80°C after the addition of one-fifth of the volume of sucrose 50% (wt/vol), NaCl (0.15 mol/L), and EDTA (0.24 mmol/L, pH 7.4) (Rumsey et al. 1992) until later GGE analysis.

For study I, two additional DGUC procedures were used. The first, described by Griffin et al. (Griffin et al. 1990), was used to obtain an LDL density gradient absorbance profile. The second, an equilibrium DGUC procedure (Chapman et al. 1988), was used to separate and isolate density-defined LDL subfractions. The density profile of the ultracentrifugation gradient was controlled by replacing LDL with a salt solution (1.040 g/L) and the densities of the subfractions thus obtained were determined by using a precision densitometer (Paar, DMA 60).

Determination of apoB content in lipoprotein fractions

The apoB-100 content in the VLDL subfractions and IDL was determined by analytical SDS-PAGE (Karpe and Hamsten 1994). In study I the total VLDL apoB-100 concentration constitutes the sum of the apoB-100 content in the small and large VLDL subfractions. The protein concentration of the isolated LDL fraction was determined according to Lowry et al. after addition of SDS to the reagent mixture to clear turbidity (Lowry et al. 1951).

Nondenaturing polyacrylamide GGE of LDL

Gel casting

Polyacrylamide gradient gels were cast using a two-chamber gradient mixer (GM-1, Pharmacia-LKB, Stockholm, Sweden) and a gel casting cassette (Hoefer Scientific, San Francisco, CA) prepared for ten gels (1.5 mm spacers, 10 well combs). The gels consisted of a short 3.0% acrylamide (Acrylamide, BioRad Laboratories, Richmond, CA) stacking gel followed by a linear 3.0 to 7.5% acrylamide gradient. Ammoniumpersulfate (10% w/v) was added to the acrylamide solutions to attain a polymerisation time of 90 min. In general, gels were stored under moist conditions at +4°C for no longer than 2 weeks.

Electrophoresis

The vertical slab gels were run in the Hoefer mighty small II apparatus equipped with an EPS 500/400 Pharmacia-LKB power supply. Pre-electrophoresis (60 min. at 50 V) and electrophoresis were carried out using Tris 180 mM, boric acid 160 mM, Na_2EDTA 6 mM, pH 8.35 as running buffer with cooling from a thermostatic circulator (Multitemp II, Pharmacia-LKB) set at 10°C. To avoid smearing, low melting point agarose (final concentration 0.4% (w/v)) was added to the sample immediately before application to the gel. A total volume of 25 µl of sample, containing approximately 3 µg LDL protein was applied to each well. Reference proteins were run in two lanes on each gel. The wells closest to the edges of the gel were not used. Electrophoresis was performed at 50 V for 60 min., followed by 100 V for 20 hours. After electrophoresis, the gels were stained for protein in glass petri dishes using a newly filtered solution of 0.04% Coomassie Brilliant Blue G-250 (Serva, Heidelberg, Germany) in 3.5% perchloric acid for a minimum of 3 hours. The gels were destained in 7% acetic acid for 20 hours with one change of destaining solution.

Reference proteins

Four different standard size reference proteins/lipoproteins were used. The largest was a lipoprotein (a) (Lp(a))

species, which was isolated by sequential overnight ultracentrifugation. An isolated LDL sample was also used as a size reference as well as a distribution pattern reference for rapid estimation of running quality and reproducibility. The Lp(a) containing fraction and the isolated LDL sample were dialysed against 1% ammoniumbicarbonate and thereafter subjected to electron microscopy (Philips EM 400) using a negative stain (1% phosphotungstic acid, pH 8.5) at 60,000 times magnification and displayed on photographic images. The calculated mean diameter of the Lp(a) particles was 25.1±1.9 nm (200 particles measured). For the LDL sample the mean particle size was 23.5±1.1 nm (115 particles measured). Aliquots of the Lp(a) fraction and the LDL sample were mixed with the sucrose-containing buffer previously described, kept frozen at -80°C, and thawed immediately before application on the gel. The smaller reference proteins were thyroglobulin (Pharmacia-LKB) and the dimer of thyroglobulin. The hydrated sizes of thyroglobulin and its dimer were calculated to be 17 nm and 21.4 nm, respectively (Rodbard and Chrambach 1971).

Densitometry and determination of LDL particle size

Gels were scanned by a laser densitometer (Ultroscan XL, Pharmacia-LKB) linked to a personal computer, and the area under the absorbance curve was calculated automatically with use of the Gelscan XL software package (Pharmacia-LKB). The migration distance of each size reference protein/lipoprotein was related to size by simple linear regression. This calculation was performed for each gel, and was used to convert migration distance on the gel into LDL particle diameter, to determine the peak particle size and calculate the predefined borders between the subfractions. The area under the curve was calculated across the LDL particle size range.

Definition of LDL subfractions

The size cut-offs for the LDL subfractions were derived from density cut-offs as described in detail in paper I. The boundaries of the total LDL particle size interval were defined as 21.0 nm > LDL particle diameter > 27.0 nm. Size cut-offs were set to 25.0 nm (corresponding to ~1.030 kg/L), 23.5 nm (corresponding to ~1.040 kg/L) and 22.5 nm (corresponding to ~1.050 kg/L). Subsequently, four LDL subfractions were defined, from the largest to the smallest particle size; LDL-I (27.0-25.0 nm, ~1.006-1.030 kg/L), LDL-II (25.0-23.5 nm, ~1.030-1.040 kg/L), LDL-III (23.5-22.5 nm, ~1.040-1.050 kg/L), and LDL-IV (22.5-21.0 nm, ~1.050-1.060 kg/L). The LDL particle size distribution was expressed with use of the defined size cut-offs, and the relative area under the absorbance curve for stainable material within each LDL subfraction was calculated by the software. Three different parameters reflecting the LDL particle size distribution were derived from the evaluation of the gel: (i) The peak particle size (nm) denoting the particle size of the predominant peak; (ii) the relative distribution fraction showing the proportion (%) of the total area under the absorbance curve accounted for by each LDL subfraction; and finally (iii) the plasma LDL subfraction concentration (mg/L), which was quantified by multiplying the total LDL protein concentration by the relative distribution fraction (ii) (Fig. 4).

The reproducibility of the GGE procedure was estimated by calculating the within subject variation. The within-subjects standard deviation was 0.033 % (LDL-I), 0.041 % (LDL-II), 0.037 % (LDL-III), and 0.017 % (LDL-IV) for the respective relative distribution of LDL

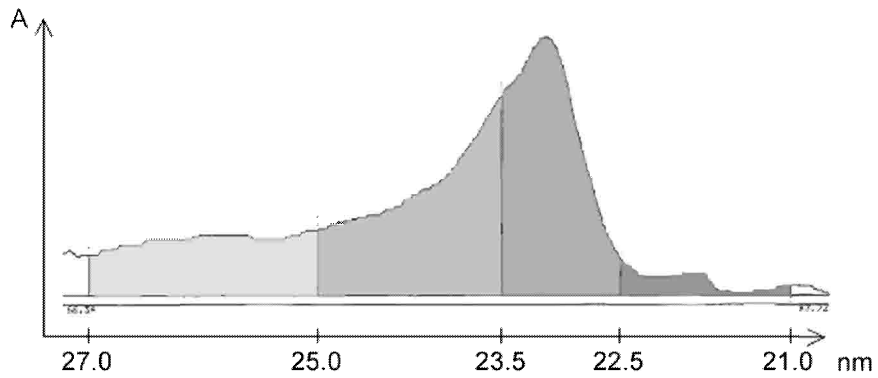

Figure 4. Absorbance curve of an LDL sample separated by 3-7.5% PA GGE.

subfractions. The within-subjects standard deviation for major peak was 0.14 nm (CV 0.006).

Determination of LDL susceptibility to oxidation

The susceptibility of LDL to *in vitro* oxidation (study II) was analysed according to Esterbauer and coworkers (Esterbauer et al. 1989). In brief, isolated LDL was kept in darkness and run on a PD-10 column (Pharmacia, Uppsala, Sweden) before analysis. The protein content of LDL was adjusted to 25 μg/ml (final concentration) by addition of PBS. The formation of conjugated dienes was monitored by spectrophotometry at 234 nm after addition of $CuSO_4$ to a final concentration of 2.5 μM at 30°C. Readings were made every fifth minute for 300 min. The lag phase was defined as the intercept of the tangent of the slope of the absorbance curve in the propagation phase (Regnström et al. 1992).

DNA preparation and genotyping

DNA was prepared from whole blood using the Qiagen DNA Kit (Qiagen Inc. Chatsworth, CA) according to the manufacturer's protocol. Genotyping was performed by PCR-based RFLP. ApoE genotypes were determined as described (van den Maagdenberg et al. 1989). Detailed descriptions of the genotyping procedures for the CETP TaqIB and -629C/A polymorphisms are given in paper III. The LPL S447X genotypes were first determined as described (Groenemeijer et al. 1997), and a second time with the forward primer 5'-TATTCACATCCATTT TCTTC and reverse primer 5'-AGTCTGG TGAGCATTCTGGGCTAAAGCTGACT GGGCATCC. PCR amplification was performed according to the former protocol. The PCR product was digested with 8 units of Hinf1 (New England Biolabs, Beverly, MA), and fragments were separated on 2.5% agarose gels. The HL -480C/T, apoB -516C/T, and MTP -493G/T genotypes were determined as described (Jansen et al. 1997; Karpe et al. 1998; van 't Hooft et al. 1999).

Carotid ultrasound examinations

Measurement of IMT was done essentially according to the ELSA ultrasound protocol (Mercuri et al. 1996) and included two components, the scanning and the reading procedures. Two certified ultrasonographers performed the scanning, with subjects in the supine position with the

head slightly turned from the sonographer. The ultrasound device used was a Biosound 2000 II s.a. (Biosound Inc., Indianapolis, IN) with an 8 MHz high-resolution annular array scanner. The far and near wall of the right and left CCA were scanned in an anterior, lateral and posterior angle, and the IMT was estimated by measuring the linear distance, perpendicular to the luminal axis, between two points defined by the ultrasonic interfaces which indicate the boundary between the lumen and the intimal surface, and the boundary between the medial and adventitial interface. CCA IMT was calculated as the mean of the right and left common carotid far wall IMT. All subjects were routinely scanned twice by the same sonographer to evaluate intra-sonographer reproducibility. To evaluate the inter-sonographer reproducibility, the two sonographers made one scanning each on the same occasion on all subjects appearing during one month at six-monthly intervals. The intra-sonographer coefficient of variation was 3.8% and 5.1%, respectively for the two sonographers. The inter-sonographer coefficient of variation was 4.7%.

Lipolysis protocols

In the *in vitro* studies (study II) a total of 4.5 ml of serum was incubated for two hours in a shaking water-bath at 37°C, with or without the addition of 65 µl (0.2 mg/ml) of bovine LPL with an activity of 560 µmol free fatty acid released/mg and min (kindly provided by Professor Gunilla Olivecrona, Department of Medical Biosciences, University of Umeå, Sweden). Lipolysis was terminated by placing the samples on ice.

For the *in vivo* part of study II, blood samples were taken before, 30 and 120 min after intravenous injection of 50 U of heparin/kg bodyweight.

Stable isotope turnover

Study subjects (recruited-by-genotype)

Six male carriers of the MTP -493G and -493T alleles, respectively, were recruited by genotype to participate in the turnover studies (study V). Of these, six were recruited from the cohort of healthy 50-year-old men described previously, and six were recruited from a previous collection of healthy men performed according to the same principles and within the same geographic area (Tornvall et al. 1993). All twelve men were identified by their genotype and subsequently asked to participate.

Tracer administration protocol

Subjects arrived in the Clinical Research Unit in the morning after a 10 h fast. Blood samples were taken at times 0, 0.16, 0.33, 0.5, 0.75, 1, 2, 3, 4, 5, 6, 8, 10, 12, 15, 24, 36 h, and after that daily in the morning before breakfast for 14 days. A bolus injection of 0.7 mg/kg body weight of tri-deuterated (d_3-Leucine) was given at time 0 followed by an infusion of 0.7 mg/kg/h for 10 hours. The subjects were asked to maintain a regular lifestyle during the following fortnight, which comprised a habitual diet without extremes, limited alcohol intake and not more exercise than was usually taken.

Lipoprotein isolation, preparation of apoB, preparation and analysis of leucine

Fractions of VLDL1, VLDL2, IDL and LDL were isolated by density gradient ultracentrifugation (Karpe et al. 1993a). After the removal of the VLDL2 fraction, the tube was sliced 29 mm from the top to isolate IDL. A further slice 57 mm from the top was made to isolate LDL. ApoB was physically isolated by isopropanol extraction (Holmquist et al. 1978) for hydrolysis and subsequent analysis of isotope enrichment by gas chromatography

mass spectrometry (GC-MS) (Demant et al. 1996).

Protein pellets were hydrolysed to amino acids at 110°C for 24 h in 6M HCl and evaporated to dryness. Amino acids were finally isolated by cation exchange chromatography using Dowex AG 50W-X8 resin (H+ form, 50-100 mesh, BioRad, Richmond, US) and eluted in 4 M NH_4OH. Samples were analysed for isotope enrichments as previously described (Demant et al. 1996).

Kinetic analysis and multicompartmental modelling

The change in tracer/tracee ratio with time of VLDL1, VLDL2, IDL and LDL apoB together with the apoB pool size was used to derive the kinetic parameters using the simulation analysis and modelling (SAAM) program II version 1.1.1 for Windows (SAAM Institute, Seattle). The multicompartmental model (Demant et al. 1996) is described in detail in paper V.

Statistical analyses

The distribution of continuous variables are presented as mean±SD or median and interquartile range (IQR). Logarithmic transformation was used to normalize skewed variables before statistical computation and significance testing. Associations between parameters were determined by linear regression analysis (study I), and results are given as correlation coefficients. Multiple stepwise regression analysis was used in study I to evaluate the determinants of CCA IMT. Allele frequencies were estimated by gene counting. Test for allelic or random association between genotypes was performed using the ASSOCIATE software (J. Ott, Rockefeller University, NY). A χ^2 test was used to test for Hardy-Weinberg equilibrium. Differences between means were tested either by Student's t-test (two groups) or by one-way analysis of variance (ANOVA), followed by the Scheffé post hoc test. ApoE genotypes were grouped according to carrier status of the e2 or e4 alleles (subjects with an e2e4 genotype were excluded from the analyses); apoE E2 (e2e2 and e2e3), apoE E3 (e3e3) and apoE E4 (e3e4 and e4e4). Gene-gene and gene-environment interactions were evaluated by two-way ANOVA. Allele frequencies were compared by gene counting followed by χ^2 analysis. Odds ratios for myocardial infarction (study IV) were calculated by comparing the number of carriers of an allele with the number of non-carriers in patients vs. controls. Statistical differences in kinetic parameters between groups (study V) were calculated using the Mann-Whitney U test.

Ethical considerations

All studies were approved by the Ethics Committee of the Karolinska Hospital and all subjects gave informed consent to participation.

Results and Discussion

Measures of LDL particle size distribution

For the studies included in this thesis a novel PA GGE procedure was developed to measure LDL particle size distribution. In general, compared with DGUC procedures, GGE techniques are less costly and labour intensive. However, traditionally, the DGUC methods have been able to separate the LDL particle spectrum with a higher resolution. In contrast, due to the steep gradient of PA, the commonly used PAA 2/16 gel separates the LDL within a short distance of the gel, and therefore the LDL particle size distribution pattern obtained by this method is often dominated by a very sharp peak.

The present GGE method was designed to improve the resolution of the LDL particle separation. This was achieved by a slow progression of the PA gradient. On the 3-7.5% PA gel, the LDL particles were separated over a distance of approximately 2-3 cm, and all samples displayed a continuous particle size distribution, without signs of discrete subclasses or sharp peaks (Fig. 5).

The GGE method described in paper I enables the evaluation of three principally different measures of LDL particle size distribution, namely the peak particle size (nm), and the relative proportions (%) as well as plasma concentrations (mg/L) of four LDL subfractions. The size cut-offs

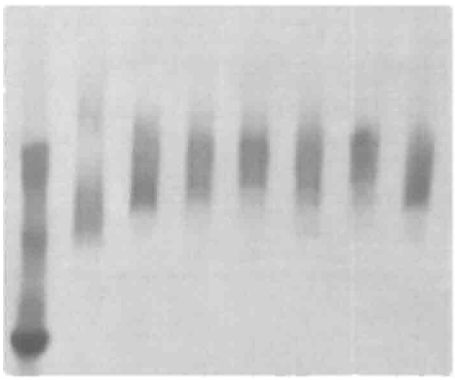

Figure 5. The 3-7.5% PA GGE gel

for the LDL subfractions were derived from density cut-offs, as described in detail in paper I. In the large cohort of healthy 50-year old men (n=377) the LDL-II subfraction (23.5-25.0 nm) was the most predominant (~50% of total LDL), and the major peak was most often found within this subfraction. The greatest heterogeneity was seen within LDL-III (22.5-23.5 nm), which constituted 27% (17-37%) of total LDL. Only a small portion (~6%) of the LDL particles were contained in LDL-IV (21.0-22.5 nm). This distribution pattern amongst the four LDL subfractions is in accordance with the distribution described in studies using DGUC procedures (Krauss and Burke 1982; Nichols et al. 1986). Furthermore, a qualitative comparison between the GGE method and an established DGUC procedure (Griffin et al. 1990) showed a good agreement between the LDL particle distribution pattern

obtained by the two different procedures. Thus, although the conversion of the LDL subfraction density to LDL particle size is approximate, the LDL subfractions obtained by this procedure proved to be appropriate.

A major advantage of the current procedure is the ability to quantify the plasma concentrations of LDL subfractions, which is obtained by multiplying the total LDL apoB by the respective proportion of stainable material within each individual LDL subfraction. The use of a continuous measure of LDL size distribution is in contrast with numerous previous studies that have used the dichotomous LDL subclass pattern A/B to describe LDL particle size heterogeneity. However, a substantial amount of information about the LDL heterogeneity is thus discarded. The value of a more comprehensive LDL particle size characterization as a measure of LDL atherogencicity was recently proven (St-Pierre et al. 2001).

LDL particle size and early atherosclerosis

The association between the small, dense LDL phenotype and the presence of coronary atherosclerosis or clinically manifest CHD is well-documented. However, the relationship between LDL particle size and early atherosclerotic changes in the vascular wall is poorly investigated. In paper I, we demonstrate that the plasma concentration of small, dense LDL (LDL-III) is strongly associated with the IMT of the CCA. Furthermore, the association is shown to be independent of plasma LDL cholesterol and TG concentrations, and alone contribute 10% to the variance in CCA IMT in a healthy population.

Carotid artery IMT is considered a good surrogate marker of early atherosclerosis, and has been shown to correlate with the presence and severity of CAD (Craven et al. 1990; Wofford et al. 1991), and also to predict coronary events (Salonen and Salonen 1993; Chambless et al. 1997). Quantification of the carotid IMT by B-mode ultrasound is noninvasive and relatively inexpensive (compared with angiography), but most importantly is able to detect early changes in the vascular wall, and may in a clinical setting be used as a tool for evaluation of CAD risk in apparently healthy individuals or subjects with subclinical diasease. At present, there is no standardized protocol for measuring IMT. For study I, the carotid IMT was measured according to the ELSA protocol (Mercuri et al. 1996), and the IMT of the CCA was calculated as the mean of the right and left CCA far-wall IMT.

In univariate analysis, the plasma concentration of LDL-III showed the strongest correlation to the CCA IMT ($r=0.42$, $p<0.001$). Of the major plasma lipids, both total cholesterol and total TG concentrations were associated with IMT ($r=0.29$, $p<0.01$ and $r=0.25$, $p<0.05$, respectively). These associations were in turn largely accounted for by the associations between LDL cholesterol ($r=0.24$, $p<0.05$), and VLDL TG ($r=0.24$, $p<0.05$) and CCA IMT, respectively. In contrast, plasma concentrations of apoB-100 in the VLDL and LDL fractions were not significantly associated with common CCA IMT, whereas IDL apoB-100 was. Regarding the different LDL particle size parameters, the major LDL peak size, as well as the relative distribution of larger LDL species (LDL-I and -II) was negatively related to IMT, but when the actual plasma concentrations of these LDL subfractions were considered, only that of LDL-I remained significantly associated ($r=-0.32$, $p<0.01$) with IMT. The opposite relations of large and small LDL to CCA

Table 5. Multivariate analysis of determinants of CCA IMT

	Model A		Model B		Model C	
	Partial correlation	r^2	Partial correlation	r^2	Partial correlation	r^2
LDL-C	0.25		0.20	0.01	f	
VLDL-TG	0.25		f	0.06	f	0.10
IDL apoB-100	0.21		0.14		f	
LDL-III	0.44	0.19	0.37	0.13	0.33	0.10
Multiple r^2		0.19		0.20		0.10

IMT are likely to explain the relative weakness of the LDL cholesterol association with IMT.

Multiple stepwise linear regression analysis was performed to identify independent determinants of CCA IMT. In the multivariate forward stepwise model (model A, Table 5), LDL-III was the first variable to enter, contributing 19% to the variation in CCA IMT. Once LDL-III had entered, none of the other lipoprotein or apolipoprotein variables remained significantly related to CCA IMT. In model B, VLDL TG was forced to enter (r^2=0.06), and subsequent stepwise forward progression allowed LDL-III to enter the model (r^2=0.13) followed by LDL cholesterol. Finally, in model C, VLDL TG, LDL cholesterol and IDL apoB-100 were forced to enter the model. Together these variables accounted for 10% of the variation in CCA IMT, which indicates a substantial degree of interaction between them. Nevertheless, LDL-III entered the model and increased r^2 by 0.10.

Several risk factors for CHD are also associated with increased carotid IMT, such as age, male sex, smoking, hypertension, diabetes, BMI, and hypercholesterolaemia (Cheng et al. 2002). Furthermore, the common apoE polymorphism has been associated with CCA IMT (Terry et al. 1996; Cattin et al. 1997). The participants in study I were homogenous with regard to age, gender and apoE genotype. In our study, neither BMI, systolic blood pressure (SBP), nor diastolic blood pressure (DBP) were significantly correlated with CCA IMT in univariate analysis. Of the major lipoprotein determinations, only VLDL TG, LDL cholesterol and IDL apoB-100 related significantly to the CCA IMT and together explained 10% of its variation, which is in agreement with an earlier study (Sharrett et al. 1994). A previous study (Hodis et al. 1997) has indicated that IDL is associated with progression of atherosclerosis measured as increase in CCA IMT. In our study, the IDL apoB-100 concentration was associated with CCA IMT in univariate analysis but failed to contribute to the variation in IMT independently of LDL cholesterol and VLDL TG.

A number of studies indicate that the total plasma or LDL apoB concentration may be more strongly associated with risk of CHD than the plasma LDL cholesterol concentration (Hamsten et al. 1986; Lamarche et al. 1996; Kwiterovich 2002; Sniderman et al. 2003), whereas the LDL cholesterol concentration seems to be a stronger predictor of the carotid IMT in healthy individuals (Sharrett et al. 1994). This discrepancy may reflect the relative contribution of different lipoprotein fractions during the development of atherosclerotic disease. Alternatively, the atherogenicity of LDL is not properly reflected by simply measuring the LDL

apoB or cholesterol concentrations. The results of our study show that the plasma concentration of small, dense LDL, which previously has been shown to be a risk factor for CHD, is strongly and independently associated with early atherosclerotic changes in the vascular wall.

Oxidative susceptibility of LDL

Oxidative modification of the LDL particle is considered an initiating step in atherogenesis, resulting in the uptake of LDL by macrophages and the subsequent development of foam cells. Furthermore, *in vitro* oxidized LDL mediates several potentially atherogenic events. The *in vitro* susceptibility of LDL to oxidation has been shown to be increased in subjects with CAD compared with healthy controls (Cominacini et al. 1993; Chiu et al. 1994), and also to correlate with the severity of CAD (Regnström et al. 1992). Several intrinsic properties of the LDL particle have been suggested to influence its ability to withstand oxidative stress, such as particle size/density, lipid composition, and content of antioxidative vitamins. However, little is known about the mechanisms or metabolic events that promote the formation of an LDL species with diminished oxidative resistance.

In study II, the influence of artificial and exaggerated lipolysis on potentially atherogenic properties of LDL was investigated in 21 healthy, normolipidemic middle-aged men (57.8±6.1 yrs). Serum and plasma triglycerides, respectively, were subjected to LPL-mediated lipolysis *in vitro* and *in vivo*. LDL was characterised by chemical composition, vitamin content, particle size and susceptibility to oxidative modification. For this study, the LDL particle spectrum was divided into two subclasses; large and small, respectively, with a size cut-off at 22.8 nm.

After a two-hour incubation of serum with bovine LPL, the LDL concentrations of free cholesterol, phospholipid, FFA and protein were increased, whereas TG and CE concentrations were unchanged compared with LDL isolated from untreated serum. When calculated as the LDL particle content (ratio of lipid to LDL protein), the FFA increased, whereas free and esterified cholesterol decreased. The LDL concentration as well as particle content of α-tocopherol increased, whereas the amount of α-carotene and co-enzyme Q10 per LDL particle decreased in serum incubated with LPL. The LDL peak particle size and the concentration of large LDL particles decreased, with a reciprocal increase in the proportion as well as absolute concentration of small LDL particles. LDL isolated from LPL-treated serum had an increased lag phase time compared with LDL from untreated serum.

In vivo, LPL lipolysis was artificially enhanced by heparin injection. No differences were found between lipid and protein concentrations in LDL isolated before and after heparin injection. In contrast, the LDL concentration as well as the particle content of α-tocopherol were increased both at 30 and 120 minutes compared with baseline. The LDL peak particle size and the distribution of large and small LDL were unchanged after heparin injection, whereas the lag phase time for oxidative modification of LDL was increased at both 30 and 120 minutes after heparin injection.

The present study demonstrates that exhaustive *in vitro* lipolysis of serum triglycerides by LPL leads to an increased number of LDL particles, suggesting that a new population of LDL particles is formed by complete catabolism of VLDL during

lipolysis. Furthermore, LPL-mediated lipolysis resulted in a decrease in LDL particle size, an increased particle content of α-tocopherol and an increase in the resistance of LDL to copper-induced oxidative modification of LDL. The decrease in LDL size was associated with a relative decrease of LDL CE and TG, likely to represent depletion of core-lipids of LDL. This is in concert with the *in vitro* findings by Lagrost and coworkers (Lagrost et al. 1994; Viens et al. 1996), who showed that a combination of lipid transfer of TG to LDL and subsequent lipolysis of LDL TG by LPL resulted in smaller LDL particles.

The observation of a decrease in LDL particle size concomitant with an increased resistance of LDL to oxidative modification is in contrast with previous studies that have shown that a small, dense LDL pattern is associated with a decreased resistance to oxidative modification (de Graaf et al. 1991; Tribble et al. 1992; Chait et al. 1993; Dejager et al. 1993; Tribble et al. 1995). However, similar results have been reported in previous *in vitro* studies (Lagrost et al. 1994; Viens et al. 1996). We speculate that *in vivo*, small, dense LDL particles, due to a longer residence time in plasma, are exposed to repeated oxidative attacks, and as a consequence gradually loose their native antioxidative capacity, which in turn may explain the observed increased oxidative susceptibility of small, dense LDL particles.

The *in vivo* studies confirmed that an enhanced lipolysis of plasma triglycerides is associated with an increased resistance to oxidative modification of LDL. However, no change in LDL particle size was seen, indicating that mechanisms other than, or in addition to, lipolysis and transfer of TG to LDL operate *in vivo* to generate LDL particles of different sizes. Furthermore, the *in vivo* studies confirmed the *in vitro* findings of an increase in the α-tocopherol content of LDL. The origin of this α-tocopherol must have been VLDL, but surprisingly, there was no increase in LDL particle number after *in vivo* lipolysis. This may indicate a simultaneously enhanced catabolism of LDL. A likely mechanism for this is the presence of large amounts of LPL on the surface of LDL particles (Goldberg et al. 1986), and enhanced binding to LRP, for which LPL is a ligand (Beisiegel et al. 1991).

In summary, the results of the present study show that an exaggerated LPL-mediated lipolysis of triglycerides results in an increased resistance of LDL to oxidative modification, which most likely is due to an increase in the particle content of α-tocopherol. In the light of the present study, it could be speculated that the rate at which VLDL forms LDL determines the antioxidant capacity of LDL and, by inference, its atherogenicity.

Determininants of LDL particle size

The different measures of LDL particle size distribution were significantly inter-related. In the large cohort of 50-year old healthy men (n=377), the LDL peak particle size was strongly and positively correlated with the proportion of LDL-II ($r=0.84$, $p<0.0001$), and inversely related to both relative proportion ($r=-0.84$, $p<0.0001$) and plasma concentration of LDL-III ($r=-0.78$, $p<0.0001$). The relative proportions of LDL-I and -III were inversely related ($r=-0.51$, $p<0.0001$), whereas the respective plasma concentrations were not ($r=0.03$, ns). Likewise, whereas the proportions of LDL-II and –III were strongly inversely related ($r=-0.84$, $p<0.0001$), the respective plasma concentrations were only weakly correlated ($r=-0.16$, $p<0.01$). In contrast, the

proportions of LDL-I and -II were unrelated (r=0.05, ns), whereas the corresponding plasma concentrations were significantly associated (r=0.39, p<0.0001).

The lipid composition of LDL was weakly associated with measures of LDL particle size distribution. In general, the LDL particle content of TG (lipid to protein ratio) was inversely related to the plasma concentrations of the larger subfractions (LDL-I and -II, r=-0.22 and r=-0.28, respectively, p<0.0001 for both), whereas the LDL particle content of cholesterol was inversely related to the plasma concentrations of the LDL subfractions containing small, dense LDL (LDL-III and -IV, r=-0.25 and r=-0.28, respectively, p<0.0001 for both). This finding is in concert with the observation that the small, dense LDL particles are relatively depleted in their cholesterol content, possibly as the result of a CETP-mediated heteroexchange of cholesterol for TG from TRL.

Major plasma lipids and lipoproteins

As previously described in numerous studies, the plasma total TG concentration was the strongest determinant of both the relative proportion as well as the actual plasma concentration of small, dense LDL (LDL-III r=0.69, p<0.0001) in the study cohort of 50-year old men (n=377). As a reflection of this relationship, both VLDL TG and cholesterol concentrations were strongly and positively associated with the plasma concentration of LDL-III (r=0.56 and r=0.54, respectively, p<0.0001 for both). The plasma HDL cholesterol concentration was inversely related to the plasma concentration of LDL-III (r=-0.39, p<0.0001), whereas the plasma LDL cholesterol concentration was strongly and positively associated with the plasma concentrations of the larger LDL particle species (LDL-I and -II, r=0.38 and r=0.64, respectively, p<0.0001 for both).

The fasting plasma concentration of subfractions of apoB-containing lipoproteins were measured in the subjects participating in study I. Of these, the VLDL1 apoB concentration was most strongly correlated with the LDL-III plasma concentration (r=0.47, p<0.0001), compared with the VLDL2 apoB (r=0.42, p<0.0001), and IDL apoB (r=0.34, p<0.001) concentrations. Also, the total plasma apoB concentration was associated with the plasma LDL-III concentration (r=0.59, p<0.0001).

Plasma activity of triglyceride lipases

The plasma HL activity was not significantly related to any measures of LDL particle size hetergeneity (n=236, study III), nor was a significant association found between the plasma HL activity and the LDL TG concentration. This is in contrast to several previous reports of an inverse relationship between HL activity and LDL particle size or buoyancy (Zambon et al. 1993; Jansen et al. 1994; Watson et al. 1994; Campos et al. 1995). These studies measured postheparin HL activity, whereas the HL activity in study III was measured in circulating plasma. However, the two measures have been described to be strongly correlated. HL activity varies considerably between individuals, and is strongly influenced by environmental factors. Thus, a possible explanaition for the lack of association between HL activity and LDL size in our study might be the study population, which was selected to be homogenous with regard to age, sex and possibly also environmental factors. However, a weak inverse relationship was seen between the plasma HL activity and the LDL particle content of TG (r=-0.14, p<0.05). In contrast, the circulating plasma LPL activity (n=18) was significantly associated with the LDL peak particle size (r=0.42, p<0.05) and the relative proportion of LDL-II (r=0.35,

p<0.05), and inversely related with both the relative proportion and plasma concentration of LDL-III (r=-0.32, p=0.05).

Genetic influence on LDL particle size heterogeneity

Plasma modulation

In plasma, the apoB-containing lipoproteins are subject to lipolysis and remodeling which alters their composition and size, and subsequently may affect their metabolic fates. Plasma factors, lipid transfer proteins and lipases with important roles in lipoprotein metabolism are thus good candidates for the regulation of the plasma concentration and heterogeneity of lipoproteins. In particular, due to the strong correlation between plasma TG and LDL particle size, proteins involved in the modulation of TRLs are likely to influence plasma LDL heterogeneity.

In study III, the separate and joint effects of the CETP (-629C/A), LPL (S447X), HL (-480C/T) and apoE (e2/e3/e4) gene polymorphisms on LDL heterogeneity were investigated in 377 healthy 50-year-old men. The study group was selected to be as homogenous as possible, and to be representative of the general population. Furthermore, to limit the effects of genetic heterogeneity, only men of North European descent were included. The general characteristics of the study population are shown in Table 6.

The polymorphisms studied here have all been suggested to influence transcriptional activity and/or protein function or activity. The less common CETP -629A allele has been associated with a decreased plasma CETP mass (Dachet et al. 2000), and may be regarded as a mild form of CETP deficiency. However, although CETP has been suggested to contribute to the formation of small, dense LDL, several investigators have been unable to find an independent effect of CETP on LDL subfraction distribution (Karpe et al. 1993b; Watson et al. 1994; Carr et al. 2002). In our study, the CETP -629A allele was associated with increased plasma LDL cholesterol, moderately increased LDL peak particle size, and an increased relative proportion and plasma concentration of LDL-II. There are no previous reports of associations between the CETP promoter polymorphism and LDL particle size distribution. With regard to the CETP TaqIB polymorphism, which is in almost complete linkage disequilibrium with the -629C/A polymorphism, previous studies

Table 6. Characteristics of 50-year old men (n=377)

Measure	Mean±SD
Cholesterol (mmol/L)	
Plasma	5.28±0.95
VLDL	0.44±0.37
LDL	3.50±0.84
HDL	1.22±0.34
Triglycerides (mmol/L)	
Plasma	1.51±0.92
VLDL	1.10±0.88
LDL	0.28±0.10
HDL	0.13±0.05
LDL peak particle size (nm)	23.5±0.4
Subfraction distribution (%)	
LDL-I	19.6±5.4
LDL-II	47.2±10.3
LDL-III	26.6±10.4
LDL-IV	6.5±3.3
Plasma concentration (mg/L)	
Total LDL apoB	860±189
LDL-I	168±55
LDL-II	403±118
LDL-III	233±118
LDL-IV	56±33
BMI (kg/m^2)	26.1±3.2
Alcohol intake (g/w)	131±121
Smoking habits	
Non-smokers	125 (33)
Ex-smokers	146 (39)
Current smokers	103 (27)

Values are mean±SD or no. of subjects (%).

have described both an increase (Ordovas et al. 2000), as well as no difference (Bernard et al. 1998; Carr et al. 2002), in LDL particle size associated with the CETP TaqIB B2 allele. The observed increase in LDL size would support the hypothesis that the LDL particle may act as a donor of CE and that the low CETP activity seen with the -629A allele favours retention of cholesterol within the LDL pool.

The functional characteristics of the LPL S447X gene variant are not yet fully elucidated. *In vitro* studies have indicated both similar (Faustinella et al. 1991; Zhang et al. 1996a), decreased (Previato et al. 1994) and increased (Kozaki et al. 1993) catalytic activity of the truncated LPL protein compared with the wild type. *In vivo*, the LPL 447X allele has been associated with increased postheparin LPL activity in CAD patients (Groenemeijer et al. 1997; Henderson et al. 1999). However, several studies also suggest that postheparin LPL activity is not affected by the S447X variant (Wittrup et al. 1999; Garenc et al. 2000). This study is the first to demonstrate an approximately 60% increased circulating plasma LPL activity in carriers of the 447X allele. Theoretically, the truncated LPL protein expressed in carriers of the LPL 447X variant could affect the endothelial binding or turnover rate of the LPL protein, which in turn could be reflected by the level of LPL activity in circulating plasma. In general, the LPL activity in circulating plasma is very low compared with postheparin LPL activity. However, we speculate that a small but consistently higher LPL activity may influence the compositional characteristics of apoB-containting lipoproteins, which could result in subtle effects on plasma TG concentration and LDL particle size. In our study, the LPL447X allele was associated with moderately increased LDL peak particle size. Although there were no significant differences in the LDL particle size distribution between the LPL S447X genotype groups, the trend seemed to be in the direction of larger LDL particles in carriers of the 447X variant, not least in a subgroup of obese people. This finding, together with the HDL raising effect of the polymorphism, would be consistent with the observed protective effect of the polymorphism on cardiovascular disease (Gagne et al. 1999).

Our study showed a decreased circulating plasma HL activity in association with the less common HL -480T allele, which is in concert with previous findings of a decreased postheparin HL activity, since these two measures of HL activity are strongly correlated. Carriers of the uncommon -480T allele had significantly increased LDL apoB concentrations, and in particular an increased plasma concentration of the largest LDL particles. However, there was no association between the HL -480C/T polymorphism and LDL peak particle size, nor with the relative distribution of LDL subfractions. This is in agreement with a previous study where the HL -480C/T polymorphism was not associated with an LDL particle size score (Couture et al. 2000).

Compared with the CETP, LPL and HL polymorphisms, the effects of the apoE polymorphism on LDL particle size distribution were pronounced (Table 7). Previous studies on the apoE polymorphism and LDL particle size have given partly contradictory results. However, in concert with our results, the majority of studies report a smaller LDL particle size in subjects carrying the apoE e4 allele (Schaefer et al. 1994; Haffner et al. 1996; Nikkilä et al. 1996). In our study, this effect was independent of plasma TG and

Table 7. Associations of the apoE polymorphism in 50-year old men (n=377)

Measure	E2	E3	E4	ANOVA
n	53	206	102	
LDL cholesterol (mmol/L)	3.16±0.90*	3.53±0.83	3.64±0.76	0.003
LDL triglycerides (mmol/L)	0.27±0.08	0.28±0.10	0.29±0.10	ns
LDL peak particle size (nm)	23.4±0.4	23.5±0.4	23.3±0.4§	<.0001
Subfraction distribution (%)				
LDL-I	21.7±6.0	19.8±5.3	18.2±4.9	0.0005
LDL-II	45.4±8.5	49.1±9.4	45.0±11.9†	0.001
LDL-III	26.4±9.3	24.9±9.9	29.9±11.0‡	0.0003
LDL-IV	6.5±3.0	6.2±2.8	6.9±3.8	ns
Plasma concentration (mg/L)				
Total LDL apoB	765±215†	864±174	902±193	<.0001
LDL-I	164±56	171±56	164±52	ns
LDL-II	344±104§	422±115	401±121	<.0001
LDL-III	209±119	217±106	275±131‡	<.0001
LDL-IV	48±22	54±28	63±41	0.03

ApoE genotype groups: E2 = e2/e2 and e2/e3, E3 = e3/e3, E4 = e3/e4 and e4/e4.
Significance levels for the Scheffé post hoc test; *p<0.05, †p<0.01, ‡p<0.001, §p<0.0001, compared with E3.

therefore appears to be driven directly by an effect of the polymorphism on the LDL trait. The mechanism for this effect is not clear but is likely to depend on the precursor pool of small dense LDL.

An interesting finding of this study is the interaction between the HL -480C/T and apoE polymorphisms on plasma concentration of small, dense LDL. In a subgroup of apoE e4 carriers, the plasma concentration of LDL-III was 26% higher in -480T carriers compared with -480C homozygotes. Due to the relatively high frequency of these genetic variants, approximately 10% of a population could be affected by this. The combined influence of background genotypes, such as the apoE polymorphism, and gender differences could also explain why an association between the HL -514T allele and increased LDL buoyancy was seen in a recent study in healthy women (Carr et al. 2002).

In summary, our results indicate that the polymorphisms studied here are associated with diverse changes in LDL particle size distribution, consistent with the current concept of the physiological functions of the respective proteins as well as their proposed effects on CHD risk.

ApoB and apoE

ApoB and apoE are important structural and functional components of TRL. A single copy of the large apoB-100 protein is present in VLDL, IDL and LDL particles, and apoB is essential for the binding and uptake of LDL by the LDLR. In contrast, the small apoE protein is often present in multiple copies per lipoprotein particle, and is readily exchanged between lipoprotein fractions. ApoE is a high affinity ligand for all members of the LDLR family, and is considered to play an important role in the removal of TRL and TRL remnants from plasma.

In study IV, we demonstrate that two common polymorphisms in the apoB and apoE genes, by means of interaction, contribute to a markedly increased plasma concentration of small dense LDL and VLDL TG in healthy subjects. Furthermore, our results show that double carriers

of the two respective uncommon alleles; apoB -516T and apoE e4, are at increased risk for myocardial infarction.

In agreement with previous findings (van 't Hooft et al. 1999), the apoB -516T allele was associated with increased plasma total and LDL cholesterol in both patients and healthy individuals. These findings support the notion that an increased transcriptional acitvity of the -516T variant (van 't Hooft et al. 1999) may lead to enhanced VLDL production and subsequent generation of LDL particles. However, the overall increase in LDL cholesterol and apoB in carriers of the apoB -516T allele was reflected primarily by an increased plasma concentration of the larger LDL species (LDL-I and -II), and no significant difference in the relative distribution of LDL subfractions was observed between the apoB -516C/T genotype groups. This indicates that an increased VLDL production does not necessarily induce a distortion in the LDL particle size distribution under 'normal' metabolic conditions.

In contrast, the increase in plasma LDL cholesterol seen in association with the apoE e4 allele, although it was of the same magnitude as for the apoB -516T allele, was primarily reflected by a significant increase in the plasma concentration of the atherogenic small, dense LDL particles. The finding of an increase in small, dense LDL in association with the apoE e4 is in agreement with two earlier studies (Haffner et al. 1996; Nikkilä et al. 1996). However, in this study, the expression of the small, dense LDL phenotype in apoE e4 carriers seemed to be dependent on the presence of the uncommon apoB promoter variant (-516T), since in apoB -516C homozygotes the phenotype of the apoE E4 group did not differ significantly from that of the apoE e3 homozygotes. Instead, the entire effect was accounted for by the apoB -516T carriers (CT and TT), among whom the apoE e4 carriers displayed an atherogenic lipoprotein phenotype, with a marked shift in the LDL size distribution towards smaller particles (Fig. 6), as well as increased VLDL TG and cholesterol concentrations.

The mechanism by which the apoE and apoB -516C/T polymorphisms interact needs further investigation. Compared with the apoE2 isoform, less is known about the functional properties of the E4 isoform. The apoE4 isoform exhibits a receptor binding capacity of comparable or increased efficiency to that of apoE3 *in vitro* (Bohnet et al. 1996), and *in vivo* data suggest that apoE4 is catabolised more rapidly than the apoE3 isoform (Gregg et al. 1986; Gregg and Brewer 1988). It is therefore reasonable to assume that the presence of an apoE4 may lead to an increased supply of lipid substrate to the liver, which in turn could augment the effect of the apoB promoter polymorphism by elevating the secretion of VLDL apoB. Alternatively, reduced clearance of VLDL is a feature of the apoE4 isoform (Knouff et al. 1999).

The e4 allele has previously been associated with a moderately increased risk for CHD on its own (Wilson et al. 1996), although this cannot be accounted for by the modest increase in plasma LDL cholesterol. Other studies have indicated that the risk associated with the e4 allele is augmented in the presence of certain environmental factors (Humphries et al. 2001). Our study shows that the apoE e4 allele is associated with an atherogenic small, dense LDL phenotype. However, this effect is only seen in subjects that are also carriers of the apoB -516T allele. Accordingly, double carrier status of the apoE e4 and the apoB -516T alleles seems

to convey a genetic risk to which the subjects have a lifelong exposure. In our study, 13% of the control group were double carriers of the apoB -516T and apoE e4 alleles, which indicates that this phenotype may have an effect at a population level.

Production of apoB-containing lipoproteins

The secretion pattern of apoB-containing lipoproteins by the liver has been suggested to be a determinant of the plasma lipoprotein heterogeneity. This relationship is illustrated for example in insulin resistance, where an overproduction of large VLDL by the liver is commonly seen in association with a preponderance of small, dense LDL in plasma. MTP plays a pivotal role in the assembly and secretion of apoB-containing lipoproteins, and the activity state of MTP may thus be a major factor in the regulation of the lipoprotein spectrum produced by the liver.

In study V, the influence of a common polymorphism in the MTP gene promoter (-493G/T) on the metabolism of apoB-containing lipoproteins was investigated. The results of this study show that the MTP -493G/T gene variant modulates the secretion pattern of apoB-containing lipoproteins in the sense that the direct production of the smaller lipoproteins (IDL and LDL) is drastically reduced in -493T carriers. The MTP -493T allele has previously been associated with decreased plasma LDL cholesterol and apoB concentrations, and the findings of this study suggest that this is due to a lower abundance of the large LDL particles.

Few previous studies have examined the role of common genetic variation in apoB production. In a study by Watts et al., apoB turnover was investigated in a cohort of 29 obese subjects, who were genotyped for variants in the apoB, apoE, CETP, HL and MTP genes (Watts et al. 2000). They concluded that variation in the apoB and CETP genes modulated VLDL production. The effect of the MTP -493G/T gene variant was not statistically significant on its own, but in combination with other genetic variants, carriers of the MTP -493T variants tended to have a lower VLDL apoB production rate. However, the production rates of the smaller apoB-containing lipoproteins were not estimated in that study.

The molecular characteristics of the MTP -493G/T polymorphism are not fully known. Previous studies using *in vitro* constructs of the MTP promoter have indicated that the -493T variant enhances transcriptional activity (Karpe et al. 1998). However, the MTP -493G/T is in strong linkage disequilibrium with another variant in the MTP gene promoter (-164T/C), and also with a polymorphism in the coding region of the MTP gene (I128T) (Ledmyr et al. 2002). Thus, the effects observed in association with the -493G/T variant may be due to changes in MTP activity or function which are caused by either of the other two polymorphisms, or a combination. Hence, we can only speculate as to how the MTP -493T variant reduces the production of the smallest apoB-containing lipoproteins. Based on the existing *in vitro* data, it could be hypothesized that high MTP activity more rapidly depletes the lipid substrate for lipoprotein production, which would lead to the intracellular degradation of poorly lipidated lipoproteins (Adeli et al. 1995). We postulate that the smallest lipoproteins would be the most vulnerable in this process.

Very little is known of how variation in apoB production influences plasma LDL heterogeneity, and to date, no turnover study has traced the apoB-containing lipoproteins down to LDL subfractions.

The MTP is a valid candidate for the genetic regulation of apoB secretion pattern, and the results of the present study show that the spectrum of apoB-containing lipoproteins secreted by the human liver is at least partly under genetic influence. However, the impact of genetic variation in MTP on plasma LDL heterogeneity appears to be rather limited. Instead, the interesting link between the apoB production rate of the various subclasses of apoB-containing lipoproteins and LDL heterogeneity is on a physiological level; reduced direct production of IDL and LDL seems to lower the absolute abundance of large LDL. This relationship suggests that a certain proportion of the large LDL particles in plasma are nascent particles that have not been formed from VLDL precursors. Although circumstantial, this is the first evidence of a genetic effect on plasma LDL heterogeneity mediated via changes in the apoB production rate.

In summary, this study shows that the direct production of the smallest apoB-containing lipoproteins (IDL and LDL) appears to be influenced by variation in the MTP gene, and that this has direct consequences for the LDL particle size distribution observed in plasma.

General Discussion

Metabolic vs genetic determinants of LDL particle size

At present, the evidence for a specific metabolic pathway leading to the generation of small, dense LDL is circumstantial, and mainly derived from correlations between LDL particle size and major plasma lipoproteins and plasma factors, such as the plasma concentration or activity of the triglyceride lipases. Stable isotope turnover studies have added valuable information about the inter-relationships between the metabolic fates of different subfractions of apoB-containing lipoproteins. However, to date, no turnover study has traced the VLDL precursors down to LDL subfractions.

There is an overwhelming consistency in the literature describing plasma TG concentration as the major determinant of LDL particle size distribution, accounting for approximately 40-60% of the variance in LDL peak particle size. Kinetic studies indicate that the plasma TG concentration is largely determined by the plasma concentration of VLDL1, which in turn is regulated mainly by its fractional transfer rate to VLDL2 (i.e. lipolysis). LPL is the rate-limiting enzyme for hydrolysis of TG in large TRL, including VLDL, and accordingly, an inefficient lipolysis (e.g. LPL deficiency) is associated with increased plasma TG, low HDL cholesterol and an abundance of small, dense LDL. We have investigated the effects of an increased LPL activity on LDL heterogeneity in an artificial model (study II), and as a consequence of a common genetic variant (study III). Although there is a substantial difference in the enhancement of LPL activity in the two studies, the common message appears to be that efficient lipolysis of plasma TG is beneficial with regard to several potentially atherogenic properties of LDL.

After adjusting for plasma TG concentration, there is still a significant contribution of the plasma HDL cholesterol concentration to variance in LDL particle size (~5%). The link between plasma HDL cholesterol and LDL particle size has been suggested to be CETP, and to some extent our results support this hypothesis. In study III, the CETP -629A allele was shown to be associated with increased plasma HDL cholesterol as well as an increase in the LDL peak particle size. After adjusting for plasma TG, this association was still significant. However, after adjustment for HDL cholesterol there was no longer a significant difference in LDL particle size between the genotype groups, indicating that the effect of the CETP -629C/A polymorphism on LDL particle size was dependent on its effect on plasma HDL cholesterol.

The strong association between plasma TG and small, dense LDL may also constitute a confounding factor in the study of the genetic regulation of LDL particle size distribution (Berneis and Krauss 2002), not least due to the fact that plasma TG levels are under heavy influence of environmental factors (e.g. diet and exercise), and elevations in plasma TG caused by such factors are likely to give rise to phenocopies. Thus, when investigating the effects of genetic variation on LDL particle size distribution, special attention must be given to the covariation in plasma TG concentration.

The role of the plasma TG concentration as a possible mediator of the genetic associations observed in our studies was evaluated by adjusting the measures of LDL particle size distribution for differences in plasma TG to yield TG-adjusted variables. In contrast to the apparently TG-independent effects of the CETP -629C/A polymorphism on LDL particle size (discussed above), the association between the LPL 447X variant and LDL peak particle size was no longer significant after adjustment for the plasma TG concentration. The HL -480C/T, apoB -516C/T, and MTP -493G/T polymorphisms were primarily associated with changes in the plasma concentration of large LDL (LDL-I), which is not significantly correlated with the plasma TG concentration on its own, and consequently, these associations were not affected by TG-adjustment.

In our studies, the most pronounced effect of a single polymorphism on the LDL particle size distribution pattern was observed in association with the apoE e4 allele. After adjustment for plasma TG, the associations between the e4 allele and LDL peak particle size and plasma concentration of LDL-III were still highly statistically signficant. Also, as previously discussed, the effects of the gene-gene interaction between the apoB and apoE polymorphisms on plasma LDL-III appeared to be independent of the plasma TG (VLDL TG) concentration.

In summary, a preponderance of small, dense LDL is often seen in association with, and perhaps as a consequence of, an increase in the plasma TG concentration. However, the results of our genetic studies indicate that other mechanisms contribute to an accumulation of small, dense LDL.

Genetic regulation of LDL particle size and CHD risk

CHD is a common complex disease, and a substantial amount of research has been aimed at identifying disease-causing gene mutations and disease-susceptibility genes. Information about the genetic causes of CHD could be used as a means to better understand CHD pathogenesis, but also ultimately to provide better preventive strategies, diagnostic testing, individually tailored treatment (pharmacogenetics), and for the development of new therapeutic drugs. Linkage and association studies are the two commonly used approaches to identify gene loci or specific alleles with an impact on CHD. Both strategies have their strengths and weaknesses. However, both linkage and association analyses have been criticized with regard to the lack of replication of initial findings (Dahlman et al. 2002; Ioannidis et al. 2003).

A genetic approach may also be used as a tool in the study of the regulation of 'intermediate phenotypes', i.e. biochemical markers or established 'risk factors', implicated in disease development. In this context, the small, dense LDL phenotype is interesting both as a risk factor for CHD on its own, and in the context of its association with other metabolic disorders, such as FCH, insulin resistance, and the metabolic

syndrome. During the last 10-15 years, numerous linkage and association studies have investigated the genetic regulation of LDL particle size heterogeneity. Taken as a whole, the results of these studies indicate that the genetic determination of LDL size is complex. Evidence of linkage between the LDL particle size or the pattern B phenotype and a number of gene loci have been demonstrated in different study cohorts. However, several studies have failed to detect linkage to a variety of candidate genes. In part this may be due to the limited ability of linkage studies to detect the contributions of 'minor' genes (that alone have a small effect on the phenotype). However, the study outcome may also have been influenced by the sensitivity or accuracy by which the phenotype was characterised, and in the case of LDL particle size, by the definition of LDL particle size as a quantitative or qualitative (pattern A/B) trait. Furthermore, in the majority of linkage studies, no measures were taken to detect or control for the joint effects of several loci, including the effects of genetic interaction, or epistasis. In addition, a large number of studies have demonstrated relatively minor contributions of common candidate gene polymorphisms to variance in LDL particle size. However, several examples of conflicting or inconsistent results can be found in the literature.

In light of the numerous previous reports of genetic contribution to LDL particle size heterogeneity, there are two major strengths of the the association studies reported here:

a) The 3-7.5% PA GGE prodecure, which has proven to be accurate and stable, and has enabled the detection of subtle differences in the LDL particle size distribution, which could not have been described in terms of a change in the LDL subclass pattern A/B.

b) The study cohort, which was relatively large and selected to be as homogenous as possible, to limit the influence of environmental factors, such as age, gender, hormonal status and medication, and still be representative of the general population.

Furthermore, the genetic polymorphisms studied here are located in genes encoding proteins with a relevant physiological function in relation to the phenotype, and have all been previously reported to be functional; that is, to increase/ decrease gene transcription, or to alter the protein function, plasma level or activity.

The most interesting finding of our studies of the genetic regulation of LDL particle size is the interactive effect of the apoB and apoE polymorphisms on the plasma concentration of small, dense LDL, which furthermore appears to confer an increased risk for CHD. Although we are cautious to infer biological interaction from statistical genetic interaction, the suggestion of a molecular basis for the interaction between the apoB and apoE polymorphisms is appealing based on the following arguments; a) the apoB and apoE proteins are tightly linked in their respective physiological functions, and b) evidence exists that the function(s) of both these genetic variants differ from those of the 'wild type', or common, variant. However, the possible biological mechanisms for an interaction need to be investigated on a molecular level.

In summary, our studies add to a large number of studies that taken together present a complex picture of the genetic determination of LDL particle size, and although there is constant progress in this research area, a challenge for the future is clearly to extend our knowledge of the genetic regulation of LDL particle size to that of the associated CHD risk.

Conclusions

I. The small, dense LDL particle species could play an important role in causing vascular change, leading to atherosclerosis, and the evaluation of LDL particle size distribution with a high-resolution GGE method may be a valuable approach to estimate the plasma levels of particularly atherogenic LDL species in asymptomatic individuals.

II. The atherogenic LDL particles are most likely produced as the result of complex metabolic pathways which cannot be mimicked *in vitro*. However, our results demonstrate that several properties inherent to the LDL particle, some of which may be determined by prior metabolic events, contribute to the capacity of LDL to withstand oxidative modification.

III. Common variants in candidate genes are associated with moderate to marked changes in the LDL particle size distribution, consistent with the current concepts of the physiological functions of the respective encoded proteins, as well as the proposed effects of the genetic variants on CHD risk.

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