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Role of Promoter C-480T Polymorphism of Hepatic Lipase in the Development of Atherosclerosis

Clinical and Autopsy Studies

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ACADEMIC DISSERTATION
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LIST OF ORIGINAL COMMUNICATIONS

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I Fan YM, Laaksonen R, Janatuinen T, Vesalainen R, Nuutila P, Koivula T, Knuuti J, Lehtimäki T. Hepatic lipase gene variation is related to coronary reactivity in healthy young men. Eur J Clin Invest 2001;31(7):574-580.

II Fan YM, Laaksonen R, Janatuinen T, Vesalainen R, Laine H, Raitakari OT, Nuutila P, Knuuti J, Rontu R, Lehtimäki T. Influence of hepatic lipase C-480T polymorphism on coronary flow reserve in young men is independent of the plasma cholesterol level. Atherosclerosis 2006;188(2):391-397.

III Fan YM, Lehtimäki T, Rontu R, Ilveskoski E, Goebeler S, Kajander O, Mikkelsson J, Viiri EL, Perola M, Karhunen PJ. The hepatic lipase gene C-480T polymorphism in the development of early coronary atherosclerosis: The Helsinki Sudden Death Study. Eur J Clin Invest 2007 (accepted)

IV Fan YM, Salonen JT, Koivu TA, Tuomainen TP, Nyyssönen K, Lakka TA, Salonen R, Seppänen K, Nikkari ST, Tahvanainen E, Lehtimäki T. Hepatic lipase C-480T polymorphism modifies the effect of HDL cholesterol on the risk of acute myocardial infarction in men: a prospective population based study. J Med Genet 2004;41(3):e28.

V Fan YM, Lehtimäki T, Rontu R, Ilveskoski E, Goebeler S, Kajander O, Mikkelsson J, Perola M, Karhunen PJ. Age-dependent association of hepatic lipase gene C-480T polymorphism and the risk of pre-hospital sudden cardiac death: The Helsinki Sudden Death Study. Atherosclerosis 2006 (in press).

VI Fan YM, Dastidar P, Jokela H, Punnonen R, Lehtimäki T. Hepatic lipase C-480T genotype-dependent benefit from long-term hormone replacement therapy for atherosclerosis progression in postmenopausal women. J Clin Endocrinol Metab 2005;90(6):3786-3792.

ABBREVIATIONS

ABCA1	ATP-binding cassette transporter A1
AMI	acute myocardial infarction
AN(CO)VA	analysis of (co)variance
apo	apolipoprotein
ASC	atherosclerosis severity score
BMI	body mass index
CAD	coronary artery disease
CE(s)	cholesteryl ester(s)
CETP	cholesteryl ester transfer protein
CFR	coronary flow reserve
CHD	coronary heart disease
CI	confidence interval
CM(s)	chylomicron(s)
DNA	deoxyribonucleic acid
EV	oestradiol valerate
EVP	oestradiol valerate plus progestin
FH	familial hypercholesterolemia
HDL	high-density lipoprotein
HL	hepatic lipase
HRT	hormone replacement therapy
HSDS	Helsinki Sudden Death Study
IAP	International Atherosclerosis Project
IDL	intermediate-density lipoprotein
IMT	intima media thickness
KIHD	Kuopio Ischemic Heart Disease
LCAT	lecithin-cholesterol acyltransferase
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
LIPC	hepatic lipase gene
LPL	lipoprotein lipase
LRP	LDL receptor-related protein

MBF	myocardial blood flow
MI	myocardial infarction
NAP	number of atherosclerotic plaques
OR	odds ratio
PET	positron emission tomography
PLTP	phospholipid transfer protein
RCT	reverse cholesterol transport
RPP	rate-pressure product
SR-BI	scavenger receptor type B class I
SCD	sudden cardiac death
SD	standard deviation
SMC(s)	smooth muscle cell(s)
TG(s)	triglyceride(s)
VLDL	very-low-density lipoprotein

Abbreviations are defined at first mention in the abstract and in the main body of the text and used only for concepts that occur more than three times.

ABSTRACT

Background Atherosclerosis is the major cause of morbidity and mortality in Western society. Atherosclerosis arises from a genetic predisposition, interacting with environmental risk factors. Hepatic lipase (HL) is a glycoprotein, functioning mainly as a lipolytic enzyme by hydrolysing triglycerides and phospholipids in almost all major classes of lipoproteins. HL may be involved in the modulation of the risk for dyslipidemia and atherosclerosis due to its essential role in both high-density lipoprotein (HDL) and triglyceride-rich lipoprotein metabolism. The HL gene (*LIPC*) has a functional promoter polymorphism at position -480, which affects transcription and leads to CC, CT and TT genotypes.

Objectives To elucidate the association between *LIPC* C-480T genotypes and coronary reactivity, the development of early and advanced atherosclerotic lesions in coronary arteries, the developing risk of acute myocardial infarction (AMI) and sudden cardiac death (SCD). Furthermore, the effect of *LIPC* genotypes on atherosclerosis progression during long-term hormone replacement therapy (HRT) was assessed.

Subjects and Methods The study was based on six study series (studies **I-VI**), comprising a total of 1,282 subjects. The subjects included 49 healthy, mildly hypercholesterolemic young men (study **I**) and 108 (included study **I** subjects) young men with varying lipid statuses (study **II**). The first two studies evaluated the association between the *LIPC* genotypes and the indices of coronary blood flow as measured with positron emission tomography. Study **III** examined the relationship between the *LIPC* genotypes and autopsy-confirmed areas of different types of atherosclerotic lesions in the coronary arteries of 700 middle-aged men. In study **IV**, 386 men who were followed up for an average of nine years were analyzed to investigate the association between the *LIPC* genotypes and the risk of developing AMI. Study **V** elucidated the association between the *LIPC* genotypes and the pre-hospital SCD in 700 male autopsy subjects. In study **VI**, 88 postmenopausal women participated in long-term HRT to determine the effect of *LIPC* genotypes on the progression of atherosclerosis severity.

Results *LIPC* -480T allele carriers had lower coronary flow reserve (CFR) than the CC homozygotes (study **I**). In study **II**, T allele carriers had lower coronary flow during hyperemia, lower CFR and higher coronary resistance during hyperemia than subjects with the CC genotype among young men with different lipid statuses, and the effect was independent of the level of plasma cholesterol. In the autopsy study **III**, men with the TT genotype had two times larger areas of fatty streaks compared to the CC homozygotes. However, this association was only significant in men < 53 years of age. In a prospective population-based nested case-control study (study **IV**), men with the CC genotype and HDL cholesterol (HDL-C) concentration in the lowest or second lowest tertile were at a higher risk of developing AMI than men in the highest HDL-C tertile. A similar effect was not found in men with the T allele. In the autopsy study **V**, TT homozygotes had an increased risk for AMI and SCD when compared to CC carriers. This association was particularly strong among men < 53 years of age, but was non-significant among older men (≥ 53 years). In the observational study **VI** on postmenopausal women during long-term HRT, the progression of atherosclerosis severity in subjects with the T allele was significantly faster in the control group than the HRT group, whereas there were no significant differences in atherosclerosis progression between the control and HRT groups in the CC genotype.

Conclusions We conclude that the *LIPC* C-480T polymorphism is an important genetic marker for atherosclerosis and responds to long-term HRT during atherosclerosis progression.

INTRODUCTION

Atherosclerosis is a disease of large and medium-sized arteries and a major cause of coronary heart disease (CHD), acute coronary syndrome and stroke. In westernized societies, such as Europe, the USA and Japan, it is the underlying cause of roughly 50% of all mortality (Ross 1999; Lusis 2000). The rising prevalence of CHD in developing countries suggests that it will become the main cause of morbidity and mortality worldwide (Reddy and Yusuf 1998). The traditional risk factors for atherosclerosis—hyperlipidemia, cigarette smoking, hypertension and diabetes—only explain half or less of CHD (Lefkowitz and Willerson 2001). Therefore, the genetic components of atherosclerosis that could increase the predictive ability and improve the accuracy of clinical decisions regarding the use of proven therapies have been actively searched for. As a consequence, there is currently convincing evidence of a genetic contribution to CHD (Myers et al. 1990; Marenberg et al. 1994). Estimates of the heritability of CHD—the proportion of variance of the disease that correlates with genetic differences—are mostly assumed to be in the range of 40%–60% (Nora et al. 1980; Koskenvuo et al. 1992; Lusis et al. 2004).

The genetic basis of atherosclerosis has been classically studied by using linkage analyses, allele-sharing methods, animal models and candidate gene association studies. In the present study, hepatic lipase gene (*LIPC*) was selected as a candidate gene that may be involved in modulating the risk for dyslipidemia and atherosclerosis because of the essential role of hepatic lipase (HL) in both high-density lipoprotein (HDL) and triglyceride-rich lipoprotein metabolism (Jansen et al. 2002). HL functions mainly as a lipolytic enzyme hydrolyzing triglycerides and phospholipids in almost all major classes of lipoproteins. It also plays a role as a ligand facilitating the binding and uptake of lipoproteins via proteoglycans and/or receptor pathways (Santamarina-Fojo et al. 1998). HL expression was also detected in macrophages (Gonzalez-Navarro et al. 2002)—revealing an another process through which HL may modulate atherogenic risk.

The *LIPC* has a functional promoter polymorphism at position -480 or -514, depending on which of the nucleotides is taken as the transcription start site (Cai et al. 1989; Ameis et al. 1990), leading to genotypes CC, CT and TT. Furthermore, the observations that patients with familial HL deficiency and absent HL activity have definite premature coronary artery disease (CAD) (Brunzell and Deeb 2001) and that high HDL levels protect against the development of atherosclerosis make *LIPC* an interesting candidate gene.

The *LIPC* genotypes modulate HL activity, but their association with early indicators of atherosclerosis—for example, coronary reactivity measured by positron emission tomography (PET) or autopsy-verified atherosclerosis lesion area, risk of acute myocardial infarction (AMI) and sudden cardiac death (SCD)—is not known. Moreover, the effect of *LIPC* genotypes on atherosclerosis progression during long-term hormone replacement therapy (HRT) has not been investigated previously.

The present thesis was based on the results from six different study series which all represent different stages of atherosclerotic disease. Two of these, both clinical series, utilized PET to examine the association between *LIPC* genotypes and coronary function and reactivity. In the third clinical series, the relationship between *LIPC* genotypes and the risk of developing AMI was examined in a population-based, nested case-control study as a part of the ongoing Kuopio Ischemic Heart Disease Risk Factor (KIHD) follow-up study. In the fourth clinical series, the relationship between the *LIPC* genotypes and progression of atherosclerosis severity in postmenopausal women during long-term HRT was studied. Furthermore, the association between the *LIPC* genotypes and the areas of early and advanced atherosclerotic lesions in coronary arteries, occurrence of AMI and pre-hospital SCD was examined in two autopsy series comprising a total of 700 subjects from the Helsinki Sudden Death study (HSDS).

REVIEW OF THE LITERATURE

1 Atherosclerosis

Atherosclerosis is a disease of large and medium-sized arteries, and it is characterized by the accumulation of lipids and fibrous elements, leading to a pathological thickening of the inner portion of the artery wall and to arterial luminal obstruction. Most commonly affected are the coronary arteries, aorta, iliac, femoral, and cerebral arteries (Ross and Glomset 1973). The atherosclerotic process begins early in life (Enos et al. 1986; Newman et al. 1986; McGill and McMahan 1998; Raitakari et al. 2003). The development of atherosclerosis includes a prolonged 'silent' period until the principal clinical complications of atherosclerosis, such as myocardial infarction (MI) and stroke, occur in middle-aged or older people (Malcom et al. 1997). The normal structure of arteries and classification of atherosclerotic lesions are briefly reviewed in the following sections.

1.1 Normal structure of arteries

Intima. The intima is the innermost layer of arteries. The lumen of the artery and the intima are separated by a monolayer of endothelial cells. The intima consists of two microscopic layers: the inner layer, subjacent to the lumen, is called the proteoglycan layer which contains an abundance of nonfibrous connective tissue identified as proteoglycan ground substance. In this layer, there are also some synthesizing-type smooth muscle cells (SMCs) and isolated macrophages. The outer layer underlying the proteoglycan layer (and adjacent to the media) is called the musculoelastic layer, which contains an abundance of SMCs, elastic fibres and more collagen than the upper layer. The internal elastic lamina separates the intima from the media (Stary et al. 1992).

Media. The media is the middle layer of the arterial wall, which is principally composed of SMCs of both synthesizing and contractile phenotype as well as an extracellular matrix consisting of elastic fibres and collagens. The muscle can contract and relax to control the blood pressure and flow in the artery. Elastic tissue and collagen increase the elasticity and strength of the wall of the artery, as the artery contracts and relaxes (Ross and Glomset 1976a). The external elastic lamina separates the media from the adventitia.

Adventitia. The adventitia, the outermost layer of arteries, is primarily composed of loose connective tissue made up of fibroblasts intermixed with SMCs, mast cells and associated collagen fibres, in addition to proteoglycans (Ross and Glomset 1976a). It also contains small blood vessels (vasa vasorum) supplying blood to the outer 2/3 of the media, lymphatic vessels and some nerves entering the media and regulating the arterial tone.

1.2 Classification of atherosclerotic lesions

In the 1990s, atherosclerotic lesions were classified by the American Heart Association Committee on Vascular Lesions of the Council on Atherosclerosis (Stary et al. 1992; Stary et al. 1994; Stary et al. 1995). The reports provided the definition of arterial intima ranges from adaptive intimal thickening present in these lesion-prone regions to type VI lesions in advanced atherosclerotic disease (Stary et al. 1992; Stary et al. 1994; Stary et al. 1995). Previously, the International Atherosclerosis Project (IAP) had examined the degree of atherosclerosis in the first large-scale autopsy survey on atherosclerosis using a standardized evaluation method in the 1960s (Guzman et al. 1968). In this previous classification, atherosclerotic lesions were stained with Sudan IV and graded visually as fatty streaks, fibrous plaques and complicated lesions. This method was used in the HSDS (study III). Table 1 demonstrates these two atherosclerosis classification methods.

In 2000, Virmani and colleagues (Virmani et al. 2000) reconsidered the current paradigm of morphological classification of lesions (Stary et al. 1994; Stary et al. 1995), in their studies of sudden death. They suggested terms for lesions that they found more simplified. However, it is still need to be tested whether this classification is useful.

Table 1. Classification of atherosclerotic lesions

Histological classification (Stary et al. 1994; Stary et al. 1995)		Macroscopic classification by the International Atherosclerosis Project (IAP) (Guzman et al. 1968)
Intimal thickening		Usually not visible or may be mistaken as a raised lesion
Early lesions		
Type I	Initial lesion	Usually not visible, fatty dots
Type IIa	Progression-prone type II	Fatty streak, fatty dots
Type IIb	Progression-resistant type II	
Intermediate lesions		
Type III	Preatheroma	Fatty streak or fibrous plaque
Advanced lesions		
Type IV	Atheroma	Fibrous plaque
Type Va	Fibroatheroma (type V lesion)	Fibrous plaque
Type Vb	Calcific lesion (type VII lesion)	Calcified plaque
Type Vc	Fibrotic lesion (type VIII lesion)	Fibrous plaque
Type VI	Lesion with surface defect, haematoma/haemorrhage or thrombosis	Complicated lesion

Type I (initial) lesion. Type I lesions are generally found in infants and children, although they can also be found in adults. Type I lesions are usually invisible to the naked eye, or only seen as small yellow dots on the intimal surface. This lesion comprises small, isolated groups of macrophages containing lipid droplets (macrophage-foam cells). In coronary arteries these cells preferentially accumulate in the regions of the intima that have an adaptive intimal thickening of the eccentric type (Stary et al. 1994).

Type II lesion. Type II lesions may be visible as yellow-coloured streaks, patches or spots on the intimal surface of arteries and stain red with Sudan III or Sudan IV. They consist primarily of macrophage-foam cells stratified in adjacent layers, rather than being dispersed in isolated groups of a few cells as seen in type I lesions. Besides macrophages, SMCs now also contain lipid droplets. Type II lesions are subdivided into a ‘progression-prone’ (type IIa) and

a ‘progression-resistant’ (type IIb) subgroup. Type IIa lesions localize at atherosclerosis-prone locations and therefore more often proceed to more advanced lesions, whereas type IIb lesions either do not progress or progress slowly. Type IIa lesions usually show adaptive intimal thickening with accumulation of more SMCs, greater accumulation of lipids and macrophages, and the deep intimal location of the foam cells and extracellular lipid droplets and particles (Stary et al. 1994).

Type III lesion (preatheroma). The type III lesion is also known as the intermediate lesion, the transitional lesion and as preatheroma. This type represents the bridge between early and advanced lesion. This lesion is characterized with an increase in extracellular lipid with progression from droplets to pools among the layers of SMCs of the intimal thickening. The lipid pools replace the intercellular matrix and by pushing SMCs apart disrupt their structural coherence. However, proliferation of SMCs or a massive accumulation of extracellular lipid has not yet developed (Stary et al. 1994).

Type IV lesion (atheroma). The type IV lesion, the first of the advanced lesions, is also known as an atheroma. It is characterized by the presence of accumulated extracellular lipids arising from the confluence of smaller isolated lipid pools, seen in type III lesions. This type of extracellular lipid accumulation is known as the lipid core. The relative thinness of the tissue between the lipid core and the endothelial surface, with a paucity of collagen fibres and the presence of macrophages, explains why type IV lesions may sometimes be susceptible to rupture, giving rise to an immediate progression to a type VI lesion. Extracellular particles and droplets have damaged and disorganized the intima by displacing structural SMCs, and SMCs have changed their morphology. At this stage, the arterial lumen is not much reduced and changes may not be visible by angiography (Stary et al. 1995).

Type V lesion. A type V lesion differs from a type IV plaque in that prominent new fibrous connective tissue is formed. A type Va lesion is present if new fibrous tissue formation is found in a lesion with a lipid core, with thickening of the tissue between the lipid core and the endothelium. This area is named the ‘fibrous cap’, and the whole lesion is often referred to as ‘fibroatheroma’. A calcified type V lesion is called type Vb or type VII lesion in which the lipid core and other parts of the lesion are calcified and in which mineralization is the dominant feature. A type Vc or type VIII lesion (fibrotic lesion, lipid poor type V lesion) is a lesion entirely or almost entirely of dense collagen and in which lipid component or core is minimal or absent, in addition to being dominated by fibrous tissue accumulation. This latter form may result from resorption of a lipid core, organization of thrombi or an extensive reparative response of the arterial wall (Stary et al. 1994).

Type VI lesion (complicated lesion). Morbidity and mortality from atherosclerosis is largely due to type IV and type V lesions in which disruptions of the lesion surface, haematoma or haemorrhage and thrombotic deposits have developed. Type VI lesions include plaques with the morphologic appearances of type IV or V lesions complicated either by disruption of the surface (type VIa), haemorrhage or haematoma (type VIb), or thrombosis (type VIc) (Stary et al. 1995).

1.3 Development of atherosclerosis

In the following sections, the most important stages in the development of early and advanced atherosclerotic lesions as well as major theories on the development of the condition are reviewed briefly.

1.3.1 Development of early atherosclerotic lesions

Endothelial function in normal and pathological conditions. In the arterial system, the endothelial cells form a continuous, smooth, uninterrupted surface and represent the principal barrier between the elements of the blood and the artery wall. As the major regulator of vascular homeostasis, the normal endothelium maintains the balance between vasodilation and vasoconstriction, inhibition and stimulation of SMC proliferation and migration, and thrombogenesis and fibrinolysis (Bonetti et al. 2003; Davignon and Ganz 2004).

Factors such as dyslipidemia, hypertension, cigarette smoking or various other conditions (risk factors, mechanical, tissue hypoxia), or their combinations, may cause an initial injury (dysfunction) to the arterial endothelium. Endothelial dysfunction has been described in high-risk subjects with no morphological atherosclerotic changes, suggesting that it is an important early event in atherosclerosis (Vita et al. 1990; Reddy et al. 1994).

It is recognized that endothelial dysfunction is a major factor contributing to the atherogenic process (Ross 1999). The individual burden of currently known risk factors can disrupt normal endothelial integrity and function and initiate the formation of the first visible signs of atherosclerosis, the appearance of small lipid droplets in the subendothelial proteoglycan-rich layer of the intima, thus leading to lesions known as fatty streaks (Ross 1999; Bonetti et al. 2003).

Low-density lipoprotein (LDL) retention. Endothelial dysfunction may allow the accumulation of lipoproteins (particles under 75 nm) in the subendothelial space of the intima.

The concentrations of LDL in the intima may grow because of increased permeability of endothelial cells and also due to the elevated plasma LDL levels (Steinberg and Witztum 1990). The retention of LDL in the vessel wall is mediated by the binding of apolipoprotein (apo) B-100 of LDL molecules to matrix proteoglycans (Boren et al. 1998).

LDL modification and formation of fatty streaks. Native LDL is taken up mainly by the LDL receptor (LDLR) pathway, which is strictly regulated by the flowing cholesterol and does therefore not lead to intracellular accumulation of cholesterol (Brown and Goldstein 1986). In the intima, different types of modifications of LDL, for example, oxidative, phospholipolytic or proteolytic, can occur (Pentikainen et al. 2000). LDL which has already been minimally modified can increase the expression of endothelial cell adhesion molecules and recruit monocytes into the vessel wall, where these cells are converted to macrophages that engulf the differentially modified LDL molecules through different types of scavenger receptors (Luoma et al. 1994; Hiltunen and Ylä-Herttuala 1998). This results in the formation of the characteristic 'foam cell' which contains massive amounts of cholesteryl esters (CEs) and which is a hallmark of the atherosclerotic plaque. The aggregation of foam cells and leukocytes within the intima produces a 'fatty streak', an early histopathological change indicating atherosclerosis. The age at which fatty streaks appear differs in the various regions of the arterial tree, but they are present in the aortas of virtually every child, regardless of race, sex or environment, by the age of 10 years. It causes little to no obstruction and no clinical symptoms (Ross and Glomset 1976a).

1.3.2 Development of advanced atherosclerotic lesions and thrombosis

The transition from the relatively simple fatty streak to the more advanced, complex fibrotic lesion is characterized by the accumulation of SMCs, lipid-laden macrophages and an SMC-derived extracellular matrix. Intimal SMCs may proliferate and take up modified lipoproteins, contributing to foam cell formation, and synthesize extracellular matrix proteins that lead to the development of the fibrous cap (Ross 1999). As atherosclerosis progresses, foam cells accumulate and the intima thickens. Subsequent foam cell necrosis due to the influence of cytotoxic oxidatively modified LDL and increased collagen synthesis by intimal SMCs leads to the established atherosclerotic lesion referred to as the fibrous plaque. The fibrous plaque compromises the lumen diameter and may impede blood flow and ultimately participate in the mechanisms that lead to the occlusion of the arteries involved.

Over time and with continued irritation, the advanced fibrous plaque progresses into a more complicated type of lesion. Although advanced atherosclerotic lesions can lead to ischemic symptoms as a result of progressive narrowing of the vessel lumen, a considerable portion of acute ischemic events are generally caused by plaque complications, such as plaque ulceration, disruption, haemorrhage and, finally, thrombosis (Fuster et al. 1992). Pathological studies suggest that plaque instability and the development of thrombus-mediated acute coronary events depend principally on the composition and vulnerability of a plaque rather than on the severity of stenosis (Libby 2001). Vulnerable plaques are defined as thrombosis-prone or at risk of rapid progression and generally have a large, necrotic lipid core, thin fibrous cap and increased numbers of macrophages, mast cells and T lymphocytes in the shoulder regions where plaque rupture clinically often occurs. By their production of metalloproteinases and other proteolytic enzymes, macrophages facilitate the degradation of the fibrous cap, making the plaque vulnerable to rupture (Galis et al. 1994).

1.3.3 Main hypotheses for the development of atherosclerosis

There are several older and new theories and hypotheses to explain the development of atherosclerosis. Although these hypotheses are not mutually exclusive, no 'universal' theory explains every observation and predicts how we can defeat atherosclerosis.

The thrombogenic hypothesis. The thrombogenic hypothesis originated from Carl von Rokitansky in the 19th century and became widely known through the work of Duguid in 1949 (Duguid 1949). This hypothesis suggests that atherosclerosis begins in the intima with a deposition of thrombus, leading to the organization of plaques by the infiltration of fibroblasts and secondary lipid deposition. The raised arterial plaques are the end result of the organization and endothelialization of mural thrombi. The main objection to this hypothesis has been the lipid accumulation, which is found in many of the naturally occurring plaques.

The monoclonal hypothesis. Benditt and Benditt proposed in 1973 that all SMCs in the lesion were of monoclonal origin (Benditt and Benditt 1973). They hypothesized that the mechanism behind the monoclonal proliferation of SMCs is a chemical mutagen or a virus. Advocates of the monoclonal hypothesis suggest that atherosclerosis is derived from a mono- or oligoclonal proliferation of SMCs, but data supporting this concept have become sparse in recent years.

The lipid infiltration hypothesis. This hypothesis emerged in 1914 when Anitschkow showed that a diet rich in cholesterol caused atherosclerosis in experimental rabbits. The validity of the lipid hypothesis was established in the landmark research entitled the Seven Countries Study, in which a 25-year follow-up found that across cultures, cholesterol is linearly related to CHD mortality and the relative increase in CHD mortality with a given cholesterol increase is the same (Keys 1980). However, this hypothesis does not explain why lipids are taken up by the macrophages and SMCs and accumulated in the intima.

The response-to-injury hypothesis. Based upon a large body of earlier data, Ross formulated the response-to-injury hypothesis, which has gone through continuous modification (Ross and Glomset 1973, 1976a, 1976b; Ross 1986, 1993). The response-to-injury hypothesis states that the initial event in the pathogenesis of atherosclerosis is injury to the endothelium. This causes the endothelium to be more susceptible to lipid accumulation and thrombus deposition. More recently, it has become clear that endothelial desquamation is not common and that an intact endothelial cell layer covers developing atherosclerotic lesions. These facts, among others, promoted the refinement of the initial hypothesis to infer that endothelial dysfunction is sufficient to initiate atherosclerosis through increased endothelial permeability to atherogenic lipoproteins (Ross 1999).

The oxidative modification hypothesis. Steinberg et al. (Steinberg et al. 1989) proposed the oxidative modification hypothesis in 1989. This hypothesis states that LDL oxidation is an early event in atherosclerosis and that oxidized LDL is readily internalized by macrophages through a so-called ‘scavenger receptor’ pathway. Oxidized LDL has a number of potentially pro-atherogenic activities by stimulating monocyte chemotaxis, preventing monocyte egress and supporting foam cell formation. Oxidized LDL also results in endothelium dysfunction and injury as well as necrotic foam cells. The role of oxidation in humans remains controversial, as several clinical trials have failed to support the ‘oxidation hypothesis’.

The response-to-retention hypothesis. This hypothesis was originally proposed by Williams and Tabas in 1995 (Williams and Tabas 1995). It states that the key event in early atherogenesis is the retention of atherogenic apo B-rich lipoproteins, mainly LDL, under the endothelium, and that the retention of these lipoproteins is both necessary and sufficient to provoke the lesion formation in artery wall (Williams and Tabas 1998).

The inflammation hypothesis. This hypothesis is actually one of the oldest etiologic theories of atherosclerosis (Nieto 1998). It is only during the past 15–20 years that attention has been given to the fact that inflammatory processes occur in the course of atherogenesis.

This belated insight is astonishing in view of many earlier reports, some dating back more than a century, which had clearly described inflammatory processes in the walls of afflicted arteries. In 1999, the role of inflammation was brought into a hypothesis by Ross (Ross 1999). In this theory, all atherosclerotic lesions represent a state of chronic inflammatory process of the intima. Dyslipidemia, hypertension, infection or various other conditions cause an initial injury to the arterial endothelium and are likely to modulate inflammation. Dysfunction of the endothelium leads to an increase in its adhesiveness and permeability, producing a number of pro-inflammatory molecules, including adhesion molecules and growth factors. As the process of injury continues, the endothelium also produces cytokines and growth factors leading to continuous inflammation, provoking more macrophages, lymphocytes and protease such as matrix metalloproteinases (Ross 1999).

2 Overview on general lipoprotein metabolism

2.1 Lipids and lipoproteins

The major lipids in the body are cholesterol (both esterified and unesterified), triglycerides (TG) and phospholipids. They participate in a wide variety of functions in living organisms. Cholesterol serves as a structural component of cell membranes and as a precursor in the synthesis of many bioactive molecules, including oxysterols, steroids, vitamin D and bile acids. TGs represent the major source of energy storage and energy use. Phospholipids are the principal architectural components of cellular membranes (Ginsberg 1998). Owing to their hydrophobic nature, lipids are not readily soluble in plasma. To facilitate their transport, lipids are packaged into particles called lipoproteins.

Lipoproteins are complex structures containing a core of hydrophobic molecules such as CE and TG surrounded by a layer of phospholipids, unesterified (free) cholesterol and amphipathic proteins called apolipoproteins. Plasma lipoproteins are heterogeneous particles, which differ in their size, density, lipid and apolipoprotein composition, metabolic function and site of origin. They are traditionally named and separated by their hydrated density at which lipoproteins float during ultracentrifugation. The major classes are chylomicrons (CM), very-low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), LDL and HDL. CMs are rich in TG and are the least dense particles ($d<0.95$g/mL). The VLDLs are also TG rich and range between 0.95–1.006 g/mL in density. IDLs have densities ranging from 1.006 g/mL to 1.019 g/mL, and their core consists of a mixture of TGs and CEs. LDLs

are more CE rich and have densities between 1.019–1.063 g/mL. HDLs, also CE rich particles, range between 1.063 and 1.210 g/mL in density. HDL can be further separated into two main subfractions: HDL_2 with densities between 1.063 and 1.125 g/mL, and HDL_3 with densities in the range of 1.125–1.210 g/mL (Ginsberg 1998). Approximately 5%–10% of HDL consists of discoidal preβ-HDL particles. Additionally, lipoproteins can be classified on the basis of particle size, electrophoretic mobility or apolipoprotein content.

2.2 Plasma apolipoproteins

Apolipoproteins (Table 2) are required for the assembly and structure of lipoproteins. Apolipoproteins also serve to activate enzymes important in lipoprotein metabolism and to mediate the binding of lipoproteins to cell-surface receptors. Apo A-I and A-II are the structural components of anti-atherogenic HDL particles. Apo B is the major structural protein of CM, VLDL, IDL and LDL; one molecule of apo B, either apo B-48 (CM) or apo B-100 (VLDL, IDL, or LDL), is present in every lipoprotein particle. Apo E is present in CM, VLDL, IDL and HDL, and it plays a critical role in the metabolism and clearance of TG-rich particles. Apo C-II participates in the activation of lipoprotein lipase (LPL) and is therefore an important regulator of TG-rich lipoprotein lipolysis (Patsch and Gotto 1996).

Table 2. Main apolipoproteins associated with lipoproteins.

Apolipoprotein	Lipoprotein association	Function
Apo A-I	CM, HDL	Structural protein of HDL, activates LCAT
Apo A-II	CM, HDL	Structural protein of HDL
Apo B-48	CM	Structural protein of CM
Apo B-100	VLDL, IDL, LDL	Principal protein in LDL, ligand for the LDLR
Apo C-I	CM, VLDL, HDL	May also activate LCAT
Apo C-II	CM, VLDL, HDL	Activates LPL
Apo C-III	CM, VLDL, HDL	Inhibits LPL
Apo E	CM, VLDL, IDL, HDL	Ligand for binding to LDLR and LRP

CM, chylomicron; LCAT, lecithin-cholesterol acyltransferase; HDL, high-density lipoprotein; VLDL, very-low-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; LPL, lipoprotein lipase; LDLR, LDL receptor; LRP, LDL receptor-related protein.

2.3 Lipoprotein metabolism and lipid risk factors for atherosclerosis

The lipoprotein metabolism is generally divided into the exogenous pathway and endogenous pathway, which are schematically illustrated in Figure 1 and reverse cholesterol transport (RCT) (Figure 2).

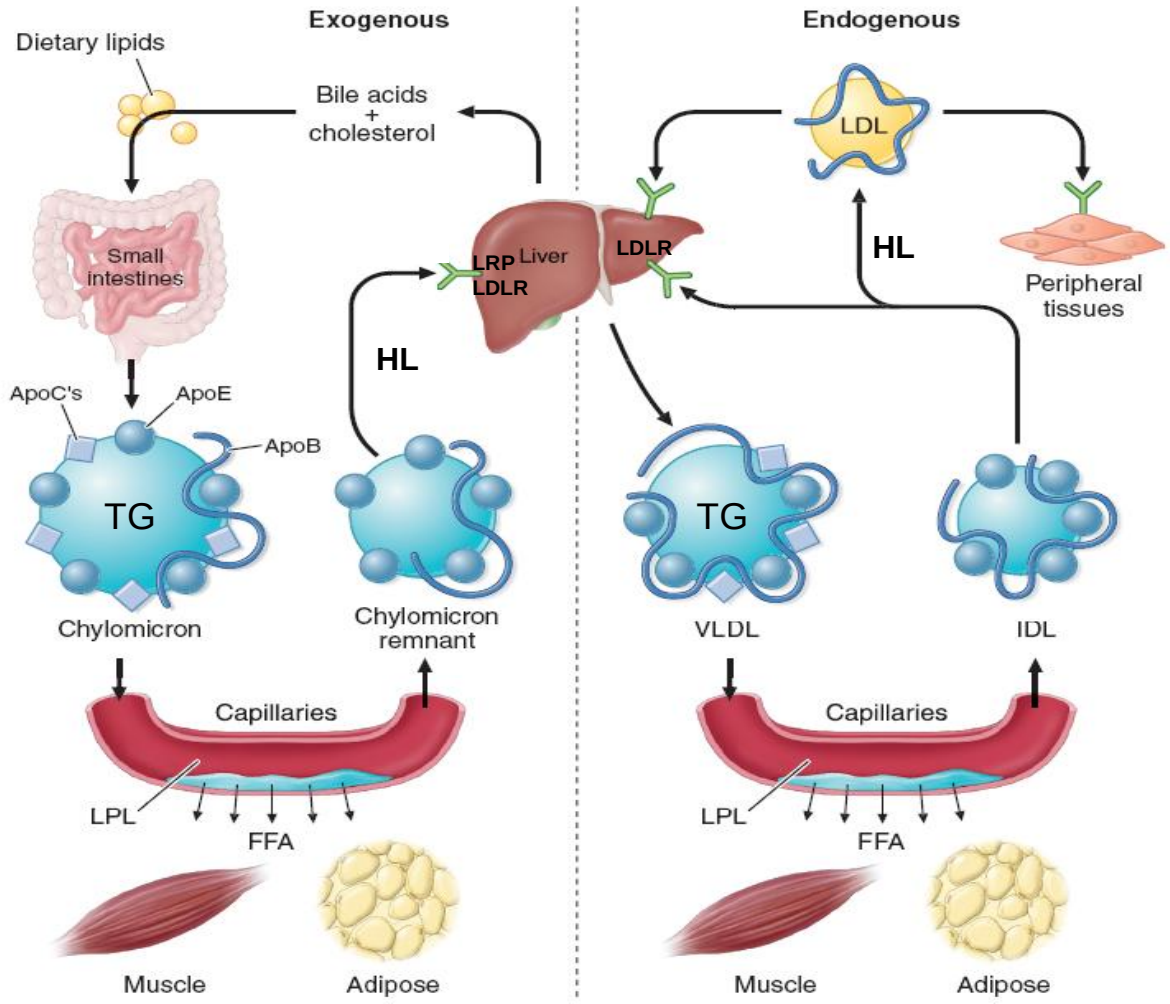

Figure 1. Exogenous and endogenous lipoprotein metabolic pathways. The exogenous pathway transports dietary lipids to the peripheral tissue and the liver. The endogenous pathway transports hepatic lipids to the periphery. FFA, free fatty acids; HL, hepatic lipase; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; LDLR, low-density lipoprotein receptor; LPL, lipoprotein lipase; LRP, low-density lipoprotein receptor protein; TG, triglycerides; VLDL, very-low-density lipoproteins (Modified from Rader and Hobbs 2006).

2.3.1 Exogenous pathway

The exogenous pathway begins with the synthesis of CMs by the intestine, following the absorption of fat from a meal. CMs are the largest lipoproteins (75–1200 nm in diameter). Their content is made up almost exclusively of TGs, which represent approximately 90% of the total mass of this particle. In contrast, proteins account for only 2% of the total mass of CMs. In the intestine, absorbed and re-esterified TG, CE and phospholipids are packed into apo B-48-containing CMs and secreted via the lymph to the circulation. In plasma, CMs first acquire apo C-II from other circulating lipoproteins, mostly from HDL. Thereafter, the TG core of CMs is rapidly hydrolyzed by apo C-II-activated LPL to free fatty acids and glycerol. This process results in the formation of smaller, cholesterol-enriched CM remnants, which are removed from the plasma mainly by the liver through the LDLR (Choi et al. 1991) and LDLR-related protein (LRP) (Rohlmann et al. 1998). Inhibition of HL activity has been demonstrated to lead to an impairment of CM remnant uptake by the liver (Shafi et al. 1994). HL may also be a co-ligand for the binding of remnants and cell surface receptors and/or heparan sulfate proteoglycans (Krapp et al. 1996; Amar et al. 1998). Under normal conditions, most of the absorbed TG carried by the CMs is used after their hydrolysis in the extrahepatic tissues, whereas nearly all cholesterol is delivered to the liver. This exogenous pathway insures that alimentary lipids are delivered to the appropriate organs.

2.3.2 Endogenous pathway

The endogenous pathway begins with the assembly and secretion of apo B-100-containing VLDL particles by the liver. TG accounts for nearly 60% and CE approximately 20% of the total mass of VLDL molecules. Proteins constitute approximately 10% of the total mass, with apo B-100 being the major apolipoprotein of the particle. VLDL TGs are hydrolyzed in peripheral tissues by LPL, and the particles are converted to smaller TG-depleted remnant particles. As VLDL remnants undergo further hydrolysis, they continue to shrink in size and become IDL. The liver removes ~40 to 60% of VLDL remnants and IDL by LDLR-mediated endocytosis via binding to apo E. The remainder of IDL is remodelled by HL to form LDL. LDLs are highly enriched in CE, which constitute about 40% of the total mass of the particles. The protein content of the particle is represented by a single apo B-100 molecule, which constitutes approximately 20% of the total mass. In normal individuals, approximately 60% to 80% of LDL can be cleared by LDLR in the liver. The remainder can be cleared via other

specific receptors, such as LRP and scavenger receptors as well as non-receptor pathways. The latter may include fluid phase endocytosis, some of which may be facilitated by the binding of lipoproteins to cell surface proteoglycans (Ginsberg 1998).

2.3.3 Reverse cholesterol transport (RCT)

The removal of excess cholesterol from the arterial wall and other peripheral tissues and its transportation to the liver for recycling or degradation is called the RCT process (Figure 2) (Fielding and Fielding 1995; Tall 1998). This function is performed by HDL particles, whose metabolism is intimately connected with both the exogenous and endogenous lipid transport pathways. The HDL particles are derived from nascent HDL secreted by the liver and small intestine as discoidal, lipid-poor apo A-I containing particles. CMs and VLDLs undergoing lipolysis may also participate in the production of these HDL precursors by releasing small vesicles or remnants with HDL properties (Musliner et al. 1991). During lipolysis, phospholipid transfer protein (PLTP) transports phospholipids to HDL, thus facilitating the formation of preβ-HDL particles as well as stabilizing HDL levels (Jiang et al. 1996; Huuskonen et al. 2001). These lipid-poor apo A-I and preβ-HDL particles acquire additional cholesterol and phospholipids from cells in the extrahepatic tissues via ATP binding cassette A1 (ABCA1)-mediated efflux, progressively generating HDL particles that are more cholesterol enriched (Lewis and Rader 2005). The enzyme lecithin-cholesterol acyltransferase (LCAT), associated with HDL particles, esterifies the free cholesterol molecules to form CEs which, as hydrophobic molecules, transfer into the core of the particle to form mature α-migrating HDL particles. They are the main mediators of the RCT system whereby cholesterol synthesized or deposited in peripheral cells is returned to the liver. Spherical HDL particles also mediate cholesterol efflux from arterial wall via the ABCG1 pathway (Wang et al. 2004). Part of the α-HDL core CE is readily exchanged with TG from apo B-48 or apo B-100-containing lipoproteins by the cholesteryl ester transfer protein (CETP), after which these transferred CEs can either be removed from the circulation by the liver or redistributed to peripheral cells. CE that remains in the α-HDL particle can be taken up selectively by the liver via scavenger receptor BI (SR-BI) (Trigatti et al. 2003). Through the action of CETP, HDL particles become TG-enriched. Conversely, VLDL and LDL particles become CE-enriched, which facilitates the catabolism of these lipoproteins by their respective receptors (LDLR and LRP) and the return of cholesterol to the liver. PLTP transfers phospholipids from TG-rich lipoproteins into HDL and mediates a variety of conversions

among HDL forms (Huuskonen et al. 2001). At the same time, TGs transferred from TG-rich lipoproteins to HDL are hydrolyzed by HL, leading to the formation of smaller and denser particles and a release of free apo A-I and lipid-poor HDL to be reused in the RCT cycle or, on the other hand, catabolized via kidney function (Moestrup and Kozyraki 2000). HDL particles can therefore be considered to serve in plasma as a reservoir of lipids and apolipoproteins for apo B-100 and apo B-48-containing lipoproteins (Chappell and Medh 1998).

Furthermore, although the efflux of cholesterol from macrophages represents only a tiny fraction (~15%) of overall cellular cholesterol efflux, it is the most important with regard to atherosclerosis, suggesting that it is specifically termed macrophage RCT (Lewis and Rader 2005). From the macrophages, cholesterol can be picked up by diffusion, or via specific ABCA1, ABCG1, SR-BI and apo E-mediated mechanisms (Linsel-Nitschke and Tall 2005; Rader 2006).

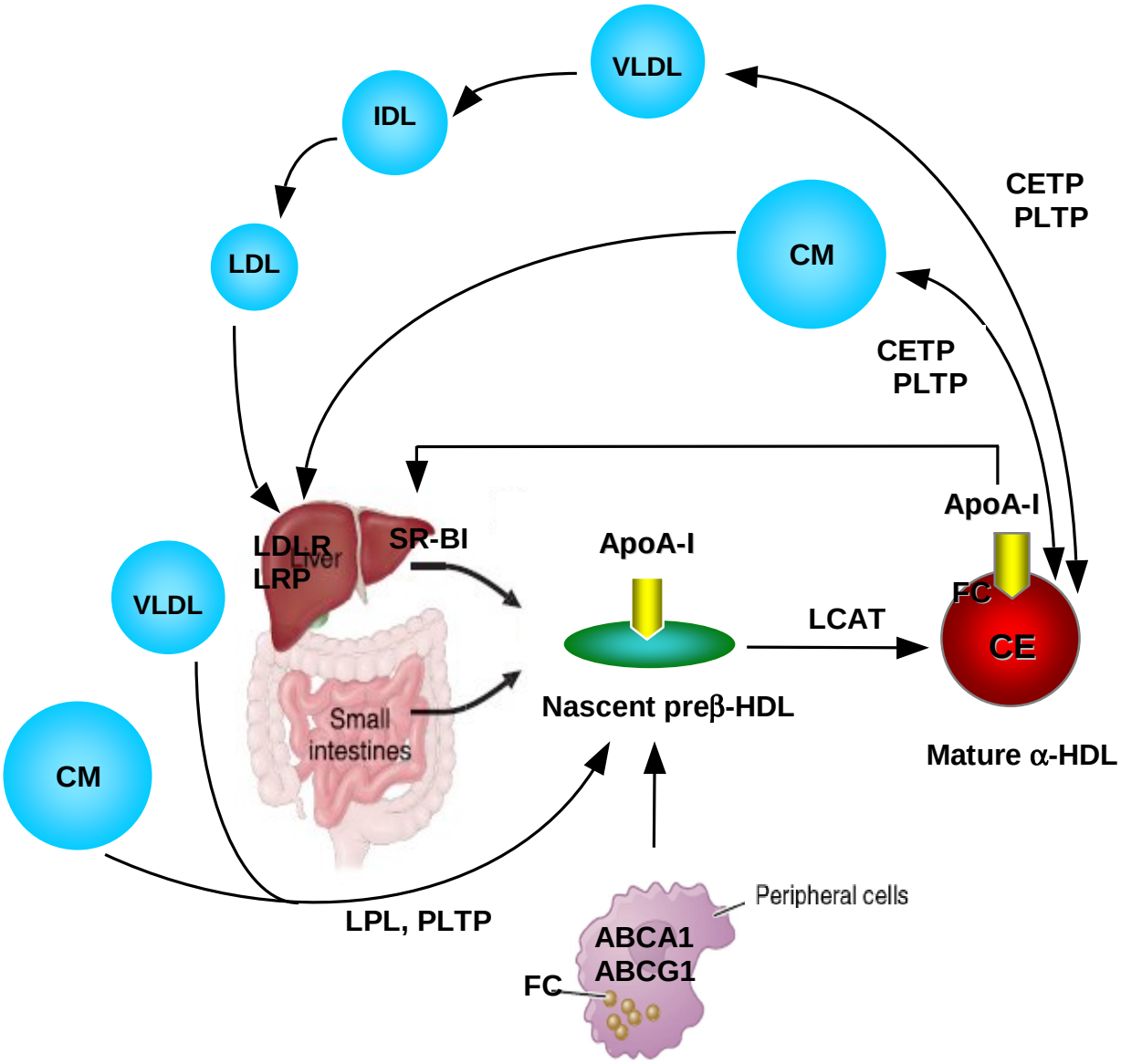

Figure 2. Reverse cholesterol transport. This pathway transports excess cholesterol from the peripheral tissue back to the liver. The liver, the intestine as well as CM/VLDL undergoing lipolysis produce nascent HDL. FC and phospholipids are acquired from peripheral tissues via transport by membrane protein ABCA1 and esterified by LCAT, forming mature HDL. HDL CEs can be selectively taken up by the liver via SR-BI. Alternatively, PLTP promotes the transfer of phospholipids from post-lipolytic CM and VLDL into HDL. CETP facilitates the exchange of CEs from HDL for TG in CM and VLDL. These CEs can then be taken up by the liver. ABC, ATP-binding cassette transporter; apo A-I, apolipoprotein A-I; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; CM, chylomicron; FC, free cholesterol; HDL, high-density lipoprotein; IDL, intermediate-density lipoprotein; LCAT, lecithin-cholesterol acyltransferase; LDL, low-density lipoprotein; LDLR, low-density lipoprotein receptor; LPL, lipoprotein lipase; LRP, low-density lipoprotein receptor related protein; PLTP, phospholipid transfer protein; SR-BI, scavenger receptor BI; VLDL, very-low-density lipoprotein.

2.3.4 Lipid risk factors for atherosclerosis and selection of hepatic lipase gene

Dyslipidemia is one of the most prominent risk factors for atherosclerosis, a finding recognized for nearly a century (Hoeg 1998). Dyslipidemia may be manifested by an elevation of total cholesterol, LDL cholesterol (LDL-C) and TG concentrations, in addition to a decrease in HDL cholesterol (HDL-C) concentrations or combinations of these alterations in the plasma. Dyslipidemia is much more commonly found in patients with CAD than in unaffected individuals (Genest et al. 1992b; Lamarche et al. 1995).

Total cholesterol. Increased plasma total cholesterol levels have been associated with CAD in many studies. Each 0.026 mmol/L (1 mg/dL) increase in total cholesterol has been associated with an approximately 1% increase in risk of MI (Stampfer et al. 1991).

LDL-C and small, dense LDL. The role of LDL in atherosclerosis is well established. This has been dramatically illustrated in individuals with familial hypercholesterolemia (FH) through the pioneer work of Brown and Goldstein (Brown and Goldstein 1986) and others (Russell et al. 1989). Epidemiological studies have consistently shown increased LDL-C to be associated with CAD (Genest et al. 1992a; Sorlie et al. 1999). It has been estimated that the majority of men with CAD have increased LDL-C (Rubins et al. 1992; Rubins et al. 1995). Furthermore, treatment which lowers LDL-C, statin therapy in particular, decreases cardiovascular events and mortality (Pedersen et al. 1998; Steinberg and Gotto 1999; Cannon et al. 2004). In addition to increased concentration of LDL-C, LDL particle size and increased density have been associated with the risk of atherosclerosis (Austin et al. 1990; Vakkilainen et al. 2003). The atherogenity of small, dense LDL is due to increased oxidative susceptibility, decreased affinity to LDLR, reduced hepatic clearance and enhanced binding to cell surface proteoglycans in the arterial wall (Chapman et al. 1998).

HDL cholesterol. In contrast to the apo B-containing lipoproteins, a low HDL-C level is an important risk factor for CAD. HDL-C was first suggested to protect against the development of CAD, independent of LDL-C levels (Miller and Miller 1975), giving it the reputation as the ‘good’ cholesterol. A strong inverse relationship between plasma HDL-C levels and CAD has since been confirmed in a large number of epidemiological studies (Gordon et al. 1977; Gordon et al. 1989; Stampfer et al. 1991). Prospective and retrospective autopsy studies have shown that HDL-C levels are inversely correlated with coronary atherosclerosis (Solberg and Strong 1983). Although low HDL-C is often seen in association with other lipid abnormalities, isolated low HDL-C is an independent risk factor for CAD

(Rubins et al. 1992; Goldbourt et al. 1997). Low plasma HDL-C is the most common lipoprotein disorder associated with premature atherosclerosis (Genest et al. 1992b), and each 0.026 mmol/L (1 mg/dL) increase in HDL-C has been associated with a 3.5% reduction in the risk of MI (Stampfer et al. 1991). Although many hypotheses have been suggested to explain the protective effects of HDL-C, the pivotal role HDL plays in RCT (Fielding and Fielding 1995; Hill and McQueen 1997) is currently its most widely accepted anti-atherogenic property.

Triglycerides. Many studies have failed to show an association between TG and CAD after correction for other risk factors, making it difficult to demonstrate an independent role for them in CAD. The demonstration that high TG levels in combination with low HDL-C levels account for roughly twice as many cases of CHD as low HDL-C alone clearly indicates a role for TG in this relationship (Castelli 1992). It has been recognized that 33% of men with CAD have increased TG (Rubins et al. 1995), and TG levels have been shown to be a significant predictor of CAD (Manninen et al. 1992; Gaziano et al. 1997). Plasma TGs are elevated in CAD patients (Genest et al. 1992b) and have been associated with increased progression of atherosclerosis (Alaupovic et al. 1997; Hodis and Mack 1998). Decreased clearance of TG following a fat-rich meal has also been identified as a risk factor (Zilversmit 1995). Furthermore, TG lowering in clinical trials has shown significant reduction in CAD (Criqui 1998), suggesting a direct relationship between TG and CAD.

Selection of *LIPC*. Twin and adoption studies have demonstrated a significant impact of genetic variation in explaining the inter-individual variation in plasma lipid levels (Heller et al. 1993; Marenberg et al. 1994). Therefore, genes involved in lipid and lipoprotein metabolism are good candidates for the development of atherosclerosis. We chose the *LIPC* as a candidate gene because of its function in lipoprotein metabolism and the well-known association of dyslipidemia (most notably, high levels of atherogenic small, dense LDL, low HDL and high TG) with atherosclerosis.

The observation that patients with familial HL deficiency and absent HL activity have definite premature CAD (Brunzell and Deeb 2001) and that high HDL levels protect against the development of atherosclerosis has stimulated considerable interest in *LIPC*. The *LIPC* has a functional promoter polymorphism at position -480 (or -514), which affects transcription and leads to genotypes CC, CT and TT. These genotypes modulate HL activity, but their role in determining the risk of atherosclerotic disease is controversial.

The studies described in this thesis focus on the relationship between *LIPC* C-480T polymorphism and atherosclerosis. The importance of HL in lipoprotein metabolism is reviewed in the following chapters.

3 Hepatic lipase (HL)

3.1 Lipase super family

Lipases are water-soluble enzymes that hydrolyze ester bonds of water-insoluble substrates, such as TG, phospholipids and CE. Lipases which affect the levels of circulating lipids have been studied for more than a century. The lipase gene family consists of multiple lipases which share structural similarities and are derived from a common ancestral gene (Hide et al. 1992). HL shows a 53% homology with LPL and a 36% homology with pancreatic lipase. However, these lipases have disparate and organ-specific expression, indicating that they may have evolved relatively specific roles. Recent studies have implicated two other lipolytic enzymes to be closely related members of this lipase gene family, namely endothelial lipase and phosphatidylserine phospholipase A1 (Wong and Schotz 2002).

Pancreatic lipase is the first reported mammalian lipase and was identified in the 19th century. The enzyme is synthesized in pancreatic acinar cells and secreted into the intestinal lumen where it hydrolyzes TG to aid in fatty acid absorption. The hydrolytic activity of pancreatic lipase is dependent on a cofactor named colipase.

The activity of LPL was first reported in 1943, when heparin infusion in dogs was shown to rapidly reduce postprandial lipemia. LPL is distributed in a variety of tissues, most notably in adipose tissue and muscle, and it is anchored to the capillary endothelium. LPL liberates fatty acids from TG-rich lipoproteins for delivery to tissues, and LPL also has non-catalytic functions, such as lipoprotein uptake from the circulation. As in the case of pancreatic lipase, LPL also requires a cofactor, namely apo C-II, for catalytic activity.

HL was first identified as a lipase released by the liver, independent of LPL activity, due to its ability to be catalytically active in the presence of high salinity (i.e. 1.0 M NaCl) (LaRosa et al. 1972). Very shortly after the LPL sequence was characterized, HL was sequenced from various species.

3.2 Synthesis, structure and function of HL

HL, a glycoprotein mostly synthesized and secreted by the liver, binds to heparan sulfate proteoglycans on the surfaces of sinusoidal endothelial cells and the external surfaces of the microvilli of parenchymal cells in the space of Disse (Doolittle et al. 1987; Sanan et al. 1997; Breedveld et al. 1997). Besides being present in the liver, HL is also found in adrenal glands (Doolittle et al. 1987), ovaries (Hixenbaugh and Paavola 1991) and kidneys (Liang and Vaziri 1997). Recently, HL expression was also detected in murine and human macrophages (Gonzalez-Navarro et al. 2002). HL, with its predominant localization in the liver, functions mainly as a lipolytic enzyme hydrolyzing TG and phospholipids in almost all major classes of lipoproteins. In addition to its role as a lipolytic enzyme, HL has a separate function as a ligand in lipoprotein metabolism, facilitating the cellular uptake of lipoproteins or lipoprotein lipids by cell surface receptors and/or proteoglycan (Santamarina-Fojo et al. 1998; Santamarina-Fojo et al. 2004).

HL has been purified from humans first by Ehnholm et al. (Ehnholm et al. 1974). HL also was isolated from rats (Kuusi et al. 1979) and other mammals. In humans, HL activity was significantly higher in newborn infants than in adults (Rovamo et al. 1984).

The human mature HL protein consists of 476 amino acids with a molecular mass of roughly 65 kDa (Martin et al. 1988; Datta et al. 1988). The crystal structure of the members of the lipase gene family is currently limited to pancreatic lipase and its related proteins. Although the structure of HL has not yet been solved, computer modelling studies based on the pancreatic lipase backbone predict that HL is also two-domain enzyme, comprising an N-terminal domain that contains an active site, surface loops and lid, in addition to a C-terminal domain that contains lipid binding, heparin binding and receptor binding sites (Wong and Schotz 2002).

3.3 Measurement of HL activity and HL mass concentration

HL is usually present as a catalytically inactive enzyme, at a low concentration, in the circulation. Heparin administration releases a large amount of HL into the circulation. Post-heparin plasma obtained in this way contains catalytically active-type HL and, therefore, is often assayed in humans as enzyme activity (Huttunen et al. 1975; Ehnholm et al. 1984). The methods which are currently widely used for measuring HL enzyme catalyzed activity are based on the hydrolysis of a gum-arabic-emulsified, radiolabelled substrate after inhibition of

LPL coexisting in post-heparin plasma with 1 M NaCl (Krauss et al. 1974) or with rabbit anti-human LPL polyclonal antibody (Ikeda et al. 1990).

The human HL mass concentration can be determined by a direct sandwich enzyme-linked immunosorbent assay with two distinct monoclonal antibodies (Ikeda et al. 1990; Nishimura et al. 2000).

3.4 HL in lipid metabolism

HL hydrolyzes TG and phospholipids of several lipoprotein classes in addition to bridging lipoproteins to lipoprotein receptors or other cell surface components. In any case, HL affects lipoprotein metabolism and mediates cellular lipid uptake. As depicted in Figure 3, HL is in several ways involved in the metabolism of HDL and apo B-containing lipoproteins (Santamarina-Fojo et al. 1998; Connelly 1999; Cohen et al. 1999; Jansen et al. 2002).

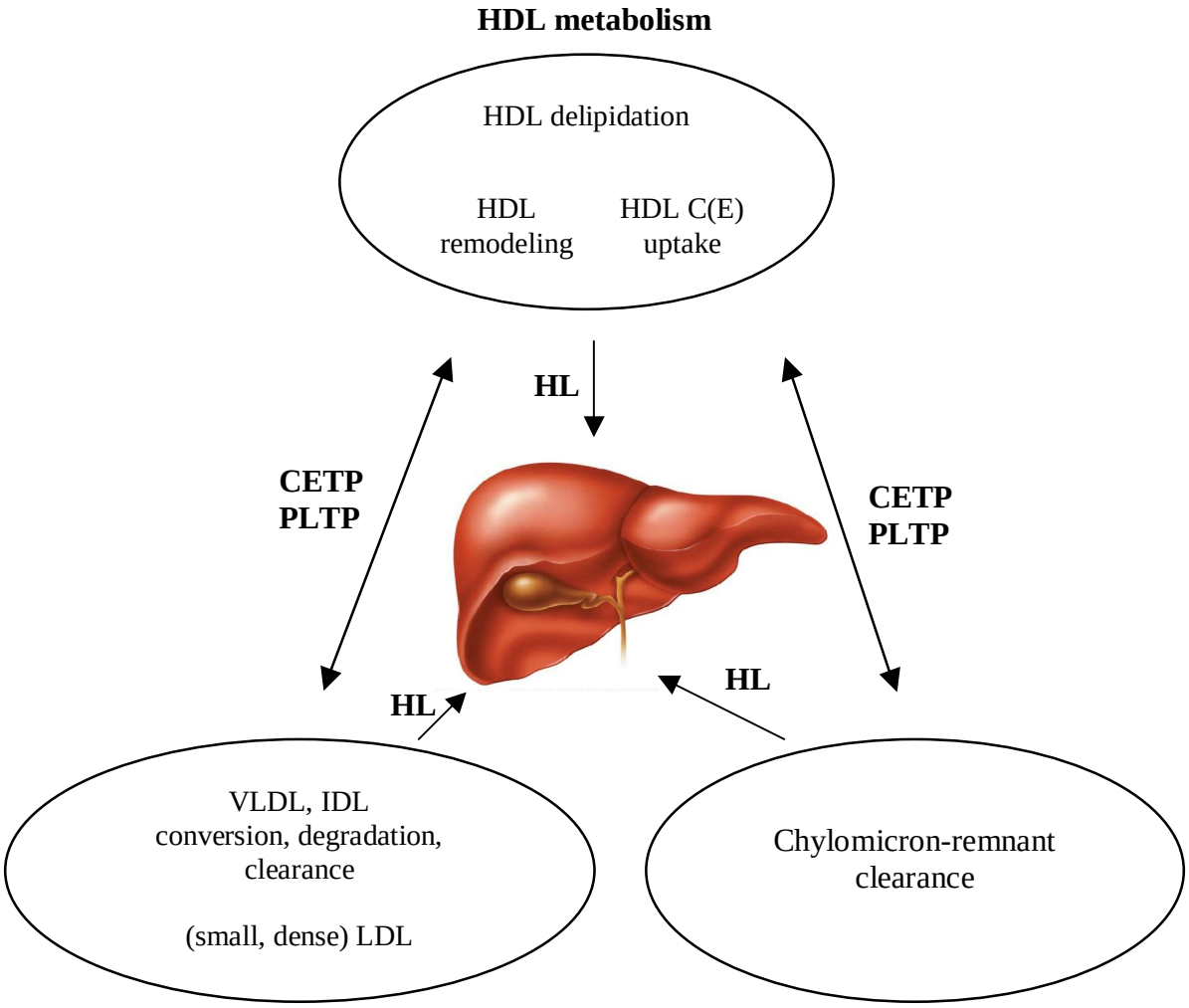

Figure 3. Schematic representation of the role of hepatic lipase (HL) in lipid metabolism. The oval panels represent the major pathways of plasma lipid transport in which HL is proposed to play a role. Between the lipoproteins of the different pathways, lipids are exchanged under the influence of transfer proteins, cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP), as represented by double headed arrows. HL interacts with the different pathways by hydrolyzing lipids, notably phospholipids and triglycerides, or by binding the lipoproteins, lipoprotein receptors or heparan sulphates on hepatocytes. In this way, HL connects plasma lipid transport to intracellular lipid metabolism in the liver (Modified from Jansen et al. 2002).

3.4.1 HL, HDL metabolism and RCT

HL hydrolyzes both TG and phospholipids of HDL and results in the formation of smaller and denser HDL particles. HL mediates the conversion of HDL_2 to HDL_3. HL also promotes the dissociation of apo A-I from the particle. This released apo A-I then goes on to form new preβ-HDL particles (Barrans et al. 1994). These small HDL particles efficiently stimulate cholesterol efflux from cells (Fielding and Fielding 2001). In humans, peripheral cholesterol once taken up by HDL and esterified by LCAT may be transferred to the liver via two distinct routes (see section 2.3.3). Both routes are connected via lipid transfer proteins. In the direct route, which involves HL, the liver takes up HDL associated CEs directly. In the indirect route, HDL CE is first transferred to the apo B-containing lipoproteins via CETP function, and is finally taken up by receptor-mediated endocytosis (e.g., LDLR). During CE transfer, HDL is depleted of CE and enriched in TG. HDL TGs are a substrate for HL. Hydrolysis of the HDL TG and phospholipids by HL results in the delipidation of HDL. Since delipidated HDL is more prone to degradation than lipid-rich HDL, HDL degradation increases (Jansen et al. 2002). In addition, HL also serves as a ligand that mediates the binding and uptake of HDL via proteoglycans and/or cell surface receptors. Adenovirus-mediated expression of catalytically inactive HL in HL-deficient mice demonstrates that catalytically inactive HL decreases plasma HDL-C concentrations by 42%, compared to a 79% reduction observed with a similar expression of the native active enzyme (Dugi et al. 2000). Both the lipolytic and nonlipolytic functions of HL are important for HDL metabolism.

3.4.2 HL and apolipoprotein B-containing lipoprotein metabolism

The role of HL in the metabolism of apo B-containing lipoproteins is well established (Figure 3). HL hydrolyzes TG and phospholipids of VLDL remnants or IDL and LDL, leading to the formation of smaller, denser lipoprotein particles (Jansen et al. 2002). HL activity promotes CM remnants uptake by the liver (Shafi et al. 1994). In addition, HL also acts as a ligand for some lipoproteins (LDL and the TG-rich lipoprotein remnants) and promotes their uptake by the liver (Krapp et al. 1996). Several studies demonstrated that inhibition of HL activity led to an impairment of CM remnant uptake by the liver. Amar et al. (Amar et al. 1998) found that inactive HL (elimination of lipolytic activity) may be a co-ligand for the binding of remnants and cell surface receptors and/or heparan sulfate proteoglycans.

3.5 HL deficiency

Familial HL deficiency is a rare autosomal recessive disorder characterized by moderate hypertriglyceridemia and premature CAD. The first patient with HL deficiency was reported in 1982 (Breckenridge et al. 1982). HL deficiency is among the rarest abnormalities of lipoprotein metabolism, with few families reported. A few patients with HL deficiency have been characterized and studied (Knudsen et al. 1997; Tilly-Kiesi et al. 2004). Most individuals who have been reported to have HL deficiency develop onset of clinical atherosclerosis in their forties and fifties (Brunzell and Deeb 2001). These patients have been shown to have elevated plasma cholesterol and TG levels, impaired remnant catabolism as well as large lipid, mostly TG and phospholipid-rich HDL and LDL particles when compared with unaffected relatives (Connelly and Hegele 1998). The unique features found in subjects with HL deficiency is a marked TG enrichment of HDL particles, and despite having increased HDL-C levels, patients with HL deficiency have often been shown to have premature atherosclerosis (Brunzell and Deeb 2001).

4 HL gene (*LIPC*)

4.1 *LIPC* structure

The human *LIPC* is located in chromosome 15q21-23. Cai et al. (Cai et al. 1989) found that the *LIPC* spans over 60 kilobase and contains 9 exons and 8 introns (Figure 4). There are many genetic polymorphisms in *LIPC* with or without effect on HL activity. Most of them are the single nucleotide polymorphism defined as a specific difference in one base at a defined location of an individual's deoxyribonucleic acid (DNA). Four polymorphisms in the proximal promoter of *LIPC* (G-216A, C-480T, T-676C and A-729G, or alternatively, G-250A, C-514T, T-710C and A-763G, depending on the nucleotide taken as the transcription start site) (Cai et al. 1989; Ameis et al. 1990), were observed to be in complete linkage disequilibrium. The allele (designated -480T or -514T) then mediates the shared genetic information by these four linked polymorphisms (Guerra et al. 1997; Vega et al. 1998). This polymorphism is an important functional polymorphism affecting HL activity (Jansen et al. 1997; Guerra et al. 1997).

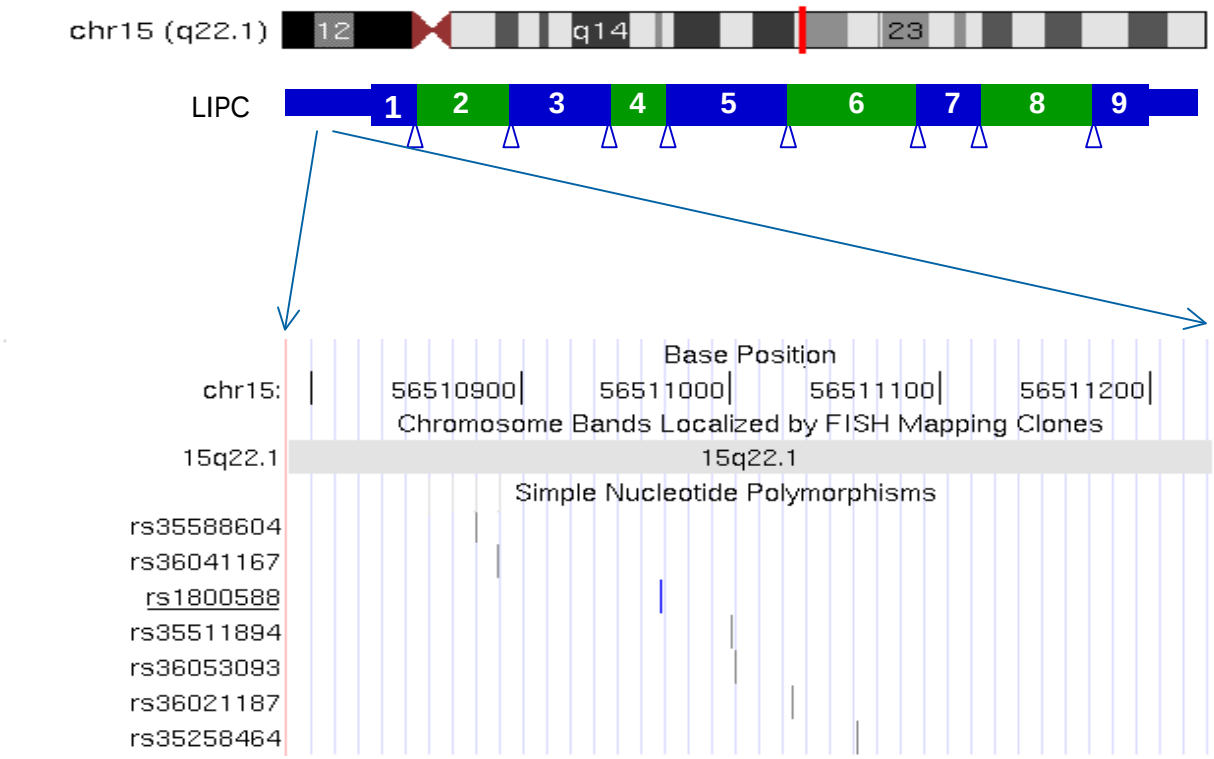

Figure 4. Schematic gene structure of hepatic lipase gene (*LIPC*, not in scale), in which the nine exons are shown as boxes and eight introns as triangles. The figure also shows the chromosomal location of the promoter polymorphisms in *LIPC* (rs 1800588). This figure was prepared with UC Santa Cruz UCSC Genome Browser http://genome.ucsc.edu. The Human March 2006 Genome Assembly was used to produce the image.

4.2 *LIPC* promoter C-480T polymorphism

Studies on twins (Kuusi et al. 1987) and nuclear families (Perusse et al. 1997) indicate that a significant fraction of the inter-individual variation in HL activity is heritable. The C-480T is the most common *LIPC* polymorphism reported to have an effect on HL activity (Jansen et al. 1997). It accounts for 20%–32% of the variability of the HL activity (Zambon et al. 1998). In a meta-analysis comprising 25 publications, Isaacs et al. observed significant decreases in HL activity for both the CT and TT genotypes when compared with the CC genotype (Isaacs et al. 2004). The -480T allele functionally drives a decreased transcriptional activity of a promoter construct in murine hepatocyte cell line, as compared with the CC genotype (Deeb and Peng 2000). It is noteworthy that the C-480T polymorphism lies at the centre of a potential binding

site (CAC*GGG, the asterisk indicates the C/T polymorphism) for specific upstream stimulatory factors. The latter are transcriptional factors involved in the regulation of glucose and lipid metabolism in the liver. Interestingly, it has been recently reported that upstream stimulatory factor proteins can bind to the -480 region and that the affinity is reduced 4-fold by the C-480T substitution (Botma et al. 2001). The -480 C-to-T substitution disrupts the upstream stimulatory factor 1 binding site present in the proximal promoter region of the *LIPC* and leads to decreased gene transcription and, thus, to decreased HL activity. The frequency of the -480T allele varies between 0.18 and 0.24 among Caucasians (Jansen et al. 1997; Andersen et al. 2003; Allen et al. 2005; Whiting et al. 2005) and between 0.45 and 0.53 among African Americans (Vega et al. 1998; Carr et al. 2004), and the figure is 0.47 among Japanese Americans (Carr et al. 2004). In Finns, like other Caucasian populations, the -480T allele frequency is in the region of 0.25 (Murtomäki et al. 1997; Tahvanainen et al. 1998).

4.3 Other reported polymorphisms and mutations of *LIPC*

Approximately 20 different mutations have been described to date in the coding, non-coding and promoter region of *LIPC* (Table 3). Most variants with defective HL activity are found in families with HL deficiency, which are very rare in the general population. Only one of these variants, A651G, is frequent in the population and associates with HL activity independently of the C-480T polymorphism (Nie et al. 1998). However, the HL activity of the A651 and G651 alleles has not been directly compared in vitro, and it is possible that the A651G polymorphism may not affect HL activity directly. The V133V, T202T and T457T are the three most common neutral polymorphisms of *LIPC* (Hegele et al. 1992; Takagi et al. 1996).

Table 3. Reported mutations and their locations in the hepatic lipase gene.

Location	Change*		Activity	Reference
Intron 1	A-13G, splice site		Defective	(Brand et al. 1996)
Promoter	T-2C		-	(Su et al. 2002b)
Promoter	T-586C		-	(Su et al. 2002a)
Exon 1	Del of promoter & exon 1		Defective	(Brunzell and Deeb 2001)
Exon 2	T230G	C53G	-	(Maruyama et al. 2004)
Exon 3	G290A	V73M	-	(Hegele et al. 1992)
Exon 4	G472T	V133V	-	(Hegele et al. 1992)
Exon 5	G593A	A174T	Defective	(Ruel et al. 2003)
Exon 5	A598G	G175G	-	(Mori et al. 1996)
Exon 5	G630T	R186H	Defective	(Knudsen et al. 1997)
Exon 5	A651G	N193S	Defective	(Nie et al. 1998)
Exon 5	C679G	T202T	-	(Hegele et al. 1992)
Exon 5	G736C	G225R	Defective	(Brunzell and Deeb 2001)
Exon 6	C873T	S267F	Defective	(Hegele et al. 1992; Hegele et al. 1993)
Exon 6	A884G	A276L	-	(Su et al. 2003)
Exon 7	A1075C	L334F	Defective	(Knudsen et al. 1996; Knudsen et al. 1997)
Exon 7	G1105A	T344T	-	(Knudsen et al. 1996)
Exon 8	C1221C	T383M	Defective	(Hegele et al. 1991)
Exon 9	C1444A	T457T	-	(Takagi et al. 1996)

*****cDNA nucleotide positions and codon numbers of mature protein (modified from Brunzell and Deeb 2001).

5 HL, lipoproteins and atherosclerosis

5.1 HL and lipid levels

There is considerable evidence that HL could affect serum lipid levels. An inverse relationship has been consistently found between post-heparin HL activity and HDL-C concentrations (Applebaum-Bowden et al. 1985; Despres et al. 1989; Kuusi et al. 1989; Blades et al. 1993). LDL size and buoyancy are also inversely associated with HL activity (Zambon et al. 1993; Watson et al. 1994).

Variation at the *LIPC* locus has been suggested to account for approximately 25% and 22% of the variation in HDL-C and apo A-I, respectively (Cohen et al. 1994). The *LIPC*

haplotype has been shown to contribute 53% and 9% influenced variation in HDL-C and LDL-C, respectively, in German families (Knoblauch et al. 2004). Moreover, the common C-480T polymorphism, CETP (TaqIB), LPL (S447X) and LCAT (S208T) contribute only by roughly 2.5% to the variance of HDL-C in the population of healthy middle-aged men (Talmud et al. 2002). According to one recent report, single nucleotide polymorphisms of several genes (ABCA1, apo A-I, apo E, CETP, *LIPC*, LCAT, LPL and SR-B1) involved in RCT explained a total of 12.4% of the variation in HDL-C in a group of Caucasian men with documented CAD (Boekholdt et al. 2006).

Dietary fat intake (Ordovas et al. 2002) and visceral obesity (St-Pierre et al. 2003) have also been shown to interact with the effect of the C-480T polymorphism on serum HDL-C. The association of the C-480T polymorphism with HDL-C is not consistent, but in a meta-analysis with more than 24,000 subjects comprising 25 publications, significant increases for both -480 CT and TT genotypes in HDL-C concentrations, compared with the CC genotype with an allele dosage effect TT>CT>CC, were observed (Isaacs et al. 2004).

The T allele of the C-480T polymorphism is also associated with the prevalence of large, buoyant LDL particles (Zambon et al. 1998). In addition, some studies found the associations of the -480T allele with higher TG or total cholesterol, but the results are inconsistent.

5.2 HL and atherosclerosis

Based on the role of HL in the metabolism of most lipoproteins, it is feasible that HL affects the risk of atherosclerosis. However, the role of HL in the development of atherosclerosis is not clear. HL has been suggested to have both pro-atherogenic and anti-atherogenic potential, which has been described in detail in several reviews (Santamarina-Fojo et al. 1998; Jansen et al. 2002; Jansen 2004; Santamarina-Fojo et al. 2004). Furthermore, recent studies have revealed that HL is present in murine and human macrophages (Gonzalez-Navarro et al. 2002; Nong et al. 2003) and that this may provide a new pathway by which HL can modulate atherogenic risk. The anti- and pro-atherogenic role of HL is shortly summarized in the following two sections.

5.2.1 Anti-atherogenic role of HL

Evidence of a more conclusive nature that HL has an anti-atherogenic role in humans can be demonstrated with the aid of HL deficiency patients. Patients with familial HL deficiency are

generally associated with accelerated CAD (Connelly and Hegele 1998). Normolipidemic (Groot et al. 1991; Johansson et al. 1991; Dugi et al. 2001) and hypercholesterolemic (Barth et al. 1987; Johansson et al. 1991; Dugi et al. 2001) CAD patients have significantly decreased HL activity, as compared to corresponding subjects without CAD. In homozygous FH patients, the low HL activity is strongly correlated with high coronary calcification (Dugi et al. 1997). In addition, normolipidemic men with symptomatic CAD and diffuse atherosclerotic narrowing of the coronary vessels have a lowered HL activity in comparison to men with normal angiograms (Barth et al. 1983). In another study, the dietary Leiden Intervention Trial, subjects with severe CAD were subjected to a strictly vegetarian diet for two years. The baseline HL values were significantly higher in the no lesion growth group than in the coronary atherosclerotic lesion progression group, and multivariate regression analysis showed that HL levels were the most important determinant of changes in coronary atherosclerotic lesions during intervention (Barth et al. 1987).

Studies with various animal models have also provided support for an anti-atherogenic role of high HL levels. Overexpression of HL in transgenic mice decreases aortic cholesterol deposition (Busch et al. 1994), markedly decreases plasma concentrations of the pro-atherogenic apo B-containing lipoprotein (Dichek et al. 1998) and increases remnant lipoprotein uptake (Gonzalez-Navarro et al. 2004; Lee et al. 2005) in spite of reduced HDL levels. Inhibition of HL activity in mice with apo A-II overexpression increases aortic cholesterol deposition, thus accelerating atherosclerosis despite increased HDL levels (Weng et al. 1999; Hedrick et al. 2001). Furthermore, overexpression of HL in transgenic rabbits also leads to a marked reduction in plasma IDL, although the HDL levels are also reduced (Fan et al. 1994).

5.2.2 Pro-atherogenic role of HL

The potential pro-atherogenic role of HL is supported by the effect of this enzyme on HDL levels and the formation of small, dense LDL, and HL activity is often high in conditions with increased atherosclerotic risk. Men exhibit higher HL activity than women (Tan et al. 1995; Despres et al. 1999; Carr et al. 2001). HL is lowered upon physical activity (Berg et al. 1994) and increased by smoking (Kong et al. 2001). In addition, HL activity is consistently inversely associated with HDL-C levels (Patsch et al. 1987; Kuusi et al. 1989; Blades et al. 1993). However, the association between high HL activity and prevalence of small, dense

LDL has been found in some (Baynes et al. 1991; Zambon et al. 1993), but not in all studies (Berk-Planken et al. 2001).

Animal studies also support the pro-atherogenic role of HL. HL deficiency increases plasma cholesterol but reduces susceptibility to atherosclerosis in apo E-deficient mice (Mezdour et al. 1997). In cholesterol-fed rabbits, HL overexpression attenuates the rise in plasma lipids, but increases lesion thickness (Taylor 1997).

5.2.3 HL and macrophages

Recently, HL expression was detected in murine and human macrophages (Gonzalez-Navarro et al. 2002; Nong et al. 2003). Macrophage HL expression in the arterial wall enhanced early lesion formation in apo E-knockout and LCAT-transgenic mice with no modification of plasma lipoprotein lipids or HL activities (Nong et al. 2003). This data implicated that HL might modulate atherogenic risk through a pathway which does not involve changes in plasma lipoprotein metabolism. However, little is known about this effect of HL at the moment.

All things considered, HL is often variably associated with atherosclerosis and CAD risk, but the extent and the direction of the association vary greatly under different circumstances. The anti-atherogenic or pro-atherogenic role of HL is likely to be modulated by the concurrent presence of other lipid abnormalities, such as hypercholesterolemia and hypertriglyceridemia, as well as by other factors involved in lipoprotein metabolism, such as CETP, LPL and LDLR (Jansen 2004). In line with clinical observations, most effects of HL on lipoprotein metabolism during hypertriglyceridemia may be interpreted as promoting atherosclerosis (formation of small, dense LDL, lowering of HDL levels), whereas most effects during hypercholesterolemia seem to be potentially anti-atherogenic (stimulation of RCT, clearing of IDL) (Jansen 2004).

5.3 *LIPC* C-480T polymorphism and atherosclerosis

Given the emerging evidence for the influence of *LIPC* C-480T polymorphism on HDL and other lipids, it is reasonable to postulate that this polymorphism contributes to atherosclerosis. The association of the *LIPC* C-480T polymorphism with CHD has been studied extensively since the identification of the association between the *LIPC* allele and HDL-C concentration in 1994 (Cohen et al. 1994).

Coronary artery disease. Studies on the association between *LIPC* C-480T polymorphism and CHD are presented in Table 4. After the *LIPC* C-480T polymorphism was discovered, a Dutch study showed that the -480T allele was more common in 782 male patients with angiographically documented CAD than in 316 asymptomatic control individuals (Jansen et al. 1997). In the Copenhagen City Heart Study involving more than 9,000 subjects, the homozygous T allele carriers exhibited a 1.7-fold higher risk of CAD than homozygous C allele carriers (Andersen et al. 2003). In men with suspected CAD, Dugi and colleagues (Dugi et al. 2001) found that the T allele was significantly associated with more severe CAD. Hokanson et al. (Hokanson et al. 2002) showed that in type 1 diabetes, the T allele is associated with coronary calcification. Recently, the T allele was found to be a susceptibility marker for CAD in a family-based association study (Allen et al. 2005). However, this finding was not reported in all related studies, including a Finnish case-control study with 395 patients who had undergone coronary bypass surgery and healthy control individuals (Tahvanainen et al. 1998). The result was also not confirmed in Koreans (Hong et al. 2000; Park et al. 2003).

There is one additional case-control study which found no overall association between the C-480T polymorphism and CHD risk in type 2 diabetes. However, the authors observed a significant interaction between this polymorphism and body mass index (BMI) in association with CHD risk (Zhang et al. 2006).

Carotid intima-media thickness. There are only four studies to date investigating the association of *LIPC* C-480T polymorphism with carotid artery atherosclerosis, measured as intima media thickness (IMT) by carotid ultrasonography. The results of these association studies have been controversial. In the first study, CC homozygote subjects were found to have a 13% higher mean IMT than T allele carriers in a multi-ethnic population containing both men and women at an average age of 70 years (Rundek et al. 2002). However, in another study on Japanese men with familial combined hyperlipidemia, T allele homozygotes had higher mean carotid IMT than other genotypes (TC+CC) (Yamazaki et al. 2004). The third study failed to demonstrate an association between *LIPC* C-480T genotypes and carotid IMT in the Diabetes Heart Study (83% of whom had type 2 diabetes mellitus) (Burdon et al. 2005). In the fourth study, T allele carriers had higher maximum IMT, for example, neointima development than the CC carriers six months after carotid endarterectomy (Zambon et al. 2006).

Cerebrovascular disease and stroke. Faggin and colleagues (Faggin et al. 2002) investigated the potential association between the C-480T polymorphism and prevalence of

inflammatory cells in the carotid plaques of 68 patients with severe carotid artery stenosis and undergoing carotid endarterectomy. In this population, a strong association was observed between CC genotype carriers and features of the unstable atherosclerotic plaque, namely an abundance of macrophages with fewer SMCs. Patients with the CC genotype and an unstable carotid plaque had a significantly higher incidence of cerebrovascular ischemic events prior to carotid surgery than T allele carriers. The percentage of the *LIPC* CC genotype was reportedly higher in patients undergoing carotid endarterectomy with previous ipsilateral events than in patients with no evidence of events (Faggin et al. 2002). No studies have been published examining the *LIPC* genotype and the risk of stroke.

Table 4. Studies on the association between hepatic lipase gene C-480T polymorphism and CHD.

Study	Design	Cases vs. controls	Sex	Age, years (range or mean ± SD)	CHD phenotype or event	Risk of CHD
(Jansen et al. 1997)	Case-control	782 vs. 316	Male	Not specified	CAD	T allele ↑
(Tahvanainen et al. 1998)	Case-control	395 vs. 194	Male	59.1±6.8 vs. 18-26	CAD	No association
(Shohet et al. 1999)	Case-control 1	317 vs. 74	Male	Not specified	CAD	No association
	Case-control 2	179 vs. 220	Male	< 60 vs. not specified	CAD	No association
(Hong et al. 2000)	Case-control (Korean)	137 vs. 124	Both	60.8±8.9 vs. 59.8±6.6	CAD	No association
(Dugi et al. 2001)	Angiography patients	200	Male	Not specified	CAD extent	T allele ↑
(Hokanson et al. 2002)	Type 1 diabetic subjects	91	Both	38±7.8	Calcification	T allele ↑
(Ji et al. 2002)	Case-control	562 vs. 642	Both	43.9±4.5 vs. 39.9±5.8	CAD or MI	T allele ↑ (male)
(Andersen et al. 2003)	Prospective	9000	Both	20-93	CAD or MI	TT ↑
	Case-control	1741 vs. 7948	Both		CAD or MI	TT ↑
(Park et al. 2003)	Case-control (Korean)	118 vs. 106	Both	Not specified	CAD	No association
(Hokanson et al. 2003)	Population-based prospective (Hispanics, non-Hispanics)	966 (397, 569)	Both	51.0±12.6, 52.4±11.3 at baseline	CHD	TT ↑
(Allen et al. 2005)	Family-based	1012	Both		CAD or MI	T allele ↑
(Whiting et al. 2005)	Case-control	3319 vs. 1385	Both	65±11 vs. 59±13	CAD	No association
(Zhang et al. 2006)	Case-control (Type 2 diabetes)	220 vs. 641	Male	59.2±7.2 vs. 55.0±8.6	CHD	T allele ↑ (obese men)
(McCaskie et al. 2006)	Case-control	485 vs. 502	Male	26-60	CHD	No association

Abbreviations: CAD, angiographically diagnosed coronary disease; CHD, angina, unstable angina, MI or angiographically diagnosed coronary disease; MI, myocardial infarction.

Note: ↑ increased risk

5.4 Environmental and other factors, HL activity and C-480T polymorphism

Diet. In rats, HL activity has been found to be inhibited by diets rich in saturated fats (Summerfield et al. 1984). Saturated fat intake has been shown to be inversely related to HL activity (Dreon et al. 1998).

Interestingly, the effect of the *LIPC* C-480T polymorphism on the response of HDL-C to dietary fat intake has been published in three larger observational studies (Ordovas et al. 2002; Tai et al. 2003; Zhang et al. 2005). The T allele was associated with higher HDL-C concentrations only in those individuals who usually derive less than 30% of their energy intake from fat. When the total proportion of fat was 30% or more of the energy intake, the mean HDL-C concentrations were lowest among those with the TT genotype, while no differences between the CT and CC genotypes were observed in the population of the Framingham study. These interactions were seen for saturated and monounsaturated fat intakes, but not for polyunsaturated fat (Ordovas et al. 2002). A second association study found that Asian Indian subjects with a total fat intake of less than 30% of their energy intake and with the TT genotype had the highest HDL-C concentrations. This interaction, however, did not apply to the Chinese or Malay subjects included in the study, and no significant interactions were found for saturated or monounsaturated fats (Tai et al. 2003). The third association study selected 780 men with confirmed type 2 diabetes: the higher HDL-C concentrations were found in men with the CT or TT genotype. However, the authors found significantly higher HDL-C concentrations in men with the CT/TT genotype who consumed large amounts of dietary fat (≥ 32% of energy intake), saturated fats and monounsaturated fats (Zhang et al. 2005).

Smoking. Smokers have been found to have increased HL activity when compared with non-smokers among MI patients (Moriguchi et al. 1991), among healthy men (Eliasson et al. 1997) and among type 2 diabetes patients (Kong et al. 2001). In addition, HL activity has been found unchanged in smokers (Freeman et al. 1998). There is also one contradictory study according to which normolipidemic smokers present with 30% lower post-heparin HL activity when compared with controls (Zaratin et al. 2004). However, no study has investigated the effect of the *LIPC* C-480T polymorphism on the relative change in HL activity in smokers.

Alcohol. Substantial evidence suggests that moderate alcohol ingestion is associated with a decreased risk of CHD, and the apparent protective effect of alcohol is mediated by increases in HDL-C (Klatsky et al. 1981; Rimm et al. 1999). HL activity is unchanged or

reduced with moderate alcohol intake and increased with heavy alcohol intake (Frohlich 1996).

Physical activity. HL activity tends to decrease with exercise (Peltonen et al. 1981; Mendoza et al. 1991). However, there is heterogeneity in this response, with some subjects showing no significant changes in HL activity with exercise (Leon et al. 2002). In the San Luis Valley Diabetes Study (Hokanson et al. 2003), 966 Caucasians were followed for 14 years and 91 CHD events occurred. The TT genotype predicted an increase in CHD, and physical activity altered this relation. The rate of CHD was significantly elevated among subjects with the TT genotype and normal levels of physical activity, but was not elevated among those with the TT genotype who participated in vigorous physical activity.

Pharmacotherapy. Synthetic anabolic steroid increases HL activity in patients with hypertriglyceridemia (oxandrolone) (Ehnholm et al. 1975) and in normotriglyceridemic men (stanozolol) (Grundy et al. 1999). Statins have been reported to decrease HL activity in men with established CAD and dyslipidemia (Zambon et al. 1999; Zambon et al. 2001). This effect was shown to be allele-specific, the -480 CC carriers displaying the most important decline in HL activity and greatest angiographic improvement in their response to lovastatin/colestipol or niacin/colestipol (Zambon et al. 2001).

Oestrogen is known to inhibit HL activity. HL activity decreases significantly with exogenous oral oestrogen (Applebaum et al. 1977). Oestrogen replacement therapy in postmenopausal women acutely decreases HL activity (Applebaum et al. 1977; Brinton 1996). Tikkanen et al. (Tikkanen et al. 1986) have shown that HL activity goes down significantly in healthy premenopausal women during the luteal phase of the menstrual cycle, when endogenous oestradiol levels are at their highest. The fall in endogenous oestrogen with menopause, again, is associated with a rise in HL activity. However, two previous studies reported that the *LIPC* C-480T polymorphism had no effect on the lipid response to HRT in postmenopausal women (Somekawa et al. 2002; Yamakawa-Kobayashi et al. 2002).

Obesity. Intra-abdominal fat deposition was positively correlated with HL activity in obese women (Despres et al. 1989). It has been shown that the relationship between central obesity and HL activity is modulated by the *LIPC* promoter polymorphism, in such a manner that the presence of the T allele seems to attenuate the increase in HL activity with high levels of intra-abdominal fat (Carr et al. 1999). In a study with 235 French-Canadian men (St-Pierre et al. 2003), TT homozygotes had the highest values of BMI, waist circumference and accumulation of visceral adipose tissue. Among men with low visceral adipose tissue, T allele carriers were characterized by significantly higher HDL_2-C levels. On the other hand, it

appears that among men with high amounts visceral adipose tissue, the effect of the *LIPC* C-480T polymorphism is attenuated.

AIMS OF THE STUDY

HL has been shown to be important in lipoprotein metabolism, and the *LIPC* promoter C-480T polymorphism has been associated with CHD. However, the role of the *LIPC* genotype in the development of different stages of atherosclerosis is still unclear. The present study used clinical and autopsy sample material to elucidate the relationship between *LIPC* genotype and coronary reactivity, autopsy-verified atherosclerotic lesions and the association of the *LIPC* genotype with AMI and SCD. The interaction between *LIPC* genotype and HRT in relation to atherosclerotic progression in postmenopausal women was also investigated. The specific aims of the present thesis are as follows:

1. To study the association between the *LIPC* genotype and the indices of coronary blood flow as measured with PET in healthy young men (I) or in young men with different serum lipid profiles (II).

2. To examine the relationship between the *LIPC* genotype and autopsy-confirmed areas of the different types of atherosclerotic lesions in the coronary artery (III).

3. To investigate the association between the *LIPC* genotype and the risk of developing AMI (IV).

4. To elucidate the association between the *LIPC* genotype and pre-hospital SCD in autopsy material (V).

5. To investigate the relationship of the *LIPC* C-480T polymorphism with the progression of atherosclerosis severity in postmenopausal women during long-term HRT (VI).

SUBJECTS AND METHODS

For more detailed information on study subjects and methods, please refer to the original articles I–VI.

1 Clinical series

1.1 Positron emission tomography (PET) study (I, II)

1.1.1 PET study in healthy, mildly hypercholesterolemic young men (I)

From the Achipelago Sea Naval Command, Achipelago Coast Guard District, Säkylä Garrison and the Turku Fire Department, 51 men were invited to participate in the study during a routine physical examination. The entry criteria were: 1) age 25 to 40 years, 2) total cholesterol level > 5.5 mmol/L, 3) clinically healthy and 4) no continuous medication or antioxidant vitamin use. The men were asked about their family history of CAD, alcohol and caffeine consumption, medication, smoking and exercise habits using a validated questionnaire. Of the total number of 51 men, 49 were included in the statistical analysis and two excluded due to technical problems with the PET measurements. The study was approved by the Ethics Committee of the Turku University Central Hospital and the University of Turku. Each subject gave written informed consent.

1.1.2 PET study in young men with different lipid statuses (II)

Between 1995 and 1999, 114 young men were examined with PET. Out of these, two were excluded from the study due to technical problems in PET and four due to unsuccessful genotyping. One hundred and eight men (aged 34 ± 5 years, range 19–44 years) comprised the final study subjects. According to their plasma total cholesterol levels, they were divided into three groups. Group 1 contained 45, group 2 contained 49 (study I) and group 3 contained 14 men with normal (4.9 ± 1.2 mmol/L), mildly elevated (5.5 ± 0.8 mmol/L) or severely elevated (7.8 ± 1.9 mmol/L, subjects with FH) plasma cholesterol levels, respectively. There were five smokers among the subjects and none suffered from diabetes. The study was approved by the

Ethics Committee of the Turku University Central Hospital and the University of Turku. Each subject gave written informed consent.

1.2 Kuopio Ischemic Heart Disease Risk Factor Study (IV)

The subjects for this study were selected from a cohort of 2,682 men from Eastern Finland, aged 42, 48, 54 or 60 years who had been examined between 1984 and 1989. A DNA sample was available for 1,263 of the men. A subpopulation of 480 men, which consisted of 160 subjects who had developed an AMI between the years 1985 and 1997, and two matched controls for each of them, were selected for this study. The average follow-up time was nine years. To ensure the comparability of the control subjects, they were drawn from the same cohort (KIHD) as the cases. The controls were matched according to age, smoking, dietary iron, dietary saturated fatty acids, dietary cholesterol and hair mercury content. In addition, the month and year of the examination and place of residence were identical for each case and the corresponding control. Because of inadequate blood samples, 94 of the men were excluded, leaving 386 subjects (126 men with AMI and 260 controls) for the final analysis. The study protocol was approved by the Research Ethics Committee of the University of Kuopio. All participants gave written informed consent.

1.3 Long-term Hormone Replacement Therapy (HRT) Study (VI)

In 1993, women attending a private outpatient clinic in Tampere for annual routine gynecological examinations were invited to participate. For the cross-sectional baseline study in 1993, 120 nonsmoking, nondiabetic postmenopausal women aged 45–71 years were enrolled. Eighty-eight of the women participated in this 5-year follow-up study from 1993 to 1998. They had no clinically evident cardiovascular diseases or hypertension and were classified into three groups based on their use of HRT. The HRT-EVP group (n=26) used oestradiol valerate (EV) at 2 mg/d for 11 d, followed by EV continued with progestin (levonorgestrel, 0.25 mg/d) for 10 d. In the HRT-EV group (n=32), the treatment was EV at 2 mg/d continuously; the control group (n=30) had never used HRT. In the HRT-EVP and HRT-EV groups, there was a pause in therapy for 7 d after each 21-d cycle. None of these women discontinued the therapy during follow-up. HRT, when used, was initiated at the time of menopause for climacteric symptoms. In the control group, the main reasons for non-use of HRT were the absence of vasomotor and other climacteric symptoms and dislike of HRT. At

baseline, the mean duration of EV and EVP treatment was 9.2 ± 3.7 and 10.9 ± 2.5 yr, respectively. The mean time from menopause in the control group was 11.9 ± 4.0 yr. The mean ages in the HRT-EVP, HRT-EV and control groups were 59.7 ± 5.5, 60.4 ± 4.8 and 61.5 ± 5.8 yr, respectively. At baseline, all women were clinically healthy and used no lipid-lowering or other chronic medication. They all gave written informed consent. The study was approved by the Ethnics Committee of Tampere University Hospital.

2 Autopsy series — The Helsinki Sudden Death Study (III, V)

The HSDS was launched to study the lifestyle and genetic risk factors predisposing Finnish middle-aged men to sudden death. The HSDS comprised two series with a total of 700 Caucasian men who had lived in Helsinki and surrounding areas and been subjected to a medicolegal autopsy at the department of Forensic Medicine, University of Helsinki. The mean age of the subjects was 53 years (range 33 to 70 years). The first series (A-series, n=400) was conducted during the years 1981 and 1982 and the second series (B-series, n=300) ten years later, in 1991 and 1992. The cause of death was cardiac in 41.1% (n=288), other diseases in 20.0% (n=140) and intoxication or other violent cause (self-inflicted or accidental) in 38.9% (n=272) of the cases.

Risk factors were determined by interviewing a relative or a close friend of the deceased. An informant was available for 500 (71%) of the cases. A detailed questionnaire included a review about past and recent smoking, drinking habits and previous illnesses. The study was approved by the Ethnics Committee of the Department of Forensic Medicine, University of Helsinki.

3 Determination of serum lipids and apolipoproteins (I, II, IV, VI)

Blood samples were obtained after an overnight fast. In studies I and II, TG, total cholesterol and HDL-C concentrations were measured with a Cobas Integra 700 automatic analyzer using the manufacturer's reagents and calibrators (Hoffmann-La Roche Ltd., Switzerland). Apo B and apo A-I concentrations were determined by an immunoturbidimetric method using specific controls (Hoffmann-La Roche Ltd., Switzerland) on the same analyzer as lipids.

In study IV, the main lipoprotein fractions were separated from fresh serum samples by ultracentrifugation and precipitation. Serum HDL-C concentration was determined after precipitation with magnesium chloride dextran sulfate. The cholesterol contents of all

lipoprotein fractions and TG were measured enzymatically (Kone Specific, Kone Ltd) (CHOD-PAP method, Boehringer Mannheim). Serum apo A-I and apo B concentrations were determined by an immunoturbidimetric method of Kone Ltd.

In study VI, lipid measurements were taken at baseline and after the 5 years' follow-up. Serum total cholesterol and TG were determined by a commercial method (Kodak Echtachem 700 XR; Eastman Kodak Co., Clinical Products Division, Rochester, NY). Serum HDL-C and its subfractions (HDL_2 and HDL_3) were separated by a dextran-sulfate-magnesium precipitation procedure, and the cholesterol content was analyzed with a Monarch 2000 analyzer (Instrumentation Laboratory, Lexington, KY) using the cholesterinoxidase-peroxidase/antiperoxidase cholesterol reagent (Roche, Mannheim, Germany) and a primary cholesterol standard (Orion Diagnostic, Helsinki, Finland). Apo A-I and apo B were determined on a Monarch analyzer by an immunoturbidimetric method (Orion Diagnostics). In the follow-up study, all lipid analyses were performed with a Cobas Integra 700 automatic analyzer with the reagents and calibrators recommended by the manufacturer (Roche Diagnostics, Basel, Switzerland).

In all four studies, LDL-C concentrations were calculated according to Friedewald's formula (Friedewald et al. 1972).

4 Evaluation of myocardial blood flow and blood flow reserve by PET (I, II)

Each participant had fasted for 6 hours before the PET studies. At the beginning, two catheters were inserted, one in the antecubital vein of the left arm for injection of $[^{15}O]H_2O$ and for adenosine or dipyridamole infusion, the other in the antecubital vein of the right arm for blood sampling. In brief, the subjects were positioned supine in a 15-slice ECAT 931/08-12 tomograph (Siemens/CTI Inc., USA). After a transmission scan, the subjects' nostrils were closed and they inhaled $[^{15}O]CO$ for 2 min through a three-way inhalation flap-valve. After the inhalation, 2 min was allowed for $[^{15}O]CO$ to combine with haemoglobin before data collection for a static scan was started. During the scan period, three blood samples were drawn at 2-min intervals, and blood radioactivity was measured. A 10-min period was allowed for the radioactive decay of $[^{15}O]CO$ before the flow measurements. Blood flow was measured at baseline and 60 sec after the beginning of intravenous administration of adenosine or 2 min after the end of intravenous administration of dipyridamole. For the blood flow measurement, $[^{15}O]H_2O$ was injected intravenously during 2 min, and dynamic scanning

was started for 6 min. Myocardial perfusion was measured twice, once at rest and once after the administration of adenosine or dipyridamole. To calculate the rate-pressure product (RPP), the subjects' heart rate and blood pressure were monitored throughout the examination.

Large regions of interest were placed on representative transaxial ventricular slices in each study covering anterior, lateral, septal and whole free wall of the left ventricle. The regions of interest were drawn on the images obtained at rest and copied to the images obtained after adenosine or dipyridamole administration. The arterial input function was obtained from the left ventricular time-activity curve using a previously validated method (Iida et al. 1992). Qualitative analysis of the PET data did not reveal any regional differences in the distribution of blood flow. Therefore, in order to enhance accuracy and statistics of flow measurements, the average flow of global left ventricular myocardium was calculated, and no detailed regional analysis was carried out. The coronary flow reserve (CFR) was defined as a ratio of overall myocardial blood flow (MBF) after adenosine or dipyridamole administration to flow at baseline. The coronary resistance values were calculated both at baseline and after adenosine or dipyridamole infusion by dividing the mean arterial blood pressure by the respective flow value. RPP-adjusted resting blood flow was calculated by multiplying the subject's basal blood flow by the mean RPP of the study population and dividing the result by the subject's RPP. The CFR adjusted for RPP was calculated as the ratio of MBF during adenosine or dipyridamole administration to RPP adjusted flow at baseline.

5 Ultrasonographic measurements of atherosclerosis severity score (VI)

Ultrasonography at baseline and follow-up were performed with Sonolayer V SSA 100 equipment (Toshiba Corp., Tokyo, Japan). In brief, transverse and longitudinal scans of the extracranial carotid arteries were carried out bilaterally at four different segments of the carotid. Only fibrous and calcified atherosclerotic lesions were taken into consideration and were defined as plaques when distinct areas of mineralization and/or focal protrusion into the lumen were identified. A far-wall IMT equal to or more than 1.3 mm at any carotid artery segment was defined as an atherosclerotic plaque (Furberg et al. 1989), and the total number of plaques (NAP) was calculated. All carotid artery examinations were made with a 5.0-MHz convex transducer probe.

Longitudinal ultrasonographs of the abdominal aorta were obtained at 1-cm intervals and transverse scans at 2-cm intervals in the area of three aortic segments. Significant aortic plaques were defined as a far-wall IMT equal to or more than 3.0 mm (Furberg et al. 1989).

All aortic examinations were performed with a 3.75-MHz convex transducer probe. The reproducibility of our ultrasonographic protocol for significant aortic and carotid plaques was also examined: 1 month after the first assessment, 20 randomly selected subjects were invited to attend a repeat examination. The repeatability of NAP between the first and second examination was 90% for the carotid artery segment areas and 100% for the aortic segments. All ultrasonographies were performed in a blinded manner by one experienced ultrasonographer and radiologist.

The atherosclerosis severity score (ASC) was constructed by dividing the atherosclerosis in the abdominal aorta and carotid arteries into three severity classes: 1=slight (1.3-2 mm), 2=moderate (2-3 mm) and 3=severe (more than 3 mm). The ASC was then calculated as the sum of the severity classes in aorta and carotid artery. The total NAP was calculated (at baseline only because 5-year data were not available) according to the NAP (criteria for plaques as above). Scoring was conducted by one person in a blinded manner, without knowledge of HRT and *LIPC* genotype status.

6 Measuring the area of atherosclerosis lesions by morphometry (III)

In the HSDS, the areas of atherosclerotic lesions were measured from the left anterior descending, left circumflex and right coronary artery. The coronary arteries were dissected free, opened, attached to a card and then fixed in 10% buffered formalin. The arteries were radiographed to detect calcifications and then stained for fat by the Sudan IV staining method. The degree of atherosclerotic lesions was evaluated according to standard protocols of the IAP (Guzman et al. 1968). The areas of fatty streaks, fibrotic lesions, complicated lesions and calcified plaques were measured with a computer-assisted planimetric technique and by radiography in the case of calcification. The areas of the different types of lesions were expressed in percentage (%).

7 Measuring coronary narrowing on silicone rubber casts of coronary arteries (V)

At autopsy, coronary angiography was performed using vulcanizing liquid silicone rubber mixed with lead oxide as the contrast medium. The proximal, middle and distal stenosis of the main trunks of the three main epicardial coronary arteries, left anterior descending, left circumflex and right coronary artery, were measured from the rubber cast model. The stenosis percentage was obtained by dividing the diameter (millimetres) of the greatest stenosis by the

diameter of the nearest proximal undamaged part of the cast model of the artery, resulting in 9 measurements on the degree of stenosis for each individual. The most severe stenosis of these measurements was used to represent the extent of coronary narrowing for each individual.

8 Determination of follow-up acute myocardial infarction (AMI) events (IV)

A prospective registry in eastern Finland collects all fatal and nonfatal AMI events among the population, which includes the present cohort. In study IV, fatal and nonfatal events were regarded as end points. Approximately half of the fatal cases were autopsied. An event was regarded as a definite AMI if at least one of the following conditions was met: definite electrocardiographic changes; typical, atypical, inadequately described symptoms combined with probable electrocardiogram and abnormal enzymes; typical symptoms and abnormal enzymes; or naked-eye appearance of fresh AMI and/or recent coronary occlusion found at necropsy regardless of other findings.

9 Characteristic and phenotypes of myocardial infarction at autopsy (V)

Coronary thrombosis and MI were recorded at autopsy and the presence of MI was confirmed by means of nitro blue tetrazolium staining and histological examination of the myocardium. The presence of neutrophil granulocytes was considered diagnostic of an AMI, and the presence of fibrous scar tissue diagnostic of an old MI. Thrombosis was defined as a reddish clot attached to the coronary wall if the clot could not be detached with saline flushing.

10 DNA extraction and *LIPC* genotyping

In the A-series of the HSDS (III, V), DNA was extracted from paraffin-embedded samples of cardiac muscle with a method described by Isola et al. (Isola et al. 1994). In the B-series of the HSDS, DNA was isolated from pieces of cardiac muscle by a standard phenol-chloroform method. In other cases, DNA was isolated from whole blood by QIAamp DNA Blood Kit (Qiagen Inc., USA).

In studies I, II, IV and VI as well as the B series of studies III and V *LIPC* C-480T genotypes were determined by PCR using primers and restriction enzyme *Nla*III (New England Biolabs, USA) digestion as described earlier (Jansen et al. 1997). After initial denaturation at 96°C for 3 min, the DNA was amplified by 32 cycles in the following

conditions: denaturation at 96°C for 1 min, annealing at 65.5°C for 1 min and extension at 72°C for 1 min, with a final elongation step of 5 min at 72°C. The PCR-products were digested for 4 h. The digested fragments were separated by electrophoresis and visualized with ethidium bromide staining under UV-light.

In the A series of studies III and V, the *LIPC* genotypes were determined by employing the 5' nuclease assay for allelic discrimination using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). PCR reaction containing genomic DNA, 2× TaqMan Universal PCR Master Mix, 900 nM of each primer and 200 nM of each probe was performed in 96-well plates using a standard protocol in a total volume of 25 µl. After cycling, end-point fluorescence was measured, and genotyping calling was carried out by the allelic discrimination analysis module. Genotyping was controlled by analyzing some random samples as duplicates and by including some known genotype and negative (water) controls in the analysis.

Genotyping was always performed without the knowledge of the clinical data.

11 Statistical methods

Discontinuous variables were compared with Pearson's χ^2 test. The t-test for independent samples, analysis of variance (ANOVA) and analysis of covariance (ANCOVA) were used to compare continuous variables. Statistical analyses of the longitudinal follow-up data were analyzed by analysis of variance for repeated measures. In the case of a significant main effect or interaction, least significant difference or Bonferroni post-hoc tests were utilized to compare the differences between groups. Non-normally distributed data was analyzed after square root or logarithmical transformation, but the results were expressed as crude.

In studies I and II, linear regression analysis was used to determine the simultaneous and independent effects of *LIPC* genotype and other variables on CFR (I, II), blood flow during pharmacological induced hyperemia (II) and coronary resistance (II). In study IV, logistic regression modelling was employed to examine associations between genotype and AMI after adjustment for other variables. In studies III and V, Bonferroni correction was used for multiple comparisons between the groups.

Data in the text are presented as mean ± standard deviation (SD) unless otherwise stated. A p-value of less than 0.05 was considered statistically significant. All statistical analyses were carried out using the Statistica for Windows version 5.1 software package (I, VI)

(Statsoft Inc., USA) or SPSS version 9.0 (I), 11.5 (IV), 12.0.1 (II) and 13.0.1 (III, V) for Windows (SPSS Inc., USA).

RESULTS

1 *LIPC* allele frequencies

The distribution of *LIPC* genotypes and allele frequencies in all studies (I–VI) are given in Table 5. The genotype distributions in all studies were in agreement with the Hardy-Weinberg equilibrium and identical in AMI and control subjects (study IV).

Table 5. Distributions of hepatic lipase gene C-480T genotypes and allele frequencies in studies I–VI.

		Genotype, n (%)			Allele frequency	
	N	CC	CT	TT	C	T
Study I	49	26 (53.1)	17 (34.7)	6 (12.2)	0.70	0.30
Study II	108	55 (50.9)	41 (38.0)	12 (11.1)	0.69	0.31
Study III	501	269 (53.7)	186 (37.1)	46 (9.2)	0.72	0.28
Study IV	386	191 (49.5)	155 (40.2)	40 (10.4)	0.70	0.30
AMI	126	67 (53.2)	47 (37.3)	12 (9.5)	0.72	0.28
Control	260	124 (47.7)	108 (41.5)	28 (10.8)	0.68	0.32
Study V	682	364 (53.4)	260 (38.1)	58 (8.5)	0.72	0.28
Study VI	88	49 (55.7)	34 (38.6)	5 (5.7)	0.75	0.25

2 The effect of *LIPC* genotype on lipids and apolipoprotein levels (I, II, IV, VI)

In studies I and II, there were no statistically significant differences in apo A-I, apo B or lipid concentrations between the -480T allele carriers and CC homozygotes (see study I, Table 1).

Serum lipids, apo A-I and apo B concentrations in study IV are shown by *LIPC* genotype in Table 6. In all subjects at baseline, *LIPC* -480T allele carriers had higher total cholesterol ($p = 0.004$), apo A-I ($p = 0.028$) and HDL-C ($p = 0.09$) concentrations than CC homozygotes. After stratification into AMI and control groups, the controls with the T allele had significantly higher total cholesterol ($p = 0.009$) and apo B ($p = 0.035$) concentrations than CC homozygotes (Table 6). After stratification, the other lipid values did not differ between *LIPC* genotype groups.

In study VI, the CC homozygotes tended to have lower HDL_3 concentrations than T allele carriers ($p < 0.05$) among all subjects; otherwise there were no statistically significant

differences between the *LIPC* genotype groups in other lipid or apolipoprotein levels at baseline (see study VI, Table 1). During the 5-year follow-up, no *LIPC* genotype differences in major serum lipid changes were observed either within the HRT or the control groups (see study VI, Table 2).

Table 6. Total cholesterol, HDL-C, apolipoprotein A-I and B levels at baseline by hepatic lipase genotype and AMI status during follow-up.

		CC homozygotes	T allele carriers	p-value*
All subjects	N	191	195	
	Total cholesterol (mmol/l)	5.89 ± 1.03	6.22 ± 1.17	0.004
	HDL cholesterol (mmol/l)	1.28 ± 0.29	1.31 ± 0.28	0.09
	Apolipoprotein A-I (g/l)	1.33± 0.23	1.37 ± 0.26	0.028
	Apolipoprotein B (g/l)	1.04 ± 0.23	1.08 ± 0.22	0.142
AMI	N	67	59	
	Total cholesterol (mmol/l)	6.10 ± 1.04	6.48 ± 1.44	0.106
	HDL cholesterol (mmol/l)	1.19 ± 0.28	1.26 ± 0.25	0.093
	Apolipoprotein A-I (g/l)	1.30 ± 0.24	1.37 ± 0.28	0.079
	Apolipoprotein B (g/l)	1.11 ± 0.22	1.12 ± 0.23	0.922
Control	N	124	136	
	Total cholesterol (mmol/l)	5.78 ± 1.00	6.12 ± 1.02	0.009
	HDL cholesterol (mol/l)	1.33 ± 0.29	1.33 ± 0.29	0.497
	Apolipoprotein A-I (g/l)	1.34 ± 0.23	1.37 ± 0.26	0.179
	Apolipoprotein B (g/l)	1.00 ± 0.22	1.07 ± 0.22	0.035

Abbreviation: AMI, acute myocardial infarction. Statistics: *ANCOVA; age, body mass index, smoking, hypertension, diabetes and family history of coronary disease as covariates.

3 *LIPC* genotype and coronary function (I, II)

Study I. Study I examined the relationship of *LIPC* genotype with coronary blood flow and reactivity. Resting MBF was not statistically different in the *LIPC* genotype groups. Adenosine infusion resulted in a slightly higher blood flow in the CC genotype group. The RPP-corrected CFR was 24% higher in men with the CC genotype than in those with the T allele (p = 0.029) (Table 7). The difference in coronary resistance between *LIPC* genotype groups was not statistically significant.

The multivariate analysis model that included *LIPC* genotype, age, BMI, total cholesterol, HDL-C, LDL-C, TG, apo A-I, apo B and smoking showed that the *LIPC* genotype remained the only significant predictor of CFR (p = 0.038) (see study I, Table 4).

Table 7. Myocardial blood flow indices according hepatic lipase genotype in study I.

	CC homozygotes	T allele carriers	p-value
Subjects (n)	26	23	
Blood flow at rest (mL×g-1×min-1)*	0.80 ± 0.15	0.88 ± 0.27	NS
Adenosine flow (mL×g-1×min-1)	3.52 ± 0.78	3.15 ± 0.88	NS
Coronary flow reserve*	4.62 ± 1.52	3.73 ± 1.08	0.029

Abbreviation: NS, nonsignificant. Statistics: ANCOVA, age and body mass index as covariates.

*Adjusted by rate-pressure product.

Study II. Study II is an extension of study I. The purpose of this study was to cover a wider spectrum of cholesterol levels to test the hypothesis that the difference in plasma cholesterol levels may not influence the effect of *LIPC* genotype on coronary reactivity, based on previous findings (Gonzalez-Navarro et al. 2002; Nong et al. 2003). There were no differences in the effect of *LIPC* genotype on the indices of MBF between the groups with different plasma cholesterol levels. Therefore, the groups were combined for further analyses.

Basal flow did not differ between the genotype groups. The subjects with the T allele had lower coronary flow during hyperemia, lower CFR and higher coronary resistance during hyperemia than subjects with the CC genotype (Table 8).

In the multivariate analysis, the effect of the *LIPC* C-480T polymorphism on the indices of MBF, was independent of other risk factors for CAD (see study II, Table 3).

Table 8. Myocardial blood flow indices by hepatic lipase genotype in study II.

	CC homozygotes	T allele carriers	p-value
Subjects (n)	55	53	
Flow during hyperemia (mL×g-1×min-1)	3.86 ± 1.26	3.20 ± 1.38	0.007
Coronary flow reserve*	4.80 ± 1.77	3.77 ± 1.43	0.001
Coronary resistance (mmHg×min×g×mL-1)	25.63 ± 9.98	35.00 ± 23.95	0.003

Statistics: ANCOVA, age and body mass index as covariates.

*Adjusted by rate-pressure product.

4 *LIPC* genotype and early and advanced atherosclerotic lesions (III)

The objective of this study was to investigate the association of the *LIPC* C-480T genotype and the areas of different types of atherosclerotic lesions in the coronary arteries. Carriers of the TT genotype tended to have larger fatty streaks when compared to CT or CC carriers. An age-by-*LIPC* genotype interaction was observed in the mean percentage area of fatty streaks using alternative models where age was used as a continuous or classified variable ($p = 0.039$ and $p = 0.014$, respectively). The interaction remained significant after adjustment for age, BMI, hypertension, diabetes, smoking, alcohol use, apo E genotype and series number.

In men < 53 years of age, TT genotype carriers had two times larger areas of fatty streaks than CC carriers (8.8% vs. 4.3%, $p = 0.009$) (Figure 5). However, this association disappeared in men over 53 years of age. In men < 53 years old, there was a dose-effect trend for the TT genotype carriers to have larger fibrotic and complicated lesions; however, this did not reach statistical significance. There were also no significant differences in the older age group between the *LIPC* genotype groups in fibrotic or complicated lesions (see study III, Table 2).

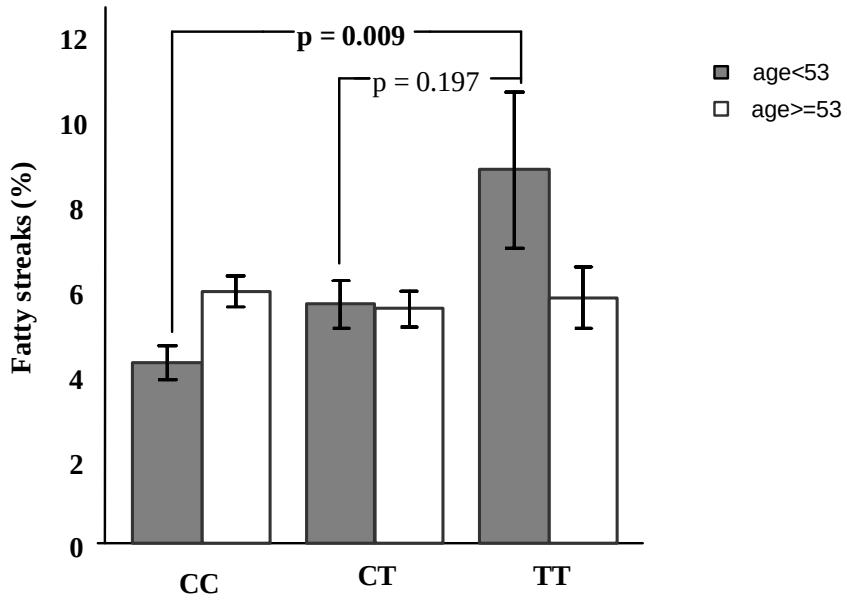

Figure 5. Area of fatty streaks in men < 53 and men ≥ 53 years by hepatic lipase genotype (age-by-genotype interaction, $p = 0.014$). Bonferroni post-hoc was used to study the difference between hepatic lipase genotypes. Values are mean ± standard error.

5 *LIPC* genotype and risk of developing AMI (IV)

In study IV, *LIPC* genotypes were related to the risk of developing an AMI according to HDL-C levels. Men with the CC genotype tend to have a higher risk for AMI than T allele carriers (relative risk, 1.6; 95% confidence interval [CI], 1.0 to 2.4; p = 0.057), after adjustment for age, BMI, smoking, hypertension, diabetes, family history of coronary disease, total cholesterol and TG (see study IV, Table 3). However, when HDL-C was added as an additional covariate in an otherwise similar model, this association disappeared. Therefore, we further explored the possible interaction between *LIPC* genotype and HDL-C levels with regard to the risk of developing an AMI. For that purpose, we divided the subjects into tertiles according to their serum HDL-C concentrations and found a significant interaction between *LIPC* genotype groups and HDL-C tertiles (p = 0.002). Men with the CC genotype and HDL-C concentration in the lowest or second lowest tertile were found to have a 4.0 to 3.3-fold risk, respectively, of developing an AMI than men in the highest HDL-C tertile, after adjusting for other risk factors. A similar effect was not found in men with the T allele (Table 9).

Table 9. Adjusted relative risk of AMI according to hepatic lipase genotype and HDL-C level status.

HDL-C (mmol/l)	CC homozygotes	T allele carriers
Subject (n)	191	195
Lowest tertile, (95% CI)	3.992* (1.656-9.627)	2.426 (0.923-6.381)
Middle tertile, (95% CI)	3.264† (1.358-7.847)	2.219 (0.920-5.356)
Highest tertile, (95% CI)	1.0	0.968 (0.376-2.488)

Abbreviation: AMI, acute myocardial infarction. Relative risk is adjusted for age, body mass index, smoking, years of hypertension, diabetes, family history of coronary disease, total cholesterol, triglycerides and energy and fat intake. Statistics: Total model < 0.001; p-value for interaction 0.002.
*p = 0.002; †p = 0.008, difference from the highest HDL-C tertile.

6 *LIPC* genotype, AMI and sudden cardiac death (V)

The aim of study V was to investigate the association between *LIPC* genotype and the risk of AMI as well as pre-hospital SCD. Men who had died of SCD were significantly older, had higher BMI and were reported to be more hypertensive as well as consume less alcohol than those who had died of other causes. Of the entire series of 682 men, 177 (26%) were found to

have had an MI with or without an old MI, and 82 (12%) men had had an AMI with or without an old MI. Of the AMI cases, 38 were associated with coronary thrombosis. Of the subjects with AMI, one had died accidentally and four had some other severe underlying disease as the cause of death. There were no significant differences in these descriptive characteristics between men with or without interview data. There were also no significant differences in cause of death, MI, AMI with or without old MI, or thrombosis between men in the A and B series.

Men with the TT genotype tended to have an increased risk for SCD and AMI when compared to CC or CT genotype carriers, and this effect was particularly strong among younger men (see study V, Table 2) as well as after adjustment of other available risk factors (see study V, Table 3).

No differences in major risk factors for CHD were found between the *LIPC* C-480T genotype groups, with the exception of age in men who had died of SCD. Of these subjects, men with the TT genotype were younger than those with the CC or CT genotype (Table 10). Furthermore, men with the TT genotype tended to be younger than those carrying the CC or CT genotype among subjects with AMI and AMI without thrombus (Table 10).

Table 10. Mean age (years) of subjects in different hepatic lipase genotypes among men with SCD, AMI, AMI without thrombus, and men who had died of non-SCD causes.

	N	CC	CT	TT	p-value
SCD	278	57.6 ± 7.8*	55.4 ± 9.5	53.5 ± 9.0	0.02
AMI	82	57.7 ± 8.6†	57.1 ± 9.3	52.5 ± 10.5	0.24
AMI without thrombus	44	59.1 ± 8.8*	58.0 ± 8.4†	51.2 ± 10.8	0.10
Non-SCD	404	51.0 ± 9.7	50.1 ± 9.2	51.8 ± 8.8	0.56

Abbreviations: AMI, acute myocardial infarction; SCD, sudden cardiac death. Statistics: ANOVA, with least significant difference post-hoc test. *$p < 0.05$ TT vs. CC, †$p < 0.1$ TT vs. CC or CT.

Due to a statistically significant *LIPC* genotype-by-age interaction ($p = 0.011$) in relation to SCD, we stratified the subjects into two groups (men < 53 and men ≥ 53) according to their mean age. The percentage of men with the TT genotype among SCD victims tended to be higher than that of the non-SCD group ($p = 0.087$). The difference in the genotype distribution was more pronounced ($p = 0.02$) among the younger men than the older age group (Table 11). The younger group also contained significantly more carriers of the TT genotype among AMI cases than among controls, in addition to containing significantly more

TT carriers among men with severe coronary stenosis (≥ 50%) than among those with less severe coronary narrowing (Table 11).

Table 11. Distribution of hepatic lipase genotypes in men with SCD, AMI and in those who had died of non-cardiac causes.

	N	All subjects				< 53 years old			
		CC	CT	TT	p-value	CC	CT	TT	p-value
Non-SCD	404	215 (53.2)	162 (40.1)	27 (6.7)		118 (52.0)	96 (42.3)	13 (5.7)	
SCD	278	149 (53.6)	98 (35.3)	31 (11.2)	0.087	37 (44.0)	34 (40.5)	13 (15.5)	0.020
AMI	82	42 (51.2)	29 (35.4)	11 (13.4)	0.23	11 (42.3)	9 (34.6)	6 (23.1)	0.018
Stenosis percentage									
< 50	381	194 (50.9)	161 (42.3)	26 (6.8)		107 (47.3)	104 (46.0)	15 (6.6)	
≥ 50	205	115 (56.1)	66 (32.2)	24 (11.7)	0.019	33 (55.9)	18 (30.5)	8 (13.6)	0.047

Abbreviation: AMI, acute myocardial infarction; SCD, sudden cardiac death. Statistics: χ^2 test. Values are n (%).

The TT genotype was associated with an increased risk of SCD in logistic regression analysis: the adjusted odds ratio (OR) versus CT was 2.3 (95% CI 1.0 to 5.3, p = 0.059), and the OR versus CC was 3.0 (95% CI 1.3 to 6.8, p = 0.011). The TT genotype was also associated with an increased risk of AMI: the OR versus CT was 2.9 (95% CI 1.2 to 7.1, p = 0.019), and the OR versus CC was 3.7 (95% CI 1.5 to 8.8, p = 0.003). The TT genotype in particular conferred an increased risk for AMI without thrombus: the OR versus CT was 3.8 (95% CI 1.4 to 10.3, p = 0.009), and the OR versus CC was 5.5 (95% CI 2.0 to 14.7, p = 0.001). There were no significant differences between men with CT and CC genotypes.

7 *LIPC* genotype and atherosclerosis progression during long-term HRT (VI)

The purpose of study VI was to establish whether the *LIPC* genotype modifies the effect of HRT on the development of atherosclerosis. At baseline, subjects with the T allele in HRT-EV tended to have an average of 45.8% lower ASC, and those in HRT-EVP had a 37.4% lower ASC than the controls (Figure 6A). After the 5-year follow-up, the corresponding differences between the HRT-EV and HRT-EVP groups and controls were 43.3% and 40.9%, respectively (Figure 6B).

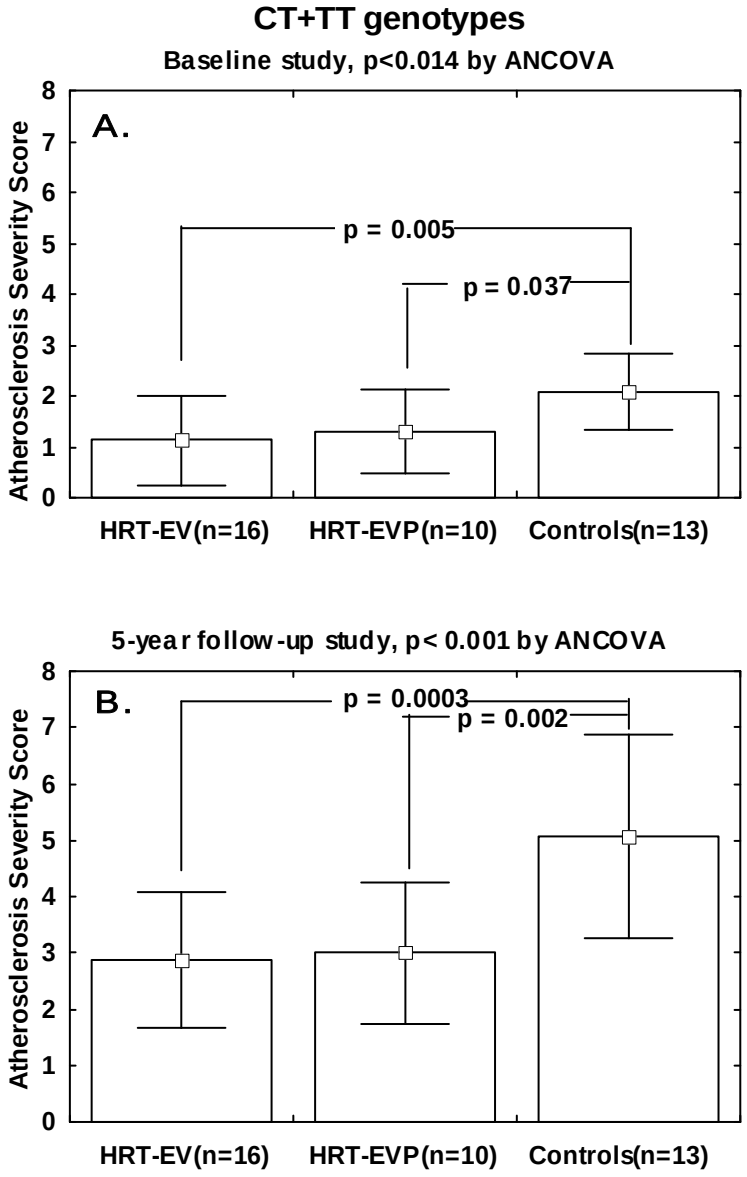

Figure 6. Atherosclerotic severity score (ASC) in postmenopausal women with T alleles by HRT group. (A) Results from the baseline study. (B) Results from the cross-sectional study after 5-year follow-up. The p values for the mean (± SD, whiskers) differences between the HRT groups and controls shown in the figure were obtained by ANCOVA, with least significance difference post-hoc test. Results were adjusted for age and BMI.

In subsequent analyses, the use of EV and EVP was combined because the results for the two therapies were similar. The *LIPC* genotype-by-HRT interaction with respect to an increase in ASC was observed (p = 0.046) after adjustment for age, BMI, changes in HDL-C during the trial and ASC at baseline.

Among the T allele carriers, the ASC progression rate differed significantly between HRT users and controls (p = 0.006). Among subjects with the CC genotype, the progression rate of ASC in HRT users and controls did not differ. The beneficial effect of HRT on atherosclerosis progression was restricted to women with the T allele, in whom the progression of ASC was slower by half (Figure 7). In postmenopausal women, the *LIPC* C-480T polymorphism conferred a genotype-specific responsiveness to HRT in atherosclerosis progression.

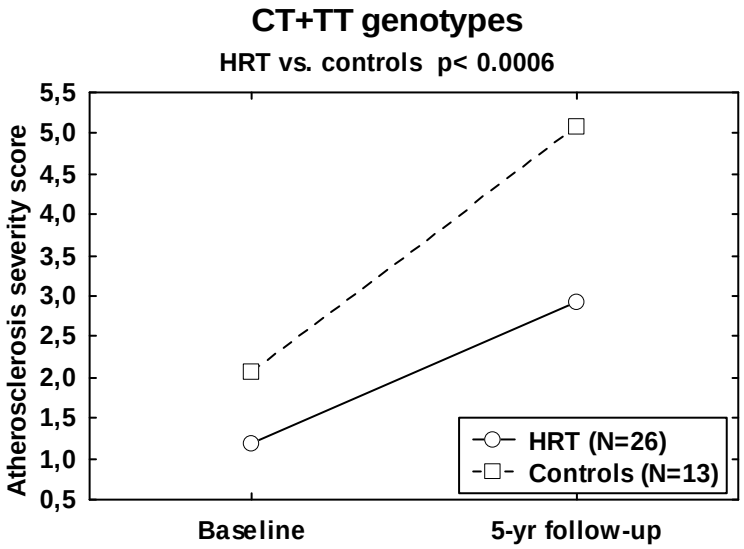

Figure 7. The effect of HRT on the progression of atherosclerosis, as measured by ASC in postmenopausal women with the T allele, compared with the progression in controls with the same hepatic lipase genotype and time elapsed from menopause but without HRT. The p values shown in the figure are from two-way ANOVA for repeated measures. ANOVA, analysis of variance; ASC, atherosclerotic severity score; HRT, hormone replacement therapy.

DISCUSSION

1 Study subjects

This thesis consists of studies I–VI including four clinical and two autopsy series. They were used to examine the relationship between *LIPC* genotype and different stages of atherosclerosis. The four series comprised 1,194 Finnish males (I–V) and 88 females (VI) who were completely independent and unrelated to each other. Based on the high prevalence and mortality of CHD in Finland (Tunstall-Pedoe et al. 1994) and the homogenous population structure caused by isolation and famines (Peltonen et al. 1995), the Finns are a particularly suitable group for genetic association studies in complex, multifactorial diseases such as atherosclerosis.

Subjects in the clinical studies. Subjects in study I were young and healthy male coast guards and firemen. They had only mildly elevated serum total cholesterol levels and normal PET measurements and, therefore, it was highly unlikely that they had significant stenosis in their coronary arteries. Due to their occupation, however, the subjects may have been healthier and in better physical condition than the population on average. In addition to the above-mentioned men, study II also included normal healthy young men and men with heterozygous FH. The FH patients had been on cholesterol-lowering therapy for several years and their serum cholesterol values at the time of the study were only moderately increased. Therefore, the present results obtained for the medically treated patients probably underestimate the effect of FH on CFR. Because only one subject in the FH group underwent coronary angiography, it is not possible to exclude the presence of mild anatomical alterations in coronary arteries not severe enough to cause clinical symptoms. It would therefore be interesting to repeat this study with a larger study population, although the PET technique is expensive and laborious and therefore not suitable for large epidemiological studies.

Study IV was a population-based prospective, nested case-control study of originally healthy middle-aged men who were followed for the risk of developing AMI for an average of nine years. The final analysis included 126 men with AMI and 260 comparable controls. To ensure the comparability of the control subjects, they were drawn from the same cohort as the cases and were matched according to age, smoking, dietary iron, dietary saturated fatty acids, dietary cholesterol and hair mercury content. In addition, the month and year of examination as well as the place of residence were identical for each case and the corresponding control.

This study had less room for bias than case-control studies because exposure data were collected during the investigation (not recalled from the past) before the disease had occurred. However, it may not provide equivalent estimates. The results from studies I, II and IV were obtained only from men and are not therefore applicable for women.

Study VI was a longitudinal study of postmenopausal women who were classified into three groups based on their use of HRT and followed for five years. The original study group consisted of 120 women who were invited by letter to participate in the study. 88 of 120 (73.3%) consented. Self-selection bias might exist here, because for some women in the control group, the main reason for non-use of HRT was dislike of HRT. The subjects represent a highly selected group and may not be representative of all women who might be prescribed HRT.

Subjects of the autopsy series. The subjects included in studies III and V were victims of sudden death or trauma who had been subjected to a medicolegal autopsy. These subjects may have more severe atherosclerosis than subjects selected randomly, and we can thus not exclude the possibility of selection bias in this study. The CHD risk factors may also be differently distributed among these subjects. This was the case at least for alcohol consumption, which was very high in the HSDS subjects. However, the *LIPC* genotype frequencies in these studies were comparable to other studies on the Finnish population (Murtomäki et al. 1997; Tahvanainen et al. 1998) and, therefore, the subjects may be considered representative samples of Finnish middle-aged men. The obvious limitation of these autopsy series was the lack of lifetime lipid risk factor and other blood sample data even though information on other risk factors was available through an interview, and it is therefore possible to consider them as confounders in statistical analysis. Again, the results were obtained from men and can therefore not be generalized to apply to women.

2 Methodological considerations

Candidate gene approach and association studies. After a landmark paper published in 1996 by Risch and Merikangas (Risch and Merikangas 1996), association studies have been proposed as a powerful means of identifying the common variants that are involved in complex human diseases, such as atherosclerosis. A candidate gene study takes advantage of both the increased statistical efficiency of association analysis of complex diseases and the biological understanding of the phenotype, tissues, genes, lipids and proteins that are likely to be involved in the disease (Tabor et al. 2002). Therefore, a re-emerging strategy in the search

for disease susceptibility genes is the evaluation of candidate genes in population-based association studies. In this approach, the genotypes of several markers in or around the candidate gene are studied in case-control, cross-sectional and prospective cohort. In the present study, *LIPC* was selected as a candidate gene because of the biological function of HL in lipoprotein metabolism and the reported association with CAD.

PET methodology. PET is based on the detection of two photons created in an annihilation reaction between a positron and a tissue electron. PET can be used to quantitate regional MBF accurately and noninvasively, and it can thus be performed on healthy volunteers. In other words, PET is also suitable for subjects who do not meet the criteria for coronary angiography. The main advantages of PET are that it is noninvasive and can accurately determine regional MBF. Measuring MBF at rest and after dipyridamole or adenosine administration allows the calculation of CFR.

Impaired CFR has been suggested as a surrogate measure of subclinical coronary atherosclerosis, providing an integrating measure of vascular endothelial function and smooth muscle relaxation (Dayanikli et al. 1994). Impaired CFR has been observed in healthy young subjects with risk factors for atherosclerosis (Pitkänen et al. 1997) or in asymptomatic men at high risk for CAD (Dayanikli et al. 1994). Impaired CFR was also found in different types of dyslipidemia (Pitkänen et al. 1996; Pitkänen et al. 1999), in diabetes (Pitkänen et al. 1998), in smoking (Campisi et al. 1998) and in hypertension (Laine et al. 1998). Two vasodilating agents, adenosine and dipyridamole, were used in the present PET studies and have been previously shown to induce comparable degrees of myocardial hyperemia (Chan et al. 1992). The coronary flow response to dipyridamole or adenosine has been found to be related to endothelium-dependent vasodilation (Leipert et al. 1992; Mayhan 1992), and it was recently found that a significant part of the adenosine response is endothelium-dependent (Lupi et al. 1997). Therefore, coronary flow response to these two agents can be regarded as an overall measure of endothelial function and vascular smooth muscle relaxation.

Ultrasound methodology. B-mode ultrasound imaging is the optimal technology for noninvasive measurement of arterial wall lesion size and extent. To date, ultrasound evaluations remain research techniques, but they hold promise as reliable noninvasive, and therefore repeatable, measures of disease and surrogate end-points for the evaluation of therapeutic interventions. The IMT of the carotid artery is associated with the prevalence of cardiovascular risk factors and disease as well as an increased risk of MI and stroke (Burke et al. 1995; O'Leary et al. 1999; Chan et al. 2003). Ultrasound imaging can detect the early arterial wall thickening and can be performed repeatedly and reliably in asymptomatic

individuals. However, ultrasonography may underestimate large or complicated plaques and lacks precision in detecting total occlusions. It cannot distinguish fatty streaks from localized IMT; dense fibrosis and calcified areas are easier to detect because they are more echogenic (Salonen and Salonen 1993). To obtain acceptable measurement reproducibility with ultrasonography, it is essential to control the effects of instrument and operator variability. In study VI, only fibrous and calcified atherosclerotic lesions were taken into consideration and defined as plaques. All ultrasonographies were performed by one experienced sonographer and radiologist, who also scored the severity of atherosclerosis in a blinded manner. The reproducibility of the ultrasonographic protocol for significant aortic and carotid plaques was also examined in the study. This method guaranteed the highest validity and reproducibility.

Classification of atherosclerotic lesions at autopsy. In living humans, atherosclerosis may be assessed invasively by clinical studies of patients undergoing coronary angiography or noninvasively by ultrasound imaging. However, these methods have limited ability to visualize the vessel wall and only provide information about the extent and characteristics of arterial lesions that are quite advanced and that significantly narrow the lumen. Therefore, the comprehensive morphological data for understanding the initiation, progression and final steps of human atherosclerotic CAD are highly dependent on autopsy material. However, the evaluation of atherosclerotic lesions at autopsy may also have its problems. In study III, the classification of atherosclerotic lesions was carried out by visual inspection after staining the arterial samples with Sudan IV according to the protocol of the IAP (Guzman et al. 1968). The first problem with this method is the classification of fatty streaks, since some of the fatty streaks that develop in regions with adaptive intimal thickening are deeper under the endothelium and may not become visible through staining (Stary et al. 1994). Another problem is that after death, some regions of adaptive intimal thickening may project into the lumen and can be mistaken for raised lesions. A new standardized histological classification method is currently available (Stary et al. 1992; Stary et al. 1994; Stary et al. 1995; Stary 2000), but this was not the case at the time of data collection for study III.

3 The effect of *LIPC* genotype on lipid and apolipoprotein levels

We did not find any major significant associations between *LIPC* genotypes and any lipid or apolipoprotein concentrations in young men (studies I, II) or in postmenopausal women (study VI). A possible reason for the negative results in these three studies might be the relatively small sample sizes and, therefore, limited statistical power. In study IV, the T allele

carriers had higher total cholesterol, HDL-C and apo A-I concentrations than men with the CC genotype at baseline among all subjects. Among men with AMI, T allele carriers tended to have higher HDL-C and apo A-I concentrations. In the control group, the T allele carriers had higher total cholesterol and apo B than CC carriers. Based on the wide spectrum of the effects of HL on lipoprotein metabolism, it might be that the *LIPC* genotypes influence lipoprotein levels through their effect on HL activity, which mediates the hydrolysis of TG and phospholipids in several lipoprotein classes (Jansen et al. 2002).

4 *LIPC* genotype and coronary function

It is recognized that endothelial dysfunction is a major contributing factor in the atherogenic process (Ross 1993). Endothelial dysfunction has been described in high-risk subjects with no morphological atherosclerotic changes, suggesting that it is an important early event in atherosclerosis (Reddy et al. 1994). Impaired CFR is one of the earliest abnormalities associated with CAD and can therefore be used as a sensitive parameter to monitor the effects of risk factor manipulation (Dayanikli et al. 1994). Impaired CFR is a marker of vascular dysfunction before the appearance of angiographic lesions. Different types of dyslipidemia have been shown to be associated with impaired coronary function in young hypercholesterolemic men (Pitkänen et al. 1996; Pitkänen et al. 1999). Based on the wide-spectrum effects of HL on lipoprotein metabolism, it is possible that the *LIPC* C-480T polymorphism affects coronary function and predisposes to endothelial dysfunction. We tested this hypothesis in study I. Furthermore, it was recently discovered that HL is present in the vessel wall and that its presence may modulate atherosclerotic risk, independent of changes in the plasma profile, by altering macrophage cholesterol accumulation (Gonzalez-Navarro et al. 2002; Nong et al. 2003). We hypothesized that the difference in the plasma cholesterol level may not influence the effect of *LIPC* genotype on coronary reactivity. To examine this, we designed a study which included young men with different lipid statuses. We tested this hypothesis in study II.

In study I, the T allele carriers had 10% higher resting MBF and 12% lower adenosine-induced hyperemia than men with the CC genotype. Accordingly, this resulted in a CFR statistically lower in T allele carriers than in men with the CC genotype. In study II, three groups with different plasma cholesterol levels were pooled together, because there were no differences in the effect of *LIPC* genotype on the indices of MBF among the groups. The subjects with the T allele had lower coronary flow during hyperemia, lower CFR and higher

coronary resistance during hyperemia than subjects with the CC genotype. The mechanisms behind the effects of *LIPC* polymorphism on MBF are not known. None of the lipid variables were in statistically significant association with *LIPC* genotypes in studies I and II. It is likely that the *LIPC* T allele-derived effect on CFR is mediated by some other lipid mechanism, such as direct effects on artery wall macrophage lipid metabolism, which was not evaluated in studies I and II. Nevertheless, the T allele may influence the cholesterol transfer between different lipoprotein fractions. The T allele may also influence preβ-HDL subfraction and clearance of TG-rich lipoproteins through the effect on HL activity (Barrans et al. 1994; Sattar et al. 1998). It is possible that the *LIPC* promoter C-480T polymorphism may make T allele carriers prone to develop endothelial dysfunction and, in turn, coronary disease. The obvious limitation of both PET studies was that we did not measure HL activity or mass.

5 *LIPC* genotype and atherosclerotic lesions

In study III, there was a significant age-dependent association between the C-480T polymorphism and the percent area of fatty streaks in the coronary arteries. The TT homozygotes had a larger mean percent area of fatty streaks than the CC homozygotes. This difference was, however, only seen in younger men (< 53 years old).

Interestingly, African Americans have been shown to have more extensive fatty streaks than Caucasian subjects in all arterial segments (McGill et al. 1997). In their case, however, the serum lipoproteins do not account for this excess of fatty streaks. Since black people have a higher frequency of the TT genotype (Vega et al. 1998; Carr et al. 2004), we speculate that the differences of frequency in the C-480T polymorphism between black and white subjects might partially account for the ethnic difference regarding susceptibility to fatty streaks.

The limitations of studies III and V include the fact that they were carried out as a post-hoc analysis of HSDS, a study not prospectively designed to assess the impact of the C-480T polymorphism on coronary atherosclerosis, and the fact that the methods of determining risk factors were limited to information obtained from relatives and that the subjects had died suddenly, most of them not having seen a doctor or had any blood samples taken prior to their death, meant that we could not analyze HL activity, either. We also did not measure the cholesterol and apolipoprotein levels in postmortem samples. Furthermore, studies III and V only enrolled Finnish men. We cannot ascertain whether the effect of the C-480T polymorphism would extend to other populations or women, as well.

6 *LIPC* genotype and the risk of developing AMI

In study IV, men with the CC genotype and HDL-C concentration in the lowest or second lowest tertile had a 4.0 and 3.3-fold higher risk of developing AMI, respectively, when compared with men in the highest HDL-C tertile. This effect was not found in men with the T allele. However, the prevalence of the T allele increased steadily and substantially with increasing HDL-C in the AMI group. This trend was not found in the control group. Our results suggest that the effect of the *LIPC* C-480T polymorphism on AMI was modified by HDL-C levels. In theory, the *LIPC* C-480T polymorphism and HDL-C levels may have synergistic effects on HL expression either in the arterial wall macrophages and/or in the liver, which may lead to varying risk for atherosclerotic diseases, such as AMI, in different individuals. Men with the CC genotype combined with low HDL-C may have higher HL activity. This could contribute to the production of a high-risk lipid profile by reducing the HDL-C pool and increasing small, dense LDL particles in the plasma, leading to impaired RCT (Zambon et al. 2003). On the other hand, men with the T allele combined with high HDL-C may have lower HL activity, leading to a lower-risk lipid profile, more effective RCT, less foam cell formation and, thus, slower progression of atherosclerosis.

7 *LIPC* genotype and sudden cardiac death

To our knowledge, study V is the first autopsy study demonstrating the association between the *LIPC* C-480T polymorphism and SCD. The TT homozygotes were at an increased risk of SCD and particularly of AMI caused by severe CAD without plaque rupture and resulting thrombosis. This association was more clearly seen among younger men (< 53 years old). The differences between younger and older men probably reflect the rate of survival. The findings may be related to the HL activity either in the liver or in artery wall macrophages.

Interestingly, in a study on out-of-hospital cardiac arrest in Chicago, Illinois, Becker et al. found that the incidence of cardiac arrest was significantly higher for blacks than for whites in every age group and that, after controlling for other recognized risk factors, the survival rate was significantly lower in blacks than in whites. The rate of SCD with stable plaque was higher in blacks (excess rate of SCD in blacks is largely due to excess in stable plaque) (Becker et al. 1993). Since blacks have a higher frequency of the TT genotype (Vega et al. 1998; Carr et al. 2004), it might be that the *LIPC* C-480T polymorphism plays a role in the ethnic difference regarding susceptibility to SCD.

8 *LIPC* genotype and atherosclerosis progression during HRT

In study VI, the T allele carriers had greater changes in ASC during a 5-year follow-up than the CC homozygotes. The T allele carriers were seen to reflect beneficial effects on atherosclerosis progression during long-term HRT. The CC homozygotes had similar atherosclerosis progression among HRT users and controls during the follow-up. There was a significant interaction between the *LIPC* C-480T polymorphism and HRT with respect to atherosclerosis progression. Our results suggest that the C-480T polymorphism confers genotype-specific responsiveness to HRT in atherosclerosis progression in postmenopausal women.

In this study, all subjects were nonsmokers, did not have diabetes, were not suffering from clinically evident cardiovascular disease or hypertension and were not on chronic medication—and they were otherwise clinically healthy. In addition, dietary analysis revealed no substantial differences in the use of saturated fats or dietary cholesterol between the HRT groups; our results are thus unlikely to have been influenced by differences in dietary habits between the HRT and control groups. Because some other factors possibly differing between users and non-users (e.g., socioeconomic status) were not accounted for, it is conceivable that some unknown factors may have biased our results. Recent studies from our group (Lehtimäki et al. 2002; Koivu et al. 2003; Mäkelä et al. 2003) have shown that the effect of HRT varies among individuals and that this variation is partly genetically determined by functional variations in candidate genes. Together with our finding, it is becoming increasingly clear that the individual response to treatment is, to a great extent, related to genetically defined differences.

It has been postulated that HL might have a direct role in the pathogenesis of atherosclerosis through a pathway not involving changes in plasma lipoprotein metabolism (Gonzalez-Navarro et al. 2002; Nong et al. 2003). The C-480T polymorphism is a key determinant of HL activity. Our finding may be related to the genotype-related HL activity or HL activity changes either in the liver or, alternatively, in artery wall macrophages during HRT treatment. The exact mechanism of why *LIPC* genotype seems to be associated with atherosclerosis progression during HRT remains to be established.

9 *LIPC* genotype in early and advanced atherosclerosis

The possible role of *LIPC* genotype in the development of atherosclerosis is presented in Figure 8, which summarizes the findings of studies I–VI.

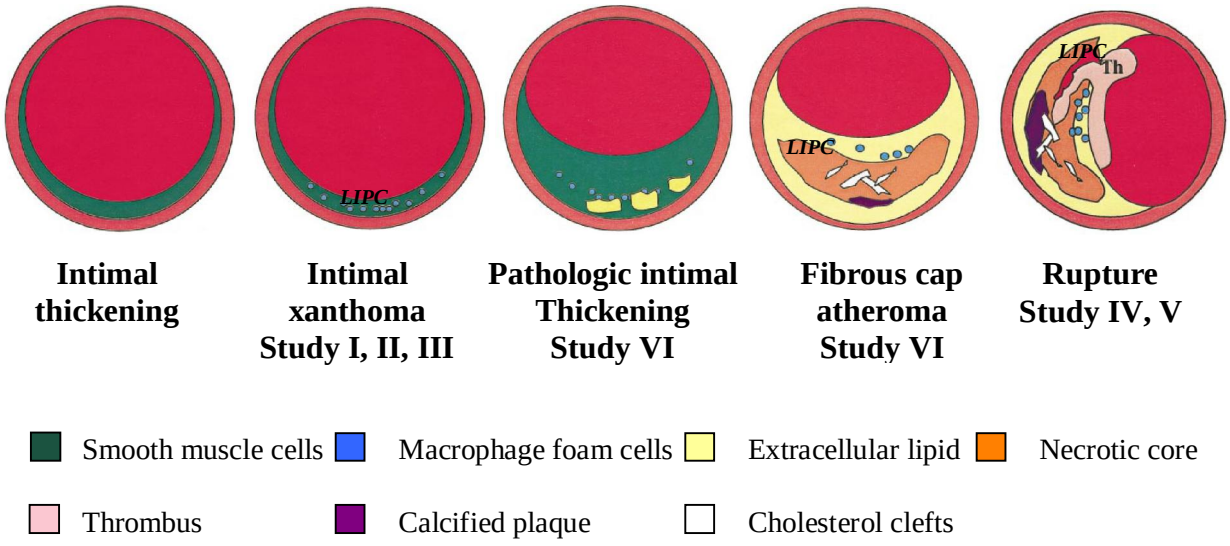

Figure 8. The potential role of hepatic lipase gene (*LIPC*) C-480T polymorphism in different stages of atherosclerosis. *LIPC* genotype seems to affect endothelial function (study I, II) and formation of intimal xanthoma (fatty streak) (study III). The effect of *LIPC* genotype on the progression of pathological intimal thickening (intermediate lesion) during long-term hormone replacement therapy is different (study VI, atherosclerotic severity score was constructed by intima-media thickness). *LIPC* genotype is associated with the fibrous cap atheroma (study VI, fibrous and calcified atherosclerotic lesions were defined as plaques). *LIPC* genotype is also involved in developing acute myocardial infarction (study IV) and sudden cardiac death (study V). Modified from Virmani et al. 2000.

10 Possible mechanisms behind the association of *LIPC* genotype with atherosclerosis

The association between *LIPC* C-480T genotype and atherosclerosis in studies I–VI is in accord with the essential role of HL activity in lipoprotein metabolism (Figure 9). First of all, HL induces the formation of smaller HDL subclasses such as HDL_3 and/or preβ-HDL. These subclasses efficiently stimulate cholesterol efflux from cells (Fielding and Fielding 2001; Jansen et al. 2002). T allele-related low HL activity may decrease RCT by reducing the production of preβ-HDL and delivery of HDL CE to the liver. Secondly, HL may promote the conversion of IDL to LDL (Connelly and Hegele 1998) and the generation of atherosclerotic

small dense LDL (Havel 2000). T allele-related low HL activity can lead to the accumulation of IDL and increased production of large, buoyant LDL. Thirdly, HL may promote CM remnant uptake (Shafi et al. 1994). T allele-related low HL activity might induce dietary hyperlipidemia. Through the above-mentioned processes, *LIPC* genotype might affect the development of atherosclerosis through changing the plasma lipoprotein metabolism.

On the other hand, since HL is expressed in the macrophages of murine and humans (Gonzalez-Navarro et al. 2002; Nong et al. 2003), HL may have a direct role in the pathogenesis of atherosclerosis without changes in the plasma lipoprotein metabolism. However, little is known about this at the moment.

All things considered, the influence of *LIPC* genotype through HL activity either in the liver or in artery wall macrophages on the above-mentioned processes may affect the development of atherosclerosis and CAD risk. However, the extent and the direction of the association is likely to be modulated by the concurrent presence of other lipid abnormalities, such as hypercholesterolemia, hypertriglyceridemia, metabolic syndrome and type 2 diabetes as well as by other genes involved in lipoprotein metabolism, such as CETP, LPL and LDLR (Jansen 2004).

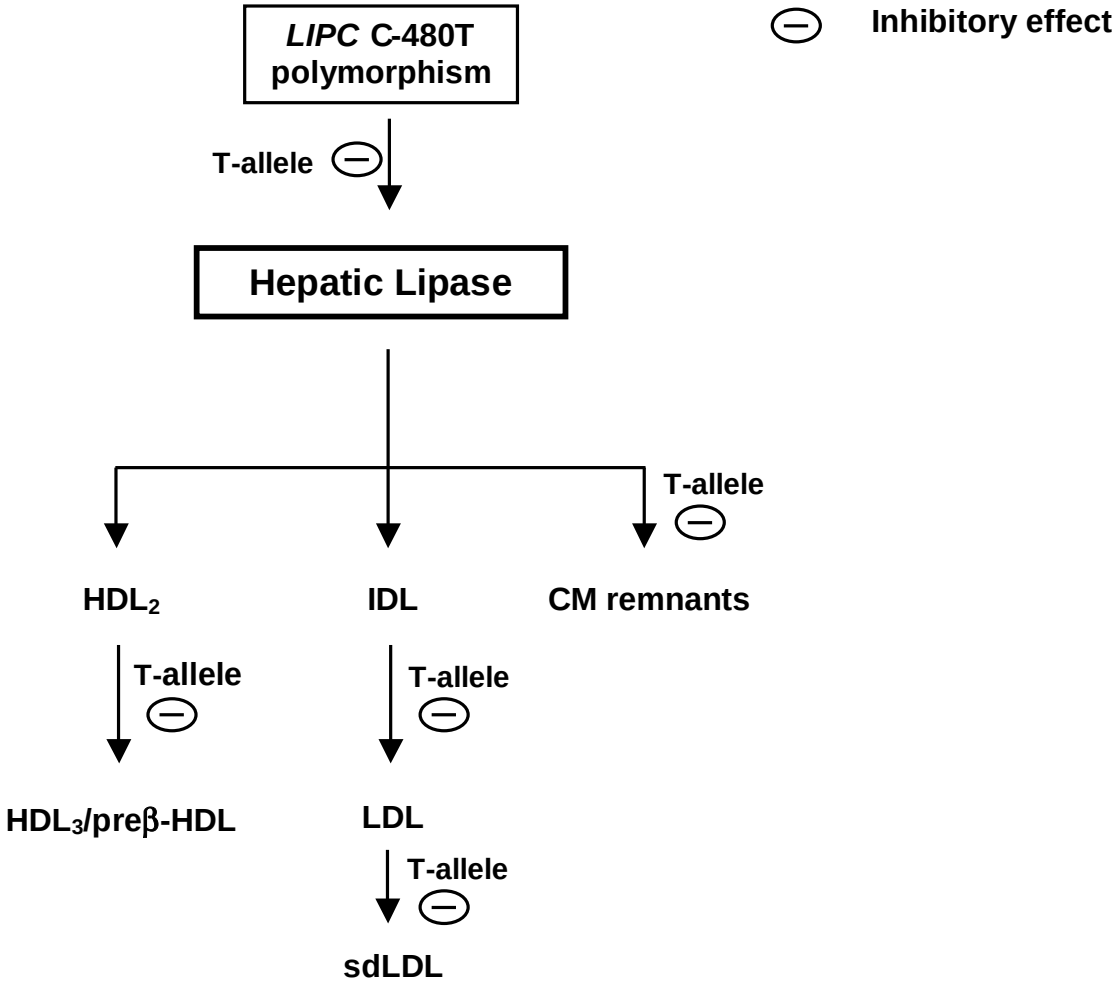

Figure 9. The possible role of *LIPC* C-480T polymorphism on lipoprotein metabolism. CM, chylomicron; HDL, high-density lipoprotein; HL, hepatic lipase; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; *LIPC*, hepatic lipase gene; sdLDL, small, dense LDL.

11 Future perspectives

The effects of the *LIPC* C-480T polymorphism have been reported to vary by age, sex, race, body mass and environmental influences. These previous studies help to illuminate the idea that gene products do not act in isolation but interact with more established influences on disease. The identified gene-environment interaction offers more hope for disease prevention. With the increasing knowledge of complex disease genes and risk factors for complex diseases, genetics—particularly genetic testing—will be increasingly applied to medicine (Shuldiner et al. 2004).

Screening for this variant in the *LIPC* promoter region will contribute to a better characterization of individual risk of coronary events, specifically in patients with qualitative,

rather than quantitative, lipid abnormalities for whom the routine lipid profile may underestimate the risk of coronary disease. Genetic analysis of the *LIPC* C-480T polymorphism therefore allows us to better characterize the actual risk of our patients beyond what has already been established by classic cardiovascular risk factors. Based on the high frequency of this polymorphism in the general population, it is possible that the combination of this polymorphism and routine parameters would provide a better diagnostic indicator for early atherosclerosis risk in young men.

Therefore, it is possible that in the future, a test for *LIPC* C-480T genotype, particularly in conjunction with family history and/or genetic testing for polymorphisms in other candidate genes, might be useful in guiding prevention and/or therapy. Even if testing for *LIPC* C-480T polymorphism does not become standard clinical practice, these research findings are useful for understanding disease pathogenesis. A better understanding of disease pathogenesis will undoubtedly lead to improved methods of treatment and prevention as well as a more rational use of existing ones.

Further studies are undeniably required to clarify the relationship of *LIPC* C-480T polymorphism and CAD as well as to explore the putative mechanisms in the cases of an increased susceptibility of *LIPC* T allele carriers to CAD.

SUMMARY AND CONCLUSIONS

Most previous studies suggest that the *LIPC* promoter -480T allele is associated with increased risk of atherosclerosis, but conflicting results have been published. In this thesis, four clinical and two autopsy studies were performed to elucidate the association between the C-480T genotypes of *LIPC* and coronary reactivity, development of early and advanced atherosclerotic plaques in coronary arteries and the developing risk of AMI and SCD. Furthermore, the effect of *LIPC* genotype on atherosclerosis progression during long-term HRT was assessed. The main findings and conclusions are as follows:

1. T allele carriers had lower CFR than CC homozygotes (study I), suggesting that the C-480T polymorphism may modify coronary reactivity and reflect differences in the early pathogenesis of coronary dysfunction in these healthy young men. This result is in line with the findings showing a larger area of fatty streaks (study III) and increased risk of AMI and SCD (study V) in subjects with the TT genotype.

2. Subjects with the T allele had lower coronary flow during hyperemia and lower CFR and higher coronary resistance during hyperemia than subjects with the CC genotype among young men with different lipid statuses (study II). This study confirmed that the *LIPC* C-480T polymorphism was associated with CFR (study I), and the effect was independent of the level of plasma cholesterol in these young men.

3. In the autopsy study III, men with the TT genotype had two times larger areas of fatty streaks than CC homozygotes. However, this association was only significant in men < 53 years of age. There was also a dose-effect trend for TT homozygotes to have larger fibrotic and complicated lesions among men < 53 years of age—however, the trend did not reach statistical significance. This finding suggests that the *LIPC* C-480T polymorphism affects the formation of early coronary atherosclerotic lesions in men in their early middle age.

4. In a prospective population-based nested case-control study (study IV), men with the CC genotype and HDL-C concentration in the lowest or second lowest tertile were at a higher risk of developing AMI than men in the highest HDL-C tertile. A similar effect was not found in men with the T allele. This suggests that the *LIPC* C-480T polymorphism might

affect AMI risk differently in men with different HDL-C levels; the atherogenicity of low concentrations of HDL-C may also be modulated diversely by different *LIPC* genotypes.

5. In the autopsy study V, TT homozygotes of the *LIPC* C-480T polymorphism had an increased risk for AMI and SCD when compared to CC carriers. This association was particularly strong among men < 53 years of age, but it was non-significant among older men (≥ 53 years). This was mainly due to a strong association between the TT genotype and AMI caused by severe coronary disease in the absence of thrombosis. TT homozygotes were more likely to have severe coronary stenosis (≥ 50%) than men with the CT or CC genotype. The results suggest that the *LIPC* C-480T polymorphism is a strong age-dependent risk factor of SCD in early middle-aged men.

6. In an observational study of postmenopausal women during long-term HRT, the progression of ASC in subjects with the T allele was significantly faster in the control group than the HRT group, whereas there were no significant differences in ASC progression between the control and HRT groups in subjects with the CC genotype. This result suggests that the beneficial effect of HRT on atherosclerosis progression was restricted to women with the T allele, in whom the progression of ASC was slower by half.

From these results we can conclude that the *LIPC* C-480T polymorphism is an important genetic marker for atherosclerosis and for the response of atherosclerosis progression to long-term HRT. The effects of *LIPC* genotype are more clearly seen in the very early stages of atherosclerosis and in the development of fatty streaks. The effects of *LIPC* genotype on the manifestation of atherosclerosis are related to HDL-C concentration and age. The beneficial effect of long-term HRT on atherosclerosis progression was restricted to women carrying the T allele. The results also indirectly refer to the importance of HL in the development of atherosclerosis.

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Hepatic lipase gene variation is related to coronary reactivity in healthy young men

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Abstract

Background Impaired coronary flow reserve (CFR) can be used to indicate vascular dysfunction before the appearance of angiographic lesions. The hepatic lipase (HL) gene has a functional promoter polymorphism at position C-480T, which affects transcription and leads to high activity (C/C) and low activity (C/T, T/T) genotypes. These genotypes modulate HL activity, but their role in coronary artery disease is controversial and the effect on coronary function has not been studied. We investigated whether HL genotypes are associated with coronary artery function in healthy young men.

Materials and methods We studied 49 healthy, mildly hypercholesterolemic men (aged 35 ± 4 years). Myocardial blood flow was measured at rest and during adenosine induced hyperaemia with positron emission tomography using [^{15}O] H_2O. HL genotype was determined by PCR and *Nla* III enzyme digestion.

Results Resting myocardial blood flow was not statistically different in subjects with high and low activity HL genotypes. However, CFR (the ratio of adenosine flow to resting flow) was 24% higher (4·62 ± 1·52 vs. 3·73 ± 1·08 mL g^{-1} min^{-1}, $P = 0{\cdot}024$) in men with the high activity genotype ($n = 26$) than in those with low activity ($n = 23$). In multivariate analysis, the HL genotype remained a significant predictor of CFR ($P = 0{\cdot}038$) after adjusting for age, body mass index, serum lipids and smoking.

Conclusions The findings of our preliminary study suggest that the C-480T polymorphism of the HL gene may modify coronary reactivity and reflect differences in the early pathogenesis of coronary dysfunction in these healthy young men. If the association between HL polymorphism and impaired CFR is also present in subjects with other dyslipoproteinemias, the HL polymorphism could be a new risk factor for cardiovascular disease.

Keywords Atherosclerosis, coronary flow reserve, hepatic lipase, polymorphism, positron emission tomography
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Introduction

Endothelial dysfunction and impairment of coronary flow reserve (CFR) are among the earliest manifestations of atherosclerosis and coronary artery disease [1–4]. CFR is an integrating marker of endothelial function and smooth muscle relaxation and therefore reflects coronary reactivity. CFR can be measured noninvasively by positron emission tomography (PET) allowing assessment of early atherosclerotic changes and vascular abnormalities already in healthy subjects who are still healthy [5,6].

Previous studies have shown that the classical coronary artery disease risk factors, i.e. diabetes [2], smoking [7],

hypertension [8], and different types of dyslipidemia [3,4] affect CFR. However, they explain only a part of the susceptibility to endothelial dysfunction. In fact, the relation between a positive family history of premature coronary artery disease and endothelial dysfunction points toward genetic determination of impaired endothelial function [9]. To extend our knowledge of the genetic risk factors underlying endothelial dysfunction, we focused on finding genes affecting coronary function and thus possibly predisposing to endothelial dysfunction.

One genetic candidate for endothelial dysfunction is a gene for hepatic lipase (HL), which is an enzyme anchored on the vascular endothelium in the liver as well as on the surface of hepatocytes, where it catalyses the hydrolysis of various lipids on lipoprotein particles [10]. The HL gene has a functional promoter polymorphism at position C-480T (or 514) affecting transcription and leading to three genotypes (C/C, C/T and T/T) [11–14]. The T allele was significantly consistently associated with lower HL activity [12,14]. But significant association of T allele with plasma high density lipoprotein (HDL) cholesterol concentration was inconsistent [12,14–16]. On the other hand, it has been reported that low HL activity is associated with severe atherosclerosis in normolipidemic subjects [17] and correlates inversely with the progression of coronary stenosis [18]. Moreover, low HL activity has been found in coronary artery disease patients with the T allele [12,13]. It is possibly that the polymorphism of HL affects coronary function and predisposes to endothelial dysfunction. However, the effect of the HL gene on coronary artery disease remains controversial [12,13]. Therefore, we undertook the present study to investigate, by using PET and [^{15}O] H_2O, whether there are abnormalities between the genotypes of HL in CFR in mildly hypercholesterolemic, otherwise healthy men.

Methods

Subjects and study design

Fifty-one men participated in the study. Forty-nine were ultimately included in the analysis and two were rejected because of technical problems in PET measurements. The mean age of the subjects was 35 ± 4 years (range 26–40 years), their mean body mass index (BMI) was 25·0 ± 2·3 kg m^{-2}. They had normal or mildly elevated serum total cholesterol levels averaging 5·5 ± 0·8 mmol L^{-1}, but were otherwise healthy. There were five smokers in the study population. Study participants were not receiving any drug therapy or antioxidant vitamins. All had normal electrocardiograms at rest during pharmacological stress induced by adenosine. All flow measurements were considered normal, suggesting that the subjects were free of atherosclerotic lesions detectable by PET. The study protocol was accepted by the Joint Commission on Ethics of Turku University and Turku University Central Hospital. Informed consent was obtained from each subject.

Production of [^{15}O] CO and [^{15}O] H_2O for PET

A low energy deuteron accelerator, Cyclone 3, was used for the production of ^{15}O (Ion Beam Application Inc., Louvain-la-Neuve, Belgium). [^{15}O] CO was produced in a conventional manner [19] and [^{15}O] H_2O using dialysis techniques in a continuously working water module [20]. Production of monoxide and water were 2·5 GBq min^{-1} and 1·7 GBq min^{-1}, respectively. Sterility and pyrogenity tests for water and chromatographic analysis for gases were applied to verify the purity of the products.

PET protocol

All PET studies were carried out after six hours fasting. Alcohol and caffeine were prohibited for 12 h prior to the study. Two catheters were inserted, one in the antecubital vein of the left hand for the injection of [^{15}O] H_2O and for the infusion of adenosine, the other in the antecubital vein of the right hand for blood sampling. The patients were positioned supine in a 15-slice ECAT 931/08–12 tomograph (Siemens/CTI Inc., Knoxville, TN, USA) with a measured axial resolution of 6·7 mm and 6·5 mm in plane. To correct for photon attenuation a 20 min transmission scan was made before the emission scans with a removable ring source containing germanium-68. After the transmission scan, the subjects' nostrils were closed and they inhaled [^{15}O] CO for 2 min through a three-way inhalation flap-valve (0·14% [^{15}O] CO mixed with room air, mean dose 3250 ± 235 MBq). After inhalation, [^{15}O] CO was allowed to combine with haemoglobin in red blood cells for 2 min *in vivo* before a static scan was initiated. During the scan period, three blood samples were drawn at 2 min intervals and the blood radioactivity concentration was measured.

Flow was measured at rest and 60 s after commencement of intravenous administration of adenosine (140 μg min^{-1} kg^{-1}); 1630 ± 115 MBq of [^{15}O] H_2O was injected intravenously for 2 min (1610 ± 115 MBq at rest and 1640 ± 120 MBq after adenosine) and dynamic scanning initiated (6 × 5 s, 6 × 15 s, 8 × 30 s). All data were corrected for dead-time, decay and photon attenuation and reconstructed in a 128 × 128 matrix. The final in plane resolution in reconstructed and Hann-filtered (0·3 cycles s^{-1}) images was 9·5 mm full width at half maximum. Heart rate and blood pressure were monitored during the studies to calculate the rate-pressure product (RPP) as an index of cardiac work.

Calculation of blood flow

Large areas of interest were placed on representative transaxial ventricular slices in each study, covering the

anterior, lateral, septal and whole free wall of the left ventricle. The regions of interest were drawn on the images obtained at rest and copied onto the images obtained after adenosine administration. The arterial input function was obtained from the left ventricular time activity curve using a previously validated method [21], in which corrections were made for the limited recovery of the left ventricular regions of interest and spillover from the myocardial signals. As no regional differences were found in myocardial blood flow, overall myocardial blood flow was used for further analyses.

The CFR was defined as the ratio of overall myocardial blood flow after adenosine to flow at rest. The resting blood flow values were corrected for resting RPP and were calculated by multiplying the subject's resting blood flow by the ratio of the mean RPP of the study population to the respective RPP of the subject. These values were then used to calculate the corrected CFR as the ratio of adenosine induced hyperaemia to RPP corrected flow at rest.

Laboratory measurements

Blood samples for biochemical analyses were collected after an overnight fast. Total cholesterol, plasma triglycerides and HDL cholesterol were measured with a Cobas Integra 700 automatic analyser with reagents and calibrators recommended by the manufacturer (Hoffmann-La Roche Ltd, Basel, Switzerland). Low density lipoprotein (LDL) cholesterol was calculated by the formula of Friedewald *et al.* [22]. Apolipoprotein B (apoB) and AI (apoAI) concentrations were determined by an immunoturbidimetric method using specific controls (Hoffmann-La Roche Ltd) on a same analyser as for lipids.

DNA extraction and HL genotyping

DNA was isolated from white blood cells using a commercial kit (Qiagen Inc, Valencia, CA, USA). To determine the HL promoter C-480T genotype, a 300-bp fragment containing the *Nla*III enzyme restriction site was amplified by PCR using the primers 5′-AAGAAGTGTGTTTACTCTAGGATCA-3′ and 5′-GGTGGCTTCCACGTGGCTGCCTAAG-3′. Thermal cycling conditions were 3 min at 96 °C followed by 32 cycles of 1 min at 96 °C, 1 min at 65·5 °C and 1 min at 72 °C, with a final elongation step of 5 min at 72 °C. The amplification cycle was performed in a PTC-225 thermal cycler (MJ Research, Waltham, MA, USA). The amplified DNA fragments were digested for 4 h with 10 units of *Nla*III (New England Biolabs, Beverly, MA, USA) and the digested fragments separated using 3% agarose gel electrophoresis containing ethidium bromide. The product can be viewed by using ultraviolet. The wild type PCR product not containing a *Nla*III restricting site yields a 300-bp band; the heterozygotes, bands of 300, 215, and 85 bp; and the homozygotes, bands of 215 and 85 bp.

Statistical analysis

Data are expressed as mean ± SD. Because the number of TT homozygotes was small, these individuals were included with the CT heterzygotes and considered as one group for the analysis. Discontinuous variables were compared using x^2-test. Means of continuous variables between HL genotypes were compared using one-way analysis of variance or analysis of covariance. Multivariate analysis was undertaken by using age, BMI, serum lipids, smoking, and promoter variants of HL as independent factors and CFR as dependent factor. All statistical analyses were performed using SPSS 9·0 for Windows 95 software (SPSS Inc., Chigaco, IL, USA) and Statistica for Windows 5·1 (StatSoft Inc., Tulsa, OK, USA). The level of significance was set at $P < 0{\cdot}05$.

Results

HL genotype, clinical characteristics and serum lipid values

In the 49 subjects, allele frequency was 0·70 for allele C and 0·30 for allele T. The frequencies of the CC, CT and TT genotypes were 0·53, 0·35 and 0·12, respectively, which were comparable to those found in Finnish population studies [14,15] and in agreement with Hardy–Weinberg equilibrium. Because the number of TT homozygous subjects was small, the samples are categorised according to the presence or absence of the T allele into high (C/C) and low activity (C/T, T/T) groups.

A summary of the characteristics and lipid profiles among men in these two genotype groups is presented in Table 1.

HL genotypes and haemodynamic findings

Heart rate and blood pressure at rest and during adenosine-induced hyperaemia are listed in Table 2. At rest, diastolic, mean arterial blood pressure and RPP were higher in the high activity genotype than in low activity group ($P < 0{\cdot}05$). After adenosine, the diastolic, mean arterial blood pressure and RPP were still higher in the high activity than in low activity group ($P < 0{\cdot}05$). Both groups showed a similar increase in RPP in response to adenosine infusion.

HL genotypes and indices of myocardial blood flow

Table 3 shows the myocardial blood flow values in the high and low activity HL genotypes. The myocardial blood flow at rest did not differ between the two groups (0·84 ± 0·17 vs. 0·81 ± 0·24 mL g^{-1} min^{-1}, $P = 0{\cdot}693$). As respective

Table 1 Characteristics and lipid profiles according to HL gene genotypes

	HL Genotype		
	High activity (C/C) ($n = 26$)	Low activity(C/T, T/T) ($n = 23$)	*P*-value
Age (years)	36·38 ± 3·38	33·96 ± 4·20	0·030
BMI (kg m^{-2})	25·29 ± 1·77	24·81 ± 2·77	0·470
Total cholesterol (mmol L^{-1})	5·71 ± 0·79	5·31 ± 0·73	0·077
Triglycerides (mmol L^{-1})	1·33 ± 0·77	1·09 ± 0·51	0·201
HDL cholesterol (mmol L^{-1})	1·40 ± 0·31	1·35 ± 0·27	0·556
LDL cholesterol (mmol L^{-1})	3·70 ± 0·66	3·47 ± 0·65	0·221
Apolipoprotein AI (g L^{-1})	1·48 ± 0·23	1·40 ± 0·22	0·237
Apolipoprotein B (g L^{-1})	1·07 ± 0·18	1·01 ± 0·17	0·249
Use of coffee (cups/day)	4·88 ± 2·92	3·85 ± 2·05	0·163
Exercise (times/week)	2·54 ± 1·54	2·85 ± 1·54	0·486
Smokers (*n*)*	4	1	0·215

BMI, body mass index; HL, hepatic lipase; HDL, high density lipoprotein; LDL, low density lipoprotein.
*x^2 test

RPP values were different, RPP corrected values were used in further analysis. The difference in RPP corrected myocardial blood flow at rest between the groups was not statistically significant (0·80 ± 0·15 vs. 0·88 ± 0·27, $P = 0·195$). Adenosine infusion resulted in a slightly higher blood flow in the high activity HL group (3·52 ± 0·78 vs. 3·15 ± 0·88 mL g^{-1} min^{-1}, $P = 0·128$). The CFR was 24% higher in the high activity group (4·62 ± 1·52 vs. 3·73 ± 1·08, $P = 0·024$ without covariates; $P = 0·029$ when age and BMI were used as covariates) than in the low activity genotypes.

In the multivariate analysis model, after entering the potential risk factors (age, BMI, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, apoA1, apoB and smoking) as independent factors, the HL genotype remained the only significant predictor of CFR ($P = 0·038$) (Table 4).

Discussion

The present results showed a significant CFR difference between HL high and low activity genotype groups in young healthy men with mild hypercholesterolemia. As previous studies have shown that an impairment of CFR is an early sign of atherosclerosis [5,6], we assume that the difference in vascular reactivity between these HL genotypes may reflect differences in susceptibility to the early atherosclerotic disease process in these young men. Earlier studies have reported that CFR is impaired in hypercholesterolemic patients [23], young adults with familial hypercholesterolemia [3], familial combined hyperlipidemia [4], type 1 diabetes mellitus [2] and borderline hypertension [8]. Our findings may extend the risk factor list for early atherosclerosis and point to the importance of genetic risk factors in the initial phases of atherosclerosis pathogenesis.

The resting myocardial blood flow here was similar in the two genotype groups. Myocardial blood flow at rest depends largely on cardiac work and resting myocardial blood flow is correlated linearly to the RPP as an index of this [24]. To account for individual differences in cardiac work, myocardial blood flow was corrected to the RPP. Therefore a more accurate and reproducible measure of myocardial blood flow is achieved by using the RPP to

Table 2 Haemodynamic data according to HL gene genotypes

	HL Genotype			
	High activity (C/C) ($n = 26$)		Low activity (C/T, T/T) ($n = 23$)	
	Rest	ADE	Rest	ADE
Heart rate (beats min^{-1})	64 ± 11	99 ± 14	59 ± 7	96 ± 12
Systolic BP (mmHg)	131 ± 15	129 ± 13	125 ± 9	123 ± 8
Diastolic BP (mmHg)	77 ± 8*	78 ± 9†	73 ± 6	70 ± 6
Mean arterial BP (mmHg)	95 ± 10*	95 ± 10†	90 ± 6	88 ± 6
RPP	6052 ± 1310*	9489 ± 1763†	5309 ± 783	8488 ± 1297
Change in RPP (%)	36 ± 9	36 ± 9	37 ± 9	37 ± 9

*$P < 0·05$ values at rest between high and low activity genotype groups.
†$P < 0·05$ values after adenosine infusion between high and low activity genotype groups.
ADE indicates adenosine; BP, blood pressure; HL, hepatic lipase; RPP, rate pressure product (heart rate × mean arterial BP).

Table 3 Myocardial blood flow indices according HL gene genotype group

Indices of myocardial blood flow	HL Genotype		
	High activity (C/C) ($n = 26$)	Low activity (C/T, T/T) ($n = 23$)	*P*-value
Blood flow at rest (mL g^{-1} min^{-1})*	0·80 ± 0·15	0·88 ± 0·27	0·195
Adenosine flow (mL g^{-1} min^{-1})	3·52 ± 0·78	3·15 ± 0·88	0·128
Coronary flow reserve (mL g^{-1} min^{-1})*	4·62 ± 1·52	3·73 ± 1·08	0·024

*Corrected by rate-pressure product

correct for variations in resting myocardial oxygen demand [24,25]. In our study, the resting myocardial blood flow corrected by RPP was 10% lower in the high activity than in the low activity genotype, while adenosine induced hyperaemia was on average 12% higher in subjects with the high activity genotype. Accordingly, this resulted in a CFR (the ratio of adenosine induced flow to RPP corrected resting flow) statistically higher in high activity than in low activity genotype subjects.

Total cholesterol concentration, RPP value and age have been reported inversely correlated to CFR [5,6,24]. Smoking is also an additional factor with an effect on CFR [26]. Although the subjects with high activity genotype have higher RPP value, borderline higher total cholesterol concentration, and older age, they still have higher CFR compared to subjects with low activity genotype. In multivariate analysis, after adjusting for age, BMI, lipids and smoking, the HL genotype remained a significant predictor of CFR. In an analysis of variance, the HL C-480T polymorphism is associated with CFR. This relationship remains significant in multivariate analysis, revealing that HL polymorphism may affect CFR. It seems that C-480T polymorphism of HL is more important than other potential risk factors involved in subjects that we studied.

Some previous studies have reported that subjects with low activity genotype have strongly higher plasma HDL cholesterol than those with high activity [12,15]. In contrast, we did not find a significant association between the HDL cholesterol and HL C-480T polymorphism that is in accordance with previous studies [14,16]. But the absence association between the HL polymorphism and HDL cholesterol can not provide any evidence on this issue due to the small sample population stratification, and differences in genetics background and/or differences in environmental factors in our mildly hypercholesterolemic but healthy subjects. There is a large number of evidence to demonstrate that HL had an effect on both HDL and triglyceride-rich lipoprotein. In our study, low activity genotype of HL gene has low CFR. It can be postulated that on one hand, because HL induces the formation of preβ-HDL [27] (which is an efficient acceptor of peripheral cell cholesterol and a key mediator in reverse cholesterol transport, although it is a minor subfraction of HDL), low HL activity could result in decreased production of preβ-HDL and decreased delivery of HDL cholesterol to the liver. On the other hand, lower HL activity is associated with the impaired clearance of lipoprotein remnants – triglyceride-rich particles such as very low density lipoprotein remnant and intermediate low density lipoprotein – which perhaps attach to the arterial endothelium to damage the endothelium [28]. It is possible that low CFR of low activity genotype might reflect arterial wall uptake and retention of the increased numbers of triglyceride-rich particles of abnormal composition and/or HDL might be defective and unable participate the reverse cholesterol transport because of compositional abnormalities. Finally, the sum of these effects may alter the risk of developing atherosclerosis in people with different risk factors. Moreover, it has been shown that HL activity is inversely correlated with the degree of calcific atherosclerosis in patients homozygous for familial hypercholesterolemia [29] and with the progression of coronary stenosis [18]. And low HL activity is associated with severe atherosclerosis in normolipidemic subjects [17]. These previous data are in line with our results showing a decreased CFR in subjects carrying the HL low activity genotypes. But the mechanism of how HL polymorphism affects CFR cannot be demonstrated in this study. Further studies are needed using large samples and relating the triglyceride-rich lipoprotein and HDL subclasses simultaneously to confirm and explain the results presented here.

In conclusion, our findings revealed that reduced CFR is already present in healthy young men with mild hypercholesterolemia together with the low activity HL genotype. These preliminary results suggest that the HL

Table 4 Relationship of CFR* with potential risk factors

Explanatory variables (independent)	Standardised coefficients	
	Beta	*P*-value
Age (years)	− 0·100	0·514
BMI (kg m^{-2})	0·141	0·390
Triglycerides (mmol L^{-1})	0·080	0·709
HDL-cholesterol (mmol L^{-1})	− 0·292	0·578
LDL-cholesterol (mmol L^{-1})	− 0·049	0·929
Apolipoprotein AI (g L^{-1})	0·090	0·803
Apolipoprotein B (g L^{-1})	0·155	0·817
Smoking	− 0·057	0·706
HL genotype	− 0·329	0·038
Entire model (R square)	0·293	0·100

*Corrected by rate-pressure product. Abbreviations are listed in legend of Table 1.

C-480T genotype may be involved in the early pathogenesis of atherosclerosis, manifesting itself as impaired coronary function. If the association of HL polymorphism with impaired CFR is also found in patients with other dyslipoproteinemias, the HL polymorphism may evolve as a new risk factor for atherosclerosis.

Study limitations

We did not perform coronary angiography, but none of the men had abnormal PET measurements. It is therefore unlikely that any of them had significant narrowing in their coronary arteries, which would have confounded our results.

Adenosine was used to test vascular function. Adenosine induced vasodilatation measures not only the endothelial function, but also smooth muscle relaxation in the artery wall, and thus our result reflects endothelial function only in part.

The number of subjects in some genotype groups studied was small and it would thus be interesting to repeat this observation with larger numbers of subjects.

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The influence of hepatic lipase C-480T polymorphism on coronary flow reserve in young men is independent of the plasma cholesterol level

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Abstract

Background: The hepatic lipase (HL) gene C-480T promoter polymorphism affects gene transcription and enzyme activity and leads to CC, CT, and TT genotypes. Recently, HL expression was detected in macrophages. It has been postulated that HL might have a direct role in the pathogenesis of atherosclerosis without changes in the plasma profile. We hypothesized that the difference of plasma cholesterol level may not influence the effect of HL genotype on coronary reactivity.

Methods: A total of 108 young men (aged 34 ± 5 years) were genotyped and divided into three groups. These groups contained 45, 49 and 14 men having either normal (4.9 ± 1.2 mmol/L), mildly (5.5 ± 0.8 mmol/L) or severely (7.8 ± 1.9 mmol/L, subjects with familial hypercholesterolemia) elevated mean plasma cholesterol level, respectively. Myocardial blood flow (MBF) was measured at rest and during adenosine or dipyridamole-induced hyperemia with positron emission tomography using [^{15}O] H_2O.

Results: The effect of HL genotype on the indices of MBF was parallel within all cholesterol groups and therefore they were combined. In all subjects, basal flow did not differ between the genotypes. However, men with CC genotype had a significantly higher hyperemic blood flow (3.86 ± 1.26 mL g^{-1} min^{-1} versus 3.20 ± 1.38 mL g^{-1} min^{-1}, $p = 0.007$), higher coronary flow reserve (CFR, 4.80 ± 1.77 versus 3.77 ± 1.43, $p = 0.001$) and lower coronary resistance during hyperemia (25.63 ± 9.98 mmHg min g mL^{-1} versus 35.00 ± 23.95 mmHg min g mL^{-1}, $p = 0.003$) than T allele carriers. In multivariate regression analysis, after adjustment for age, body mass index, serum lipids, blood pressure, adenosine or dipyridamole administration, and study group, HL polymorphism was an independent predictor of blood flow during hyperemia ($p = 0.016$), coronary resistance ($p = 0.014$), and CFR ($p = 0.005$), respectively.

Conclusions: The HL C-480T polymorphism is associated with CFR, which is an early indicator of atherosclerosis, independently of the level of plasma cholesterol in young men.

Keywords: Coronary flow reserve; Hepatic lipase; Myocardial blood flow; Polymorphism; Positron emission tomography

1. Introduction

Hepatic lipase (HL) is a multifunctional enzyme involved in lipid metabolism that hydrolyzes various lipids on lipoprotein particles and serves as a ligand that mediates the interaction between lipoproteins and cell surface receptors and/or

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proteoglycans [1]. HL is primarily synthesized in the liver, but it is also found from the adrenal glands and ovaries. Recently, HL expression was detected also in macrophages [2,3].

The HL gene has a functional C → T promoter polymorphism at position −480 or 514 depending on which of the nucleotides is taken as the transcription start site [4,5], leading to three genotypes (CC, CT and TT). The T allele has been shown to be consistently associated with lower HL activity than the C allele [6,7]. It has been reported that low HL activity is associated with severe atherosclerosis in normolipidemic subjects [8] correlating inversely with the progression of coronary stenosis [9]. Low HL activity has been found in coronary artery disease (CAD) patients with the T allele [6,10] and has been found to be associated with coronary artery calcification in type 1 diabetes [11]. Therefore, T allele is considered as a risk factor for CAD [6,12,13]. Recently, one family-based association study showed that the evidence of linkage and association for the T allele and CAD [14].

In our preliminary pilot study published in 2001 [15], we have shown that the T allele is associated with a lower coronary flow reserve (CFR) measured by positron emission tomography (PET) in mildly hypercholesterolemic but otherwise healthy young men as compared with subjects carrying the CC genotype [15]. After this finding [15], however, González-Navarro et al. [2] and Nong et al. [3] showed in their experiments that the HL is present in the macrophages of the vessel wall and, due to that finding, the investigators postulated that HL might have a direct role in the pathogenesis of atherosclerosis, independently of the changes in the plasma lipid profile. Inspired by their work [2,3], we hypothesized that the difference in plasma cholesterol levels may not influence the effect of HL genotype on coronary reactivity. We thus extended our preliminary sample of mildly hypercholesterolemic subjects [15] with a group of normocholesterolemic subjects (as defined according to the recommendations of European Guidelines on Cardiovascular Disease prevention in clinical practice [16]) as well as a group of patients with familial hypercholesterolemia (FH) and extremely high cholesterol levels. The purpose was to cover a wider spectrum of plasma cholesterol levels as well as to double the preliminary study population in order to increase the statistical significance of the study [15].

To examine this, we investigated whether the HL C-480T polymorphism is associated with CFR assessed by PET in a total of 108 young men covering a wider spectrum of plasma cholesterol levels.

2. Materials and methods

2.1. Study subjects

Indices of myocardial blood flow (MBF) of 114 young men were studied with PET between years 1995 and 1999. From these men two were excluded from the study due to technical problems in PET and four due to unsuccessful genotyping. Total of 108 young men (aged 34 ± 5 years, range 19–44 years) with an average body mass index (BMI) of 25.0 ± 2.4 kg/m^2 formed the final study group and were divided into three groups according to their plasma total cholesterol levels. Group 1 contained 45, group 2 contained 49 and group 3 contained 14 men having either normal (4.9 ± 1.2 mmol/L), mildly elevated (5.5 ± 0.8 mmol/L) or severely elevated (7.8 ± 1.9 mmol/L, subjects with FH) plasma cholesterol levels, as described previously [15,17]. There were five smokers among these men and none had diabetes. Written informed consent was obtained of all study subjects before the study. The study protocol was approved by the Joint Ethics Committee of the Turku University and the Turku University Central Hospital.

2.2. Laboratory measurements

Venous blood samples were obtained after an overnight fast. Total cholesterol, plasma triglycerides and HDL cholesterol concentrations were measured as described elsewhere [15,17]. The LDL cholesterol concentration was calculated by the formula of Friedewald et al. [18]. Apolipoprotein B and apolipoprotein A–I concentrations were measured as described previously [15,17].

2.3. PET protocol

Each participant underwent myocardial perfusion measurement twice after a 6 h fast, once at rest and once after administration of adenosine or dipyridamole, as described previously [15,17]. The flow marker was [^{15}O] H_2O, which was administrated intravenously at rest and separated either 2 min after the beginning of an intravenous infusion of adenosine or 6 min after the beginning of administration of dipyridamole. Heart rate and blood pressure were monitored during the studies to calculate the rate-pressure product (RPP) as an index of cardiac work. Electrocardiography was followed throughout the studies.

2.4. Calculation of blood flow

Large regions of interest were placed on representative transaxial ventricular slices covering the anterior and lateral free wall of the left ventricle. Qualitative analysis of the images did not reveal any regional differences in the distribution of blood flow. Therefore, in order to enhance accuracy and statistics of flow measurements, the average flow of the global left ventricular myocardium was calculated.

The CFR was defined as the ratio of overall MBF after adenosine or dipyridamole administration to flow at rest. The coronary resistance values were calculated both at rest and after adenosine or dipyridamole infusion by dividing the mean blood pressure by the respective flow value (expressed

as mmHg min g mL^{-1}). The resting blood flow adjusted for RPP was calculated by multiplying the subject's resting blood flow by the ratio of the mean RPP of the study population to the respective RPP of the subject. These values were then used to calculate the corrected CFR as the ratio of adenosine or dipyridamole induced hyperemia to RPP corrected flow at rest.

2.5. DNA extraction and HL genotyping

DNA was isolated from white blood cells using a commercial kit (Qiagen Inc., CA, USA). The C-480T polymorphism of the HL gene was identified by PCR as described [15].

2.6. Statistical analysis

Statistical comparisons were made by analysis of covariance (ANCOVA), using age and BMI as covariates. χ^2-test was used to test frequency differences among study groups and to test whether the genotypic distribution of alleles were in Hardy–Weinberg equilibrium. The study group by HL genotype interaction in relation to MBF indices was analyzed by two-way analysis of variance. In our statistical analysis, CFR was used as a primary dependent variable and the other myocardial blood flow variables as supportive explorative variables. A multiple linear regression analysis was performed to determine the simultaneous and independent effects of promoter variants of HL, age, BMI, serum lipids, blood pressure, adenosine or dipyridamole administration, and study group on blood flow during pharmacological induced hyperemia, coronary resistance, and CFR. Statistical analyses were performed using SPSS 12.0.1 for Windows software (SPSS Inc., Chicago, IL, USA). The level of significance was set at $p < 0.05$. Data are expressed as mean ± S.D. unless otherwise stated.

3. Results

Clinical characteristics, lipid and apolipoprotein values and genotype distributions of the study participants are shown in Table 1 according to the cholesterol group. There were statistically significant differences among groups in age, levels of plasma total, LDL, HDL cholesterol, and apolipoprotein B. Of the 108 subjects, 55 (50.9%) were CC homozygotes, 39 (36.1%) were heterozygotes, and 14 (13.0%) were TT homozygotes. The genotype distribution followed the Hardy–Weinberg equilibrium. Because the number of TT homozygous subjects was small and there was only one subject with the TT genotype in the group with FH, the TT and CT genotype groups were combined for the further analysis.

In our study, adenosine and dipyridamole were used for 49 and 59 subjects, respectively. The blood flow values were similar in these groups, which is in agreement with the previous studies [19].

MBF indexes according to HL genotype in different study groups are shown in Table 2. Within groups 1 and 2, we found that T allele carriers had higher CFR than men with CC genotype after adjustment for age, BMI, and HDL cholesterol ($p = 0.033$ and 0.018). In men with FH, the T allele carriers tend to have lower CFR than men with CC genotype, although it did not reach significant difference. Within group 1, the T allele had lower hyperemic blood flow ($p = 0.053$), higher coronary resistance during hyperemia ($p = 0.038$) than men with CC genotype. The flow during hyperemia appeared to be lower in T allele carriers than in men with CC genotype, although this tendency did not reach a statistically significant difference in groups 2 and 3.

The effect of HL genotype on MBF indices was parallel within all study groups and there were no statistically significant interactions between the study group and HL genotype. Therefore, groups were combined for fur-

Table 1
Characteristics of study subjects divided according to the degree of plasma cholesterol level (groups 1–3)

	Group 1 ($n = 45$)	Group 2 ($n = 49$)	Group 3 ($n = 14$)	p-Value for trend
Age (years)[a]	34.8 ± 3.6	35.2 ± 3.9	30.2 ± 7.1	0.001
Body mass index (kg/m^2)[a]	24.7 ± 2.2	25.1 ± 2.3	26.0 ± 2.8	NS
Total cholesterol (mmol/L)	4.91 ± 1.20	5.52 ± 0.78	7.76 ± 1.92	<0.001
Triglycerides (mmol/L)	1.10 ± 0.58	1.22 ± 0.66	1.23 ± 0.28	NS
HDL cholesterol (mmol/L)	1.25 ± 0.43	1.37 ± 0.29	1.00 ± 0.20	0.008
LDL cholesterol (mmol/L)	3.18 ± 1.12	3.59 ± 0.66	6.19 ± 1.82	<0.001
Apolipoprotein A–I (g/L)	1.52 ± 0.22	1.45 ± 0.23	1.36 ± 0.16	NS
Apolipoprotein B (g/L)	0.89 ± 0.29	1.05 ± 0.18	1.49 ± 0.35	<0.001
HL C-480T polymorphism, n (%)				NS[b]
CC	23 (51.1)	26 (53.1)	6 (42.9)	
CT	17 (37.8)	17 (34.7)	7 (50.0)	
TT	5 (11.1)	6 (12.2)	1 (7.1)	

Analysis of co-variance (ANCOVA), age and body mass index as covariates. Abbreviations: HL, hepatic lipase; NS, not significant. *Note*: the groups having either normal (group 1), mildly (group 2) or severely (group 3, subjects with familial hypercholesterolemia) elevated plasma cholesterol level.

[a] Analysis of variance.

[b] χ^2-test.

Table 2
Myocardial blood flow indices according to HL genotype and study group

	Group 1	Group 2	Group 3
Flow at rest (mL g^{-1} min^{-1})[a]			
CC	0.83 ± 0.15	0.80 ± 0.15	1.03 ± 0.13
CT, TT	0.81 ± 0.23	0.89 ± 0.28	0.86 ± 0.19
Flow during hyperemia (mL g^{-1} min^{-1})			
CC	4.14 ± 1.63	3.52 ± 0.78	3.94 ± 1.41
CT, TT	3.38 ± 1.70[†]	3.15 ± 0.88	2.82 ± 1.63
CFR			
CC	5.11 ± 2.23	4.58 ± 1.49	3.90 ± 1.54
CT, TT	4.16 ± 1.83[*]	3.68 ± 1.03[*]	3.19 ± 1.43
CR (mmHg min g mL^{-1})			
CC	24.60 ± 13.17	28.36 ± 6.89	21.94 ± 11.06
CT, TT	36.43 ± 31.06[*]	30.34 ± 9.09	43.87 ± 29.55

Abbreviations: CFR, coronary flow reserve; CR, coronary resistance; HL, hepatic lipase. Groups are listed in Table 1.
[a] Corrected by rate pressure product. ANCOVA between HL genotype groups (age, BMI, HDL cholesterol as covariates).
[*] $p < 0.05$ vs. CC genotype, same group.
[†] $p = 0.053$ vs. CC genotype, same group.

ther analysis. In all subjects, blood flow at rest was 0.85 ± 0.21 mL g^{-1} min^{-1}, coronary blood flow during hyperemia 3.52 ± 1.36 mL g^{-1} min^{-1}, CFR 4.27 ± 1.70, and coronary resistance during hyperemia 30.4 ± 18.8 mmHg min g mL^{-1}. Basal flow did not differ between the genotype groups. However, men with the CC genotype had a significantly higher hyperemic blood flow (3.86 ± 1.26 mL g^{-1} min^{-1} versus 3.20 ± 1.38 mL g^{-1} min^{-1}, $p = 0.007$), higher CFR (4.80 ± 1.77 versus 3.77 ± 1.43, $p = 0.001$) and lower coronary resistance during hyperemia (25.63 ± 9.98 mmHg min g mL^{-1} versus 35.00 ± 23.95 mmHg min g mL^{-1}, $p = 0.003$) than the T allele carriers. Adenosine or dipyridamole infusion induced significant and parallel increases in heart rate and RPP in both HL genotype groups (data not shown) and the hemodynamic variables were similar between HL genotype groups at rest and after adenosine or dipyridamole infusion (data not shown).

In multivariate analysis, after adjustment for age, BMI, serum lipids, blood pressure, adenosine or dipyridamole administration, and study group, HL genotype remained significant, independent predictor of coronary flow during hyperemia ($p = 0.016$), coronary resistance ($p = 0.014$) and CFR ($p = 0.005$), respectively (Table 3).

No interactions were found between HL genotype and other lipid parameters, such as HDL cholesterol or triglycerides, on indices of MBF (data not shown).

4. Discussion

In this study we investigated whether the HL C-480T polymorphism is associated with indices of MBF assessed by PET in three groups with different lipid status in young asymptomatic Finnish men. Since there were no differences in the effect of HL genotype on the indices of myocardial blood blow among the groups having different plasma cholesterol levels. Therefore, the groups were combined for further analyses. The subjects with the T allele had lower coronary flow during hyperemia, lower CFR, and higher coronary resistance during hyperemia than subjects with the CC genotype. In multivariate analysis, the effect of HL C-480T polymorphism on the indices of MBF was independent of other risk factors for CAD.

Previously, abnormal CFR has been demonstrated to be an early manifestation of atherosclerosis and CAD. Impaired CFR has been observed in healthy subjects with risk factors for CAD [20,21], and in patients with FH and other dyslipidemias [17,20,22]. In our study, the T allele carriers had lower blood flow during hyperemia, lower CFR and higher coronary resistance than men with the CC genotype. This is in line with our previous study showing that the HL

Table 3
Multivariate analysis for the associations of coronary risk factors and HL genotype with myocardial blood flow indices measured by positron emission tomography

Variables	Flow during hyperemia		Coronary resistance		Coronary flow reserve	
	Beta	*p*-Value	Beta	*p*-Value	Beta	*p*-Value
Age	−0.129	0.24	0.249	0.018	−0.123	0.24
Body mass index	−0.107	0.32	0.142	0.16	0.069	0.50
Total cholesterol	−2.159	0.38	2.609	0.26	−2.030	0.38
Triglycerides	0.559	0.25	−0.797	0.084	0.698	0.13
HDL cholesterol	0.287	0.42	−0.465	0.17	0.197	0.56
LDL cholesterol	1.006	0.32	−3.865	0.12	2.640	0.29
Apolipoprotein A–I	0.131	0.55	0.005	0.98	0.061	0.77
Apolipoprotein B	−0.580	0.31	1.436	0.009	−0.779	0.15
Ade or Dip infusion	0.084	0.64	−0.352	0.039	0.358	0.037
Groups	−0.165	0.29	0.186	0.21	−0.253	0.088
HL genotype	−0.241	0.016	0.233	0.014	−0.269	0.005
Entire model		0.188		0.006		0.009

Beta, standardized regression co-efficient from mulivariate linear regression analysis; Ade, adenosine; Dip, dipyridamole; HL, hepatic lipase. Group refers to study groups with different cholesterol levels; units for all lipids are (mmol/L), for apolipoproteins A–I and B (g/L) and body mass index (kg/m^2).

C-480T polymorphism was associated with CFR in mildly hypercholesterolemic, otherwise healthy young men [15]. The present study extended the result of our earlier study, since in this larger material the HL polymorphism was associated with all the three indices of MBF, i.e., blood flow during hyperemia, coronary resistance, and CFR. Moreover, the results were similar in men with normal cholesterol level to men with severely elevated total cholesterol level.

The mechanisms behind the effects of HL polymorphism on MBF are not known. In our pooled data, none of the lipid variables were in statistically significantly association with CFR in univariate analysis. Therefore, it is likely that the HL T allele derived effect on CFR is mediated with some other lipid mechanism such as direct effects on artery wall macrophage lipid metabolism, which was not evaluated in the present study. One of the mechanisms might involve HL activity, which is associated with the HL C-480T polymorphism [6,7] and is shown to correlate with the progression of atherosclerosis and CAD [9,23,24]. HL is expressed within the macrophages of atherosclerotic plaques, and it has been speculated that HL might have a direct role in the pathogenesis of atherosclerosis through a pathway that does not involve changes in plasma lipoprotein metabolism [2,3]. Our results are in line with this assumption since the effect of HL genotype on coronary reactivity seems to be independent of the levels of plasma cholesterol. In this study, we were not able to find a significant association between HL C-480T polymorphism and HDL cholesterol or other lipids which is in consistent with several previous studies [7,10,25,26] but not all [13,27]. Moreover, our multivariate analysis supports the independent role of HL C-480T polymorphism in the early atherosclerotic changes. Nevertheless, the T allele may still act through some other lipid mechanisms, which were not evaluated in the present study. HL may also influence the cholesterol transfer between different lipoprotein fractions. HL activity influences the pre-beta-HDL subfraction [28], which is an efficient acceptor of peripheral cell cholesterol and a key mediator in reverse cholesterol transport. Therefore, low HL activity associated with the T allele may decrease the production of pre-beta-HDL, and impair reverse cholesterol transport and thus increase cholesterol levels in artery wall. On the other hand, low HL activity may reduce the clearance of triglyceride-rich lipoproteins, which could impair endothelial function [29] and possible also MBF.

In line with our findings, it has been shown that HL activity is inversely correlated with the amount of calcified atherosclerotic changes in patients homozygous for FH [30]. Moreover, Hokanson et al. [11] found that the T allele was associated with coronary calcification in type 1 diabetes and Andersen et al. reported in the Copenhagen Heart Study almost two-fold higher risk of CAD in homozygote T allele carriers relative to homozygote C allele carriers [12]. Low HL activity was also found to be associated with severe atherosclerosis in normolipidemic subjects [9]. In addition, the normolipidemic men with symptomatic CAD and diffuse atherosclerotic narrowing of the coronary vessels have a lowered HL in comparison with men with normal angiograms [24]. In another study, multivariate regression analysis showed that, the HL activity was the most important determinant of changes in coronary atherosclerotic lesions, with the lower HL values in the lesion progression group [9]. Recently, Dugi et al. [23] reported that low HL activity is a novel risk factor for CAD, and that subjects with T allele of C-480T polymorphism have significantly greater extent of CAD than subjects with the CC genotype. All these previous studies support our findings showing a decreased coronary reactivity and MBF in subjects carrying the T allele.

In this study, two vasodilating agents, adenosine and dipyridamole were used in PET studies and are previously shown to induce comparable degrees of myocardial hyperemia [31]. In our study, the blood flow values were similar in these groups, which is in agreement with the previous studies [19]. Increased shear stress associated with increased flow is found to induce release of vasodilating substances from endothelial cells [32], and thus elicit more prominent vasodilation in vessels with normal endothelium than in vessels with a damaged endothelium. Indeed, the coronary flow response to dipyridamole or adenosine has been found to be related to endothelium-dependent vasodilatation [33,34] and recently it was found that a significant part of the adenosine response is endothelium dependent [35]. Therefore, coronary flow response to these two agents can be regarded as an overall measure of endothelial function and vascular smooth muscle relaxation. The use of a male study population rather than a population including both sexes is one of our study's strengths rather than a limitation, since gender is the strongest predictor of MBF during hyperemia [36]. Whether the same results can be extrapolated to female subjects remains to be shown. Also, it would have been interesting to use a larger sample size, but this was impossible due to expensive and laborious nature of the PET technique.

In conclusion, our results suggest that the effect of the HL C-480T polymorphism on the indices of MBF is independent of different levels of plasma cholesterol and other traditional risk factors for CAD. This study is likely to have important implications for diagnostic testing and for early preventive therapy. Although risk factor analysis allows for the better characterization of high-risk groups, early detection of CAD is desired in order to provide guidelines for aggressive therapy. It is clear that genetic factors acting directly on vascular cells contribute importantly susceptibility to CAD. In fact, studies on genetic susceptibility to atherosclerosis in mice suggest that such factors may be more significant than those influencing the traditional risk factors. The identification of these genetic factors is expected to provide an understanding of new pathways and lead to new treatments and diagnostic tests for CAD. Based on the rapid progress being made, an individual's genotype may well play an important clinical role in the assessment, prevention, and treatment of atherosclerosis. Screening for this variant in the HL gene promoter region will allow a better characterization of individual risk of early atherosclerotic disease process in young men.

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The hepatic lipase gene C-480T polymorphism in the development of early coronary atherosclerosis: the Helsinki Sudden Death Study

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Abstract

Background: The T allele of the hepatic lipase (HL) C-480T polymorphism was previously found to be associated with lower post-heparin plasma HL activity, atherosclerosis and risk of coronary artery disease. We studied the association of HL C-480T polymorphism with the extent of atherosclerosis at vessel-wall level in an autopsy series of middle-aged men.

Materials and methods: An autopsy cohort of 700 Caucasian Finnish men aged 33 to 70 years (mean 53 years), which comprised two autopsy series, collected 10 years apart during 1981 to 1982 and 1991 to 1992, were analyzed. Areas of coronary wall covered with fatty streaks and fibrotic and complicated lesions were measured using computer-assisted planimetry and related to HL C-480T genotypes (CC, CT, and TT).

Results: There was a significant age-by-genotype interaction on the mean percentage area of fatty streaks (P=0.01). The HL C-480T polymorphism was a significant explanatory factor for fatty streak area in men under 53 years of age with or without age, body mass index, hypertension, diabetes, smoking, alcohol consumption, apolipoprotein E genotype, and series number as covariates. Men carrying the TT genotype had two times larger areas of fatty streaks compared to the CC carriers (8.8% vs. 4.3%, P=0.009). However, this association disappeared in men over 53 years. The areas of more advanced atherosclerotic lesions did not vary significantly among the genotype groups.

Conclusions: Our results suggest that the HL C-480T polymorphism affects the formation of early coronary atherosclerotic lesions in men in their early middle age.

Key words: atherosclerosis, autopsy study, fatty streak, hepatic lipase, polymorphism

Introduction

Human hepatic lipase (HL) plays a central role in lipid metabolism and atherosclerosis [1]. HL is a lipolytic enzyme synthesized mostly by the liver [2] and also by macrophages [3]. HL hydrolyzes triglycerides and phospholipids on lipoprotein particles and may also serve as a ligand that mediates the binding and uptake of lipoproteins via proteoglycans and/or receptor pathways [4].

The HL gene, located on chromosome 15 (15q21-23), spans over 60 kb and contains 9 exons and 8 introns. The HL gene has a functional promoter polymorphism, designated alternately as the -480 C→T or the -514 C→T [5, 6], affecting transcription and leading to three genotypes (CC, CT, and TT). A recent meta-analysis of 25 studies showed the HL -480 T allele to be associated with lower HL activity and higher plasma high-density lipoprotein (HDL) cholesterol concentration [7]. In the last few years, several laboratories have reported the results of their studies investigating the relationship between HL C-480T polymorphism and the presence of coronary artery disease (CAD). The findings of these studies have been inconsistent [8-12]. Recently, in a family-based study of 1,012 patients from 386 families, the -480T allele was found to be associated with CAD [13].

Given the central role of HL in lipid metabolism, it can be hypothesized that HL C-480T polymorphism may affect the pathogenesis process of atherosclerosis, which can be measured at the vessel-wall level. To our knowledge, there are no previous reports on the relation between HL C-480T polymorphism and autopsy-verified atherosclerosis. It has been previously shown that the apolipoprotein E (apoE) ε4 allele is associated with a larger area of coronary atherosclerosis in early middle-aged men [14]. Recently, it was shown that the risk of ischemic heart disease associated with the T allele of HL C-480T polymorphism was more obvious among carriers of the apoE ε4/3 genotype [8]. In this paper, we report the results of an autopsy study where we related the HL C-480T genotypes with the severity

of atherosclerosis in the coronary arteries with or without adjustment for apoE genotype and classical risk factors of CAD.

Materials and Methods

Subjects

The Helsinki Sudden Death Study (HSDS) was designed to investigate the risk factors for sudden out-of-hospital cardiac death in Finnish middle-aged men who had lived in Helsinki and surrounding areas. The HSDS study comprised two consecutive series of a total of 700 white Finnish men subjected to a medicolegal autopsy at the Department of Forensic Medicine, University of Helsinki. The first series took place in 1981 to 1982 (A series, n=400), and the second ten years later, in 1991 to 1992 (B series, n=300). The mean age of subjects in both series was 53 years (range 33 to 70 years). The distribution of subjects by cause of death was as follows: cardiac causes in 41.1% (n=288), other diseases in 20.0% (n=140), and suicides or accidents in 38.9% (n=272) of the cases. The study was approved by the Ethics Committee of the Department of Forensic Medicine, University of Helsinki.

DNA extraction and genotyping

In the A series, DNA was extracted from paraffin-embedded samples of cardiac muscle, and in the B series from frozen (-70ºC) cardiac muscle samples. The method used for genotyping the HL C-480T polymorphism for the B series was based on PCR amplification, restriction enzyme analysis and DNA electrophoresis, the procedure has been described previously [15]. For the series A, DNA samples were genotyped by employing the 5’ nuclease assay for allelic discrimination using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). PCR reaction containing genomic DNA, 2 × TaqMan Universal PCR Master Mix, 900 nM of each primer, and 200

nM of each probe was performed in 96-well plates according to standard protocol in a total volume of 25 µl. Water controls and known control samples previously typed by restriction fragment length polymorphism-PCR analysis were run in parallel with unknown samples. To show that the genotyping results from these two methods match perfectly, twelve samples were randomly selected and analyzed in both methods, revealing exactly the same genotype results. After cycling, end-point fluorescence was measured, and genotype calling was carried out by the allelic discrimination analysis module. The genotyping results were the same for the samples run in both methods. Genotype information was obtained for 682 men.

ApoE genotyping was performed as described previously [14].

Measuring the area of atherosclerosis by morphometry

At autopsy, the proximal parts of the left anterior descending coronary artery, right coronary artery, and left circumflex coronary artery were collected for analysis. Coronary arteries were dissected free, opened, attached to a card, and then fixed in 10% buffered formalin. The arteries were stained for fat by the Sudan IV staining method. The measurements of the atherosclerotic lesions were based on the protocols of two international studies: the International Atherosclerosis Project, Standard Operating Protocol 1962 [16], and the World Health Organization Study Group in Europe [17]. Any flat or slightly elevated intimal lesion that stained distinctly with Sudan IV and showed no apparent change beneath it was classified as a fatty streak. A raised lesion not exhibiting ulceration, hemorrhage, necrosis or thrombosis was regarded as a fibrous lesion. A complicated lesion, in turn, was considered to include one or several of the above-mentioned changes, with or without calcium deposits. Coronary artery wall areas covered by fatty streaks and fibrous and complicated lesions were measured with a

computer-assisted planimetric technique. The percentage area of the various atheromatous changes was obtained by dividing the lesion area by the total surface area of the coronary segment studied. The mean proportional area of each particular atherosclerotic change in the three coronary arteries was used for statistical analysis.

Of the series of 700 men, arterial samples for the planimetric measurements were available from 511 men for the analysis of three coronary arteries. Complete data on HL genotype and autopsy data were available in 501 cases; this autopsy cohort constituted the final study population.

Risk factors for CAD

A spouse, relative, or close friend of the deceased could be interviewed in 500 cases (71.4%). Questions delineated past and recent smoking and drinking habits as well as hypertension, diabetes, and previous illnesses. On the basis of these interviews, the subjects were classified as smokers or non-smokers. For statistical analysis, ex-smokers were included in the class of smokers. Complete data on all risk factors in addition to HL genotype and autopsy data were available in 276 cases, which comprised the adjusted study population.

Statistical analysis

Data analysis for areas of atherosclerotic changes was based on analysis of variance (ANOVA), and analysis of covariance (ANCOVA), in which the possible confounding effects of age, body mass index (BMI), diabetes, hypertension, smoking, alcohol use, apoE genotype, and series number were taken into account by including them in the model as covariates. HL genotype status and age subgroup were used as factors in the two-way ANOVA and ANCOVA. Differences between specific genotype groups were analyzed

further with pairwise comparisons using Bonferroni correction for multiple comparisons. Categorical variables were compared with the χ^2-test. Non-normally distributed data of the plaque areas was analyzed after square-root transformation, but the results are expressed as crude. All statistical analysis was performed using the SPSS Version 13.0.1 for Windows.

Results

Of all 501 men with the genotype and autopsy data available, 269 (53.7%) carried the CC genotype, 186 (37.1%) had the CT genotype, and 46 (9.2%) were TT homozygotes. To study the effect of age, the subjects were divided according to the mean age of 53 years, a cut-off point previously used in the same autopsy series [14]. The background characteristics and the HL genotype frequencies for both age groups are shown in Table 1. The frequencies of C and T alleles were 0.70 and 0.30 for men < 53 years and 0.74 and 0.26 for men ≥ 53 years. There were no significant differences in genotype or allele distributions between these two age groups with or without interview data. The distribution of apoE genotype has been described previously [14]. The distributions of the genotypes were in accordance with the Hardy-Weinberg equilibrium. There were no significant differences between genotype groups regarding age, BMI, prevalence of hypertension and diabetes, smoking habits or alcohol use in the two age subgroups (Table 1).

We tested the statistical interaction between the HL genotype and age on the percentage area of fatty streaks using alternative models where age was used as a continuous or classified variable. Both interactions were significant (P=0.039 and P=0.014, respectively). As shown in Table 2, men < 53 years with the TT genotype had, on average, a 56% and 106% increase in the area of fatty streaks compared to the carriers of the CT or CC genotypes (P=0.009 and P=0.197, respectively). In contrast, no statistically significant

associations of HL genotype with areas of fatty streaks were found among men ≥ 53 years (Table 2).

In ANCOVA involving age, BMI, hypertension, diabetes, smoking, alcohol use, apo E genotype, and series number as covariates, the age-by-genotype interaction remained significant regarding the areas of fatty streak lesions (P=0.005, R^2=0.161). Men < 53 years with the TT genotype had a larger percentage area of fatty streaks compared to the carriers of other genotypes (P=0.001 by ANCOVA, R^2=0.201).

In men < 53 years old, there was also a dose-effect trend for the TT genotype carriers to have larger fibrotic and complicated lesions; however, this did not reach statistical significance. There were also no significant differences in the older age group between the HL genotype groups in fibrotic or complicated lesions (Table 2).

Discussion

This is the first study to investigate the association between a common polymorphism in the promoter region of the HL gene and the severity of atherosclerosis using arterial wall samples obtained at autopsies. In this study, we found an age-dependent association between the HL gene promoter C-480T polymorphism and the percentage area of fatty streaks in the coronary arteries. This association was independent of apoE genotype as well as classical CAD risk factors available. Carriers of the TT genotype had a statistically significantly larger mean percentage area of fatty streaks than carriers of the CC genotype. This difference was, however, only seen in men under the age of 53 years. This result agrees with our previous study reporting that the HL C-480T polymorphism affects the risk of sudden cardiac death especially among younger men [18].

Previous studies have reported that the HL C-480T polymorphism is associated with lower HL activity and higher HDL cholesterol level [7]. Recent *in vivo* and *in vitro* studies

suggest that HL may modulate the development of cardiovascular disease through its catalytic activity and independently of ligand-binging function pathways [19]. In addition, HL has been identified to be present in the vessel wall where it modulates atherogenic risk in apoE-deficient and lecithin-cholesterol acyltransferase transgenic mice [20]. These multiple functions of HL, which facilitate not only plasma lipid metabolism but also cellular lipid uptake, can be anticipated to have a major and complex impact on atherogenesis. Therefore, the C-480T HL polymorphism, by affecting HL synthesis and activity, may confer atherosclerosis susceptibility through multiple mechanisms that, in fact, maybe synergistic. There is ample evidence to demonstrate that HL affects both HDL cholesterol and triglyceride-rich lipoproteins [21, 22]. In our study, TT genotype carriers had a larger percentage area of fatty streaks. It can be postulated that TT genotype with low HL activity could induce the decreased production of preβ-HDL and delivery of HDL cholesterol to the liver [23]. In addition, TT genotype with low HL activity is associated with an impaired clearance of lipoprotein remnants – triglyceride-rich particles. It is possible that the larger percentage area of fatty streaks in TT carriers might reflect arterial wall uptake and retention of the increased numbers of triglyceride-rich particles and their HDL might be defective and unable to participate in the reverse cholesterol transport. On the other hand, HL is expressed in the macrophages of mice and human, and in mice this enhances early lesion formation in mice without modification of plasma lipoprotein lipids or HL activities. HL might, therefore, have a direct role in the pathogenesis of atherosclerosis through a pathway that does not involve changes in plasma lipoprotein metabolism [3, 20].

Moreover, the previous data are in line with our results which show the high incidence of CAD among patients with HL gene mutations leading to complete HL deficiency [1]. Patients with HL deficiency have often been shown to develop premature

atherosclerosis [24-27]. Furthermore, our results regarding the TT genotype carriers having larger areas of fatty streaks are in agreement with our previous paper which determined that the T allele is associated with impaired coronary blood flow reserve in healthy mildly hypercholesterolemic young men [15]. Recently Allen *et al.* found evidence of an association between the HL -480T allele and CAD when the family-based pedigree disequilibrium test was used in a well-defined Irish population [13]. This is in agreement with a Swedish twin study [28], showing that important genetic factors in the pathogenesis of atherosclerosis exert their strongest effect in youth or in the early middle-age.

Interestingly, African Americans have been shown to have more extensive fatty streaks than Caucasian subjects in all arterial segments [29]. In their case, however, the serum lipoproteins do not account for this excess of fatty streaks. Since black people have a higher frequency of the TT genotype, we speculate that the differences in HL C-480T polymorphism between black and white subjects might partially account for the ethnical difference regarding susceptibility to fatty streaks.

In our study, the association between HL C-480T genotype and the area of fatty streaks in coronary arteries was more evident in men under 53 years of age. We have shown a similar age-dependent association for another CAD candidate gene: apo E [14]. These findings support the evidence that at older age, other known risk factors for CAD may be more important in the process of atherosclerosis than genetic background [28]. Because Finns are genetically a quite homogeneous population [30], it is unlikely that the associations revealed in this study could be due to a stratification error in sampling. The missing association among older men probably reflect survivorship due to men carrying the TT genotype dying from sudden cardiac death at earlier age, as was observed in our recent study [18].

The limitation of our study includes the fact that this is a post hoc analysis of HSDS, a study not prospectively designed to assess the impact of the HL C-480T polymorphism on coronary atherosclerosis and the fact that lack of detailed investigation of risk factors due to the sudden death of the victims and the fact that the methods of determining risk factors were limited to information obtained from relatives and that the subjects had died suddenly, most of them not having seen a doctor or had any blood samples taken prior to their death, meant that we could not analyze the HL activity either. We also did not measure the cholesterol and apolipoprotein levels in postmortem samples. Furthermore, this study only enrolled Finnish men. We cannot ascertain whether the effect of the HL C-480T polymorphism would extend to other populations or women, as well.

In conclusion, our results suggest that HL C-480T polymorphism affects the formation of early coronary atherosclerotic lesions in men in their early middle age. The TT genotype of HL C-480T polymorphism occurred in approximately 10% of the men included in our study. This genotype might, therefore, be one piece along with other genes (e.g., apoE) and risk factors in the puzzle that explains why some individuals may develop premature CAD that can predispose them to a sudden pre-hospital cardiac death.

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Table 1. Characteristics of the men included in the Helsinki Sudden Death Study by hepatic lipase genotype and age group

	All subjects			< 53 years old			≥ 53 years old		
	CC	CT	TT	CC	CT	TT	CC	CT	TT
Number of subjects	269	186	46	103	85	20	166	101	26
Age, years	54.6 ± 9.3	53.2 ± 9.7	52.5 ± 8.6	44.5 ± 5.1	44.0 ± 4.8	44.3 ± 5.4	60.7 ± 4.7	60.5 ± 4.8	59.4 ± 4.6
BMI, kg/m^2	24.7 ± 5.0	25.0 ± 5.1	25.4 ± 4.7	24.7 ± 4.8	25.0 ± 4.8	24.7 ± 5.0	24.7 ± 5.1	24.9 ± 5.4	25.9 ± 4.5
Alcohol use, ≥60 g/d [a]	77 (46.4)	56 (48.3)	13 (54.2)	35 (54.7)	30 (63.8)	4 (50.0)	42 (41.2)	26 (37.7)	9 (56.3)
Coronary risk factor									
Diabetes [b]	39 (21.9)	32 (27.8)	9 (33.3)	10 (14.5)	11 (23.9)	3 (27.2)	29 (26.6)	21 (30.4)	6 (37.5)
Hypertension [b]	52 (29.2)	32 (27.8)	7 (25.9)	16 (23.2)	11 (23.9)	3 (27.2)	36 (33.0)	21 (30.4)	4 (25.0)
Smoking [c]	158 (84.9)	109 (84.5)	22 (88.0)	60 (85.7)	42 (79.2)	8 (88.9)	98 (84.5)	67 (88.2)	14 (87.5)

Values are mean ± SD or n (%). Abbreviation: BMI, body mass index.

[a]Data available in 306 cases. [b]Data available in 320 cases. [c]Data available in 340 cases.

Table 2. Mean percent area of atherosclerotic lesions in three coronary arteries by hepatic lipase (HL) genotype and age groups

	< 53 years old				≥ 53 years old				P, ANOVA		
	CC	CT	TT	P	CC	CT	TT	P	HL	Age	Interaction
	N=103	N=85	N=20		N=166	N=101	N=26				
Fatty streak	4.27 ± 4.05	5.64 ± 5.20	8.81 ± 8.20†	**0.006**	5.94 ± 4.74	5.53 ± 4.29	5.79 ± 3.70	0.616	**0.043**	0.717	**0.014**
Fibrotic	2.89 ± 3.97	3.14 ± 4.27	4.09 ± 4.80	0.615	4.44 ± 4.56	4.43 ± 3.81	4.53 ± 3.63	0.945	0.623	0.001	0.845
Complicated	0.56 ± 1.55	0.74 ± 2.16	1.33 ± 2.43	0.197	1.83 ± 2.81	2.01 ± 4.49	2.49 ± 4.40	0.836	0.347	<0.001	0.738

ANOVA, analysis of variance

Values are mean ± SD.

†P=0.009 for TT vs CC and P=0.197 for TT vs CT in Bonferroni post hoc test.

ELECTRONIC LETTER

Hepatic lipase C-480T polymorphism modifies the effect of HDL cholesterol on the risk of acute myocardial infarction in men: a prospective population based study

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Previous studies have revealed an inverse association between high density lipoprotein cholesterol (HDL-C) levels and the risk of acute myocardial infarction (AMI).[1 2] HDL-C level is modulated by genetic factors as well as environmental factors such as obesity, smoking, and physical exercise. Hepatic lipase (HL) is a lipolytic enzyme in lipoprotein metabolism, functioning as a phospholipase, an acylglycerol hydrolase, and a ligand of cell surface glycosaminoglycans, hydrolysing triglyceride-rich lipoprotein particles.[3] Recently, it has been reported that HL is synthesised by macrophages.[4] The HL gene variation has a significant effect on the variability of HDL-C in the population.[5 6] The functional HL promoter C-480T transition, also referred to as (-514C/T), leads to three common genotypes: CC, CT, and TT. The C and T alleles are associated with high and low HL activity, respectively.[7–9] However, the common polymorphisms of HL (-480T), cholesterol ester transfer protein (CETP) (TaqIB), lipoprotein lipase (S447X), and lecithin cholesterol acyl transferase (S208T) contribute only about 2.5% to the variance of HDL-C in the population.[10] This suggests that the HL C-480T polymorphism and HDL-C levels are different factors, and studying their interaction is justified. One previous study has shown that there might be an interaction between CETP gene polymorphism and HDL-C on the risk of myocardial infarction.[11] This result raises the possibility that other polymorphisms associated with HDL-C—for example, HL gene polymorphism—might interact with HDL-C and thus modify the risk of AMI. In fact, an effect of the C-480T polymorphism on coronary artery disease (CAD) has been sought in several studies with both negative[7 12] and positive findings.[13–15] One possible reason for the mixed results may be the interaction between HL C-480T genotype and HDL levels on CAD, a hypothesis not studied previously. To address this question, and to prospectively examine the relationship between the C-480T polymorphism of the HL gene and subsequent occurrence of AMI, we conducted a population based study in a cohort of Finnish men of middle age and with no previous history of coronary disease. We also explored the interaction between C-480T polymorphism and HDL-C levels on the risk of developing AMI.

Key points

- Decreased high density lipoprotein cholesterol (HDL-C) level is a well known risk factor for acute myocardial infarction (AMI). Hepatic lipase (HL) promoter C-480T transition, affecting gene transcription and leading to genotypes CC, CT, and TT, has been shown to be associated with HL activity and HDL-C concentration. We examined the relationship between C-480T polymorphism and subsequent occurrence of AMI in a prospective population based cohort of 126 men who developed AMI during follow up and 260 matched controls.
- Men with CC genotype tend to have a higher risk for AMI compared with T allele carriers, after adjustment for age, body mass index, smoking, years of hypertension, diabetes, family history of coronary disease, total cholesterol, triglycerides, and total energy and fat intake. However, when HDL-C is added additionally as covariate in an otherwise similar model, this association disappears.
- Men with CC genotype and HDL-C concentration in the lowest or second lowest tertile were found to have a 4/1 and 3.3/1 risk of developing AMI, respectively, compared with men in the highest HDL-C tertile, after adjusting for risk factors. A similar effect was not found in men with the T allele.
- The HL C-480T polymorphism might affect AMI risk differently in men with different HDL-C levels; the atherogenicity of low concentrations of HDL-C may be modulated diversely by different C-480T genotypes as well.

MATERIALS AND METHODS

Study subjects

The study subjects were from the Kuopio Ischaemic Heart Disease Risk Factor Study (KIHD). The study protocol was approved by the Research Ethics Committee of the University of Kuopio. A total of 2682 men from Eastern Finland, aged 42, 48, 54, or 60, were examined from 1984 to 1989. A DNA sample was available for 1263 of the men. A subpopulation of 480 men, which consisted of 160 men who developed AMI between the years 1985 and 1997, and two matched controls for each of them, was selected for this study. The average follow up time was nine years. To ensure the comparability of the control subjects, they were drawn from the same cohort (KIHD) as the cases. The controls were matched according to age, smoking, dietary iron, dietary saturated fatty acids, dietary cholesterol, and hair mercury content. In addition, examination year and month and the place of residence were identical for each case and the corresponding control. Because of inadequate blood samples, 94 of the men were excluded, leaving 386 men (126 men with AMI and 260 controls) for the final analysis. All participants gave written informed consent.

Examination protocol

The KIHD examination protocol and measurements have been described previously.[1] Subjects arrived to give fasting

venous blood samples in the morning. They had been instructed to abstain from alcohol for the three preceding days, and from smoking and eating for 12 hours. Blood was drawn after 30 minutes of supine rest. Body mass index (BMI) was calculated as weight (kg)/height^2 (m^2). The consumption of foods was assessed at the time of blood sampling, with instructions for food recording using household measures over four days. The instructions were given and the completed food records were checked by a nutritionist. The intake of nutrients and total energy intake were estimated by means of Nutrica software. The Nutrica databank uses mainly Finnish values for measuring nutrients. HL promoter C-480T genotype was determined by PCR and restriction enzyme *Nla*III digestion.[16]

Statistical analysis

Differences of risk factors between the cases and controls were tested for significance with Student's *t* test. To evaluate the relationship between HL genotypes and dependent variables, we used one way analysis of covariance (ANCOVA). Discontinuous variables, and the trend for the prevalence of genotypes according to HDL-C tertiles in the AMI and the control groups, were analysed using χ^2 tests. Logistic regression modelling was employed to examine associations between genotype and AMI, adjusted for age, BMI, smoking, years of hypertension, diabetes, family history of CAD, total cholesterol, triglycerides, and energy and fat intake. Finally, we further explored in a multivariate analysis the interaction between HL C-480T polymorphism and HDL-C tertiles on the risk of developing AMI. All statistical analyses were performed using SPSS version 11.5.

RESULTS

Table 1 shows the baseline characteristics for the AMI and the control groups. Age, smoking status, and total energy and fat intake did not differ significantly in the two groups, because they were matched for these factors. The AMI group, however, had significantly higher serum total cholesterol, LDL cholesterol, and apolipoprotein B, and lower HDL-C, than the control group. Diabetes and family history of CAD were more prevalent in the AMI group than in the control group.

Of the cases, 67 (53.2%) were CC homozygous, 47 (37.3%) were heterozygous, and 12 (9.5%) were TT homozygous. Of the controls, 124 (47.7%) had CC genotype, 108 (41.5%) had CT genotype, and 28 (10.8%) had TT genotype. Because the number of TT homozygous subjects in the AMI group was small, and there was no statistically significant difference between the T allele carrier groups in any background characteristics, the allele T carriers (CT, TT) were combined into one group which was compared with the group of CC homozygous participants.

In all subjects, the T allele carriers had higher total cholesterol ($p = 0.004$), apolipoprotein AI ($p = 0.028$), and HDL-C ($p = 0.09$) than men with CC genotype (table 2). In the AMI group, the T allele carriers tended to have higher HDL-C ($p = 0.093$) and apolipoprotein AI ($p = 0.079$) than men with CC genotype (table 2). In the control group, the T allele carriers had higher total cholesterol ($p = 0.009$) and apolipoprotein B ($p = 0.035$) than men with CC genotype (table 2).

Men with CC genotype tended to have a higher risk for AMI (relative risk, 1.5; 95% CI, 1.0 to 2.4; $p = 0.07$) as compared with T allele carriers after adjustment for age, BMI, smoking, years of hypertension, diabetes, family history of coronary disease, total cholesterol, triglycerides, and energy and fat intake. However, when HDL-C was added as covariate in an otherwise similar model, this association disappeared (table 3). Therefore, we further explored the interaction between C-480T polymorphism and HDL-C levels on the risk of developing AMI. For that purpose we divided the subjects into tertiles according to their serum HDL-C concentrations. The lowest tertile had HDL-C below 1.14 mmol/l, whereas the

Table 1 Baseline characteristics of subjects who developed AMI during follow up, and controls

Characteristic	AMI (n = 126)	Controls (n = 260)	p Value
Age	54.5 (3.8)	54.3 (4.6)	0.697
Body mass index (kg/m^2)	27.3 (3.7)	26.6 (3.1)	0.074
Proportion of smokers	36.5% (46)	29.6% (77)	0.200
Years of hypertension	3.1 (5.2)	3.0 (5.8)	0.793
Diabetes	12.7% (16)	5.0% (13)	0.012
Family history of coronary disease	57.1% (72)	45.4% (118)	0.039
Total fat intake (g, 4d mean)	112.5 (40.4)	118.6 (36.7)	0.142
Total energy intake (kJ, 4d mean)	10751 (3166)	11170 (2826)	0.190
Serum total cholesterol (mmol/l)	6.28 (1.25)	5.95 (1.02)	0.007
Serum LDL cholesterol (mmol/l)	4.39 (1.03)	4.05 (0.99)	0.002
Serum HDL cholesterol (mmol/l)	1.22 (0.27)	1.33 (0.29)	<0.001
Serum triglycerides (mmol/l)	1.40 (0.76)	1.37 (0.98)	0.251
Serum apolipoprotein B (g/l)	1.12 (0.22)	1.03 (0.22)	0.001
Serum apolipoprotein AI (g/l)	1.33 (0.26)	1.36 (0.24)	0.316

Data are mean (SD) or per cent (number of participants).
AMI, acute myocardial infarction; n, number.

Table 2 Baseline characteristics of subjects according to HL C-480T genotype and AMI status, during follow up

	HL C-480T genotype		
	CC	CT, TT	p Value
All subjects			
Number	191	195	
Serum total cholesterol (mmol/l)	5.89 (1.03)	6.22 (1.17)	0.004
Serum LDL cholesterol (mmol/l)	4.09 (1.00)	4.22 (1.03)	0.270
Serum HDL cholesterol (mmol/l)	1.28 (0.29)	1.31 (0.28)	0.090
Serum apolipoprotein B (g/l)	1.04 (0.23)	1.08 (0.22)	0.142
Serum apolipoprotein AI (g/l)	1.33 (0.23)	1.37 (0.26)	0.028
Serum triglycerides (mmol/l)	1.28 (0.69)	1.47 (1.08)	0.210
Subjects with AMI			
Number	67	59	
Serum total cholesterol (mmol/l)	6.10 (1.04)	6.48 (1.44)	0.106
Serum LDL cholesterol (mmol/l)	4.37 (0.98)	4.42 (1.09)	0.732
Serum HDL cholesterol (mmol/l)	1.19 (0.28)	1.26 (0.25)	0.093
Serum apolipoprotein B (g/l)	1.11 (0.22)	1.12 (0.23)	0.922
Serum apolipoprotein AI (g/l)	1.30 (0.24)	1.37 (0.28)	0.079
Serum triglycerides (mmol/l)	1.33 (0.62)	1.46 (0.90)	0.596
Control group			
Number	124	136	
Serum total cholesterol (mmol/l)	5.78 (1.00)	6.12 (1.02)	0.009
Serum LDL cholesterol (mmol/l)	3.94 (0.98)	4.14 (0.99)	0.125
Serum HDL cholesterol (mmol/l)	1.33 (0.29)	1.33 (0.29)	0.497
Serum apolipoprotein B (g/l)	1.00 (0.22)	1.07 (0.22)	0.035
Serum apolipoprotein AI (g/l)	1.34 (0.23)	1.37 (0.26)	0.179
Serum triglycerides (mmol/l)	1.25 (0.72)	1.47 (1.15)	0.272

Values are mean (SD). Significance based on ANCOVA. with age, body mass index, smoking, years of hypertension, diabetes, and family history of coronary disease as covariates.
AMI, acute myocardial infarction.

Table 3 Relative risk of acute myocardial infarction according to HL C-480T genotype status

	HL C-480T genotype		
Relative risk	CT, TT	CC	p Value
Number of participants	195	191	
Number of cases of AMI	59	67	
Number of controls	136	124	
RR	1.0	1.245 (0.813–1.907)	0.313
Age and smoking adjusted RR	1.0	1.289 (0.839–1.982)	0.247
Multivariate adjusted RR*	1.0	1.558 (0.986–2.459)	0.057
Multivariate adjusted RR†	1.0	1.531 (0.965–2.429)	0.070
Multivariate adjusted RR‡	1.0	1.405 (0.878–2.248)	0.156

AMI, acute myocardial infarction; RR, relative risk (95% confidence interval).
*Adjusted for age, body mass index, smoking, years of hypertension, diabetes, family history of coronary disease, and total cholesterol and triglycerides.
†Additionally adjusted for energy and fat intake.
‡Additionally adjusted for HDL cholesterol.

highest tertile had HDL-C greater than 1.39 mmol/l. The frequency of the T allele carriers increased according to HDL-C tertiles within AMI group. The T allele carriers were found in 40% of the 53 men with lowest HDL-C, in 48% of the 45 men with middle value HDL-C, and in 60% of the 25 men with highest HDL-C tertile (χ^2 test, p = 0.094 for trend). However, this trend was not found in the control group (table 4).

The subgroup analysis (table 5) revealed a significant interaction between HL C-480T polymorphism and HDL-C tertiles (p = 0.002). Those men who had CC genotype and whose HDL-C concentration was in the lowest or in the second lowest tertile had a 4/1 (95% CI, 1.7 to 9.6; p = 0.002) and 3.3/1 (95% CI, 1.4 to 7.8; p = 0.008) risk of developing AMI, respectively, compared with those in the highest HDL-C tertile after adjusting for age, BMI, smoking, hypertension, diabetes, family history of coronary disease, total cholesterol, triglycerides, and energy and fat intake.

DISCUSSION

The present study examined the role of HL C-480T polymorphism and serum HDL-C on a nine year follow up risk of AMI in originally healthy middle aged men. In this prospective study, we found that men with CC genotype had a slightly higher risk of developing AMI than did T allele carriers. Moreover, in secondary data analysis we found a highly significant interaction between the HL C-480T polymorphism and HDL-C levels in predicting development of AMI. This interaction revealed that men with the CC genotype and whose HDL-C was in the lowest tertile

Table 4 Distribution of HL C-480T genotypes according to HDL-C tertile

	Lowest tertile		Middle tertile		Highest tertile		p Value
Group	CC	CT, TT	CC	CT, TT	CC	CT, TT	for trend
AMI	32 (60%)	21	25 (52%)	23	10 (40%)	15	0.094
Control	39 (52%)	36	35 (42.7%)	47	50 (48.5%)	53	0.722

Data are numbers of subjects (per cent).
AMI, acute myocardial infarction.

Table 5 Adjusted relative risk of acute myocardial infarction according to hepatic lipase C-480T genotype status and levels of HDL-C

HDL-C (mmol/l)	HL C-480T genotype		
	CC (n = 191)	CT, TT (n = 195)	All (n = 386)
Lowest tertile	3.992†	2.426	3.378*
95% CI	(1.656–9.627)	(0.923–6.381)	(1.779–6.412)
Middle tertile	3.264‡	2.219	2.748*
95% CI	(1.358–7.847)	(0.920–5.356)	(1.506–5.017)
Highest tertile	1.0	0.968	1.0
95% CI		(0.376–2.488)	

Relative risk is adjusted for age, body mass index, smoking, years of hypertension, diabetes, family history of coronary disease, total cholesterol, triglycerides, and energy and fat intake. Total model <0.001; p value for interaction, 0.002.
CI, confidence interval. *p<0.001; †p = 0.002; ‡ p = 0.008, difference from the highest HDL-C tertile.

(<1.14 mmol/l) appeared to have a higher risk of developing AMI than did other HDL-C and HL genotype combinations.

There is evidence for an interaction of HL C-480T polymorphism with dietary fat intake,[17] and with medications that lower lipids.[18] In our study, such medication was used by only one subject, and it therefore could not have had a significant effect on our results. Dietary saturated fatty acids and dietary cholesterol were similar in men in the AMI and the matched control groups, which diminished the possibility of diet affecting our study. We also added total energy and total fat intake as covariates in multivariate analysis, which did not make any notable change, suggesting that energy and fat intake had no major effect on our results.

The previous findings as to the relationship between HL C-480T polymorphism and CAD are inconsistent.[7 12–15] One possible reason for the mixed results may be the interaction between HL C-480T genotype and HDL-C levels on CAD. However, no earlier data are available on whether the relation between HL C-480T polymorphism and AMI risk is modified by HDL-C levels. Thus, our results may explain some earlier ambiguous results on the association between HL C-480T genotype and CAD, and can be interpreted in two different ways. First, they may suggest that the HL C-480T polymorphism affects AMI risk differently in men with different HDL-C levels; or secondly, that the atherogenicity of low concentrations of HDL-C maybe modulated diversely by different C-480T genotypes. This finding is not fully comparable with the findings of Liu *et al*,[11] who found a similar interaction between CETP TaqIB polymorphism and HDL levels in predicting first myocardial infarction. However, our results support the idea that assessing the effect of HDL-C on AMI risk may require taking additional factors, such as HL C-480T or CETP TaqIB polymorphisms, into account.

We do not have any plausible explanation for how C-480T polymorphism interacts with HDL-C levels in predicting the risk for AMI. However, the C-480T polymorphism is a key determinant of HL levels, accounting for up to 38% of HL level variability.[19] The T allele has been shown to decrease the transcriptional activity of the HL gene also in the laboratory.[9] Our findings may be related to the HL activity either in the liver or alternatively in artery wall macrophages.[4 20]

Since HL is expressed within the macrophages of atherosclerosis plaques,[4 20] it has been postulated that HL might have a direct role in the pathogenesis of atherosclerosis without changes in plasma lipoprotein metabolism.[4 20] In theory, C-480T polymorphism and HDL-C levels may have synergistic effects on HL expression either in the arterial wall macrophages and/or in the liver, which may lead to varying risk for atherosclerotic diseases, such as AMI, in different individuals. Men with CC genotype together with low HDL-C

may have higher HL activity. This could contribute to the production of a high risk lipid profile by reducing the HDL_2 cholesterol pool and increasing small, dense LDL particles in the plasma, leading to impaired reverse cholesterol transport.[21] In addition, high HL expression in arterial macrophages may lead to enhanced foam cell formation within atherosclerotic plaques and thus faster progression of atherosclerosis.[4 20] The latter idea is supported by the fact that two independent atherosusceptible mouse models have shown that the localised production and accumulation of HL by macrophages present in the vessel wall contribute significantly to aortic lesion formation.[20] On the other hand, men with the T allele together with high HDL-C may have lower HL activity, leading to a lower risk lipid profile,[21 22] more effective reverse cholesterol transport,[21] less foam cell formation, and thus slower progression of atherosclerosis.[20]

In summary, our findings suggest that HL CC genotype, previously associated with high HL activity,[7–9 12 14] together with a low HDL-C level, may increase the risk of developing AMI. Our study does not determine the effect of HL genotype on the risk of developing AMI alone, but our data suggest the possibility that genetic screening for HL C-480T polymorphism together with determining the serum HDL-C level maybe helpful in identifying persons at high risk for AMI. Nonetheless, more research is still needed to clarify the complex role of HDL-C and HL C-480T polymorphism in the development of CAD.

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Age-dependent association between hepatic lipase gene C-480T polymorphism and the risk of pre-hospital sudden cardiac death: The Helsinki Sudden Death Study

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Abstract

Objective: We investigated the association between hepatic lipase (HL) C-480T polymorphism and the risk of acute myocardial infarction (AMI) as well as pre-hospital sudden cardiac death (SCD).

Methods: Seven hundred sudden or unnatural pre-hospital deaths of middle-aged (33–70 years, mean 53 years) Caucasian Finnish men were subjected to detailed autopsy (Helsinki Sudden Death Study). Genotype data were obtained for 682 men.

Results: In logistic regression analysis with age, body mass index, hypertension, diabetes, smoking and alcohol consumption as covariates, men with the TT genotype had an increased risk for SCD and AMI compared to CC carriers (OR = 3.0, $P = 0.011$; and OR = 3.7, $P = 0.003$). There was a significant age-by-genotype interaction ($P < 0.05$) on the risk of SCD. Compared to CC genotype carriers, the association between the TT genotype and SCD was particularly strong ($P = 0.001$) among men <53 years of age, but this association was non-significant among older men. This was mainly due to a strong association between the TT genotype and AMI due to severe coronary disease in the absence of thrombosis. Carriers of the TT genotype were more likely to have severe coronary stenoses (≥50%) than men with the CT or CC genotype ($P = 0.019$).

Conclusions: The results suggest that HL C-480T polymorphism is a strong age-dependent risk factor of SCD in early middle-aged men.

Keywords: Acute myocardial infarction; Hepatic lipase; Polymorphism; Sudden cardiac death

1. Introduction

Sudden cardiac death (SCD) is the most common manifestation of coronary heart disease in early middle age and accounts for approximately 50% of the estimated annual cardiovascular deaths. Despite decreased overall cardiac mortality, SCD rates appear to be rising in concert with escalating global prevalence of coronary disease. While many victims have a history of angina pectoris, myocardial infarction (MI) or previous cardiac arrest, a major proportion of events, especially in middle age, occur in subjects without any history of cardiac disease [1]. In addition to coronary heart disease with or without MI, SCD may also be due to ventricular arrhythmias associated with non-coronary heart disease. At present, there are no useful parameters to estimate the risk of pre-hospital SCD in an asymptomatic individual. Among those who died of acute MI (AMI) within 28 days of the event, the only factor that was associated with the risk of death was

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the serum cholesterol concentration in North Karelia, Eastern Finland [2]. Those with serum cholesterol of 310 mg/dl or more had a 3.8 times greater age-adjusted probability of dying within 1 h than those with serum cholesterol 310 mg/dl or less [2]. Family history of SCD has also been found to be an independent predictor of the risk of SCD [3,4], which suggests the influence of genetic factors.

Hepatic lipase (HL) is anchored on the vascular endothelium in the liver as well as on the surface of hepatocytes, where it functions as a lipolytic enzyme which hydrolyses triglycerides and phospholipids of circulating plasma lipoproteins particles [5]. HL also serves as a ligand facilitating lipoprotein uptake by cell surface receptors and proteoglycans, thereby directly affecting cellular lipid delivery [6]. Recently, it has been reported that HL is synthesised in mouse and human macrophages [7,8] and that its presence in aortic lesions in mice markedly alters lesion formation even in the absence of changes in plasma lipids [7,8]. These multiple functions of HL – which facilitate not only plasma lipid metabolism but also cellular lipid uptake – can be anticipated to have a major and complex impact on atherogenesis.

Recently, low HL activity has been suggested to be a novel risk factor for coronary artery disease (CAD) [9]. The HL gene has a functional C-to-T promoter polymorphism at position −480 (or −514) affecting transcription and leading to three genotypes (CC, CT and TT). The T allele has been found to associate with lower HL activity [10–13]. The findings of the association between the T allele and HDL cholesterol as well as total cholesterol concentration have been inconsistent [10,11,13–17]. In a recent meta-analysis [18], however, carriers of the TT genotype had an elevated total and HDL cholesterol level. In patients undergoing coronary angiography, the T allele associated with more severe CAD [9], despite the fact that not all studies on the possible association of HL C-480T polymorphism with the risk of CAD have produced similar results [9,11,13,19,20]. Recently, the T allele was found to be a susceptibility marker for CAD in a family-based association study of 1012 CAD patients from 386 families [21]. There are no studies on the association between the HL C-480T polymorphism and pre-hospital SCD.

In the present study, we investigated whether the HL C-480T polymorphism is associated with the risk of SCD and AMI in middle-aged Finnish men who had died suddenly. The Finns present a particularly suitable group for genetic association studies due to the homogenous population structure caused by isolation and famines [22].

2. Materials and methods

2.1. Subjects

The Helsinki Sudden Death Study (HSDS) was launched to investigate the lifestyle and genetic factors predisposing Finnish middle-aged men to sudden pre-hospital cardiac death. The HSDS study was comprised two consecutive series of a total of 700 White Finnish men who had lived in Helsinki and surrounding areas and who were subjected to a medicolegal autopsy at the Department of Forensic Medicine, University of Helsinki. The first series (A series, $n = 400$) was conducted during the years 1981 and 1982 and the second series (B series, $n = 300$) 10 years later, in 1991 and 1992. The mean age of subjects was 53 years (range 33–70 years). In the area of Helsinki, a forensic autopsy is performed for 42% of all persons who died at under 65 years of age with the following indications: sudden pre-hospital death without previous health records, accidental death, suspected intoxication, suicide or homicide. The cause of death was cardiac in 41.1% ($n = 288$), other diseases in 20.0% ($n = 140$) and intoxication or other violent cause (self-inflicted or accidental) in 38.9% ($n = 272$) of the cases. SCD referred to all cardiac-related deaths occurring out of hospital, or deaths in which the decedent was found dead on arrival. The study was approved by the Ethics Committee of the Department of Forensic Medicine, University of Helsinki.

2.2. Determining the MI phenotype at autopsy

Coronary thrombosis and MI in the series were recorded at autopsy, and the presence of MI was confirmed by nitro blue tetrazolium staining and by histological examination of the myocardium. The presence of neutrophil granulocytes was considered diagnostic of an AMI and the presence of fibrous scar tissue diagnostic of an old MI. Thrombosis was defined by a reddish clot attached to the coronary wall if the clot could not be detached with saline flushing.

2.3. Measuring the percentage of stenosis in silicone rubber casts of the coronary arteries

At autopsy, coronary angiography was performed using vulcanising liquid silicone rubber [23]. The proximal, middle and distal stenosis of the main trunks of the three main epicardial coronary arteries (left anterior descending, left circumflex and right coronary artery) were measured from the rubber cast model. The stenosis percentage was obtained by dividing the diameter (millimeters) of the greatest stenosis by the diameter of the nearest proximal undamaged part of the cast model of the artery, resulting in nine measurements on the degree of stenosis for each individual. The most severe stenosis was used to define the extent of coronary narrowing for each coronary artery. These measurements were available in 670 men.

2.4. DNA extraction and genotyping

In the A series, DNA was extracted from paraffin-embedded samples of cardiac muscle, and in the B series from frozen cardiac muscle samples. In the B series, genotyping was based on PCR amplification, restriction enzyme analysis and DNA electrophoresis; the procedure has been described previously [24]. In the A series, DNA samples were

Table 1
Characteristics of the study subjects according to cause of death

	All subjects			*P*-value		
	SCD	Violent death	Other diseases	ANOVA	SCD vs. violent death	SCD vs. other diseases
Number of subject, *n*	278	265	139			
Age (years)	56.4 ± 8.7	49.3 ± 9.4	53.4 ± 8.8	<0.001	<0.001	0.005
Body mass index (kg/m^2)	25.9 ± 5.2	23.9 ± 4.1	23.5 ± 4.8	<0.001	<0.001	<0.001
Average alcohol consumption (g/day)	64 ± 86	116 ± 106	112 ± 111	<0.001	<0.001	0.001
Smokers	171 (83.4)	138 (78.9)	90 (87.4)	NS	NS	NS
Hypertension	61 (31.1)	23 (13.8)	19 (19.6)	<0.001	<0.001	<0.05
Diabetes	54 (27.6)	31 (18.6)	25 (25.8)	NS	<0.05	NS
AMI	77 (27.8)	1 (0.4)	4 (2.9)	<0.001	<0.001	<0.001
Old MI	118 (42.4)	15 (5.7)	13 (9.4)	<0.001	<0.001	<0.001

Analysis of variance or χ^2 test. *Abbreviations*: AMI, acute myocardial infarction; NS, not significant; SCD, sudden cardiac death. Values are mean ± S.D. or *n* (%).

genotyped by employing the 5′ nuclease assay for allelic discrimination using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). PCR reaction containing genomic DNA, 2× TaqMan Universal PCR Master Mix, 900 nM of each primer and 200 nM of each probe was performed in 96-well plates using standard protocol in a total volume of 25 μl. Water controls and known control samples previously typed by RFLP-PCR analysis were run in parallel with unknown samples. Twelve samples randomly selected from the B series were rerun in parallel with unknown A series samples. After cycling, end-point fluorescence was measured, and genotype calling was carried out by the allelic discrimination analysis module. The genotyping results were the same for the samples run in both methods. Genotype information was obtained for 682 men, who then constituted our final study population.

2.5. Collection of risk factor data

Risk factors were sought for by interviewing a relative or close friend of the deceased. An informant was available for 500 (71%) of the cases. The questionnaire included a review of risk factors including hypertension, diabetes, past and recent smoking, drinking habits and previous illnesses. On the basis of these interviews, men were classified as smokers or non-smokers. Ex-smokers were included in the class of smokers for statistical analysis. Average daily alcohol consumption of the deceased was calculated from information given by the interviewed persons. On the basis of questions on previous illnesses, 103 men had suffered from hypertension and 110 men from diabetes.

2.6. Statistical analysis

Statistical analysis was performed using SPSS Version 13.0.1 (SPSS Inc., Chicago, USA). One-way analysis of variance (ANOVA) was used to test for mean differences in continuous variables among different groups. Differences between specific groups were further analysed with pairwise comparisons using Bonferroni correction for multiple comparisons. Categorical variables were compared with Pearson χ^2 test. We analysed the interaction between genotypes and age groups using logistic regression. Logistic regression analysis was also used to determine adjusted odds ratios for SCD and AMI according to HL genotype group. In this analysis, age, body mass index (BMI), smoking, diabetes, hypertension and alcohol consumption were used as covariates.

3. Results

3.1. Characteristics of the subjects

Table 1 shows descriptive data stratified by causes of death. Men who died of SCD were significantly older ($P < 0.001$), had higher BMI ($P < 0.001$) and were reported to be more hypertensive ($P < 0.05$) as well as consume less alcohol ($P < 0.001$) than those who died of other causes. Of the entire series of 682 men, 177 (26%) were found to have had a MI with or without an old MI, and 82 (12%) men had had an AMI with or without an old MI. Of the AMI cases, 38 were associated with coronary thrombosis. Of the subjects with AMI, one had died accidentally and four had some other severe underlying disease as the cause of death. There were no significant differences in these descriptive characteristics between men with or without interview data (data not shown). There were also no significant differences in cause of death, MI, AMI with or without old MI, and thrombosis between men in the A and B series (data not shown).

Overall, there were 364 subjects with the CC genotype (53.4%), 260 heterozygote subjects (38.1%) and 58 carriers of the TT genotype (8.5%). Allele frequencies of C and T were 0.72 and 0.28, respectively. The allele frequencies did not differ significantly between the two autopsy series or the subpopulations with or without interview data. The genotype distribution was in Hardy–Weinberg equilibrium and similar to distributions reported previously among the Finnish population [11,17] and some other populations [19,25].

The major risk factors for coronary heart disease did not differ between the HL C-480T genotype groups among men

Table 2
Distribution of hepatic lipase (HL) genotypes in men with SCD, AMI, old MI and in those who had died of non-cardiac causes

		All subjects				<53 years old				≥53 years old			
HL genotype	*N*	CC	CT	TT	*P*-value	CC	CT	TT	*P*-value	CC	CT	TT	*P*-value
Non-SCD	404	215 (53.2)	162 (40.1)	27 (6.7)		118 (52.0)	96 (42.3)	13 (5.7)		97 (54.8)	66 (37.3)	14 (7.9)	
SCD	278	149 (53.6)	98 (35.3)	31 (11.2)	0.087	37 (44.0)	34 (40.5)	13 (15.5)	**0.02**	112 (57.7)	64 (33.0)	18 (9.3)	0.66
AMI	82	42 (51.2)	29 (35.4)	11 (13.4)	0.23	11 (42.3)	9 (34.6)	6 (23.1)	**0.018**	31 (55.4)	20 (35.7)	5 (8.9)	0.99
Old MI	146	81 (55.5)	49 (33.6)	16 (10.9)	0.29	14 (50)	10 (35.7)	4 (14.3)	0.46	67 (56.8)	39 (33.1)	12 (10.1)	0.71
Stenosis percentage													
<50		194 (50.9)	161 (42.3)	26 (6.8)		107 (47.3)	104 (46.0)	15 (6.6)		87 (56.1)	57 (36.8)	11 (7.1)	
≥50		115 (56.1)	66 (32.2)	24 (11.7)	**0.019**	33 (55.9)	18 (30.5)	8 (13.6)	**0.047**	82 (56.2)	48 (32.9)	16 (10.9)	0.45

χ^2 test. *Abbreviations:* AMI, acute myocardial infarction; SCD, sudden cardiac death. Values are *n* (%).

who died of SCD, with the exception of age—men with the TT genotype were slightly younger than those with the CT or CC genotype (53.5 ± 9.0 years versus 55.4 ± 9.5 years versus 57.6 ± 7.8 years) ($P = 0.02$).

3.2. HL C-480T polymorphism and SCD

In univariate analysis, HL C-480T polymorphism, age, BMI, alcohol consumption and hypertension were significant predictors of SCD in all study subjects. In multivariate logistic regression analysis, they still remained significant predictors of SCD. The results were similar for the younger (<53 years) and the older age group (≥53 years), except for the fact that the HL C-480T polymorphism was a significant predictor of SCD only in the younger age group (data not shown). We tested the interaction between the HL genotype and age when age was as a continuous or classified variable, respectively. Both interactions were significant after adjusting for other covariates (age, BMI, hypertension, smoking, alcohol consumption and diabetes) ($P = 0.011$ and 0.018, respectively). The percentage of men with the TT genotype among SCD victims tended to be higher compared with that in the non-SCD group ($P = 0.087$). The difference in the genotype distribution was more pronounced ($P = 0.02$) among the younger men than the older age group (Table 2). In the whole study series, TT genotype was associated (OR 3.0, 95% confidence interval [CI] 1.3–6.8, $P = 0.011$) with an increased risk of SCD when compared with subjects carrying the CC genotype. This association was especially strong in younger men (OR 14.9, 95% CI 3.1–72, $P = 0.001$). In these younger subjects, the risk was also statistically significantly increased among TT homozygotes when compared to CT heterozygotes (OR 5.2, 95% CI 1.2–23.8, $P = 0.032$), whereas there were no significant differences between men with CT and CC genotypes. In older men, the association between the T allele and SCD weakened to non-significant.

3.3. HL C-480T polymorphism and AMI

The genotype distribution was similar among cases of AMI and controls ($P = 0.23$, Table 2) in the whole series. In the younger age group, however, there were significantly more carriers of the TT genotype among AMI cases than among controls ($P = 0.018$) (Table 2). With regard to the older men, the distribution of the TT genotype was similar in both groups. There were more carriers of the TT genotype among men with severe coronary stenoses (≥50%) than among men with less severe stenosis ($P = 0.019$, Table 2). This association again remained stronger among younger men ($P = 0.047$, Table 2).

TT homozygotes had an increased risk of AMI when compared to both CC homozygotes (OR 3.7, 95% CI 1.5–8.8, $P = 0.003$) and to CT heterozygotes (OR 2.9, 95% CI 1.2–7.1, $P = 0.019$) (Table 3). This association between AMI and the TT genotype was again more pronounced among younger men (OR 14.2, 95% CI 3.1–64.7, $P = 0.001$)

Table 3
Adjusted odds ratios by hepatic lipase genotype among men who died of SCD

	Number of subjects	OR (95% CI) TT vs. CT	*P*-value	OR (95% CI) TT vs. CC	*P*-value
All men					
SCD	278	2.3 (1.0–5.3)	0.059	3.0 (1.3–6.8)	**0.011**
AMI	82	2.9 (1.2–7.1)	**0.019**	3.7 (1.5–8.8)	**0.003**
AMI with thrombus	38	1.6 (0.3–8.2)	0.55	1.7 (0.4–8.4)	0.49
AMI without thrombus	44	3.8 (1.4–10.3)	**0.009**	5.5 (2.0–14.7)	**0.001**
Men < 53 years					
SCD	84	5.2 (1.2–23.8)	**0.032**	14.9 (3.1–72.0)	**0.001**
AMI	26	7.5 (1.7–32.0)	**0.007**	14.2 (3.1–64.7)	**0.001**
AMI with thrombus	12	1.1 (0.1–13.9)	0.94	0.7 (0.1–9.0)	0.81
AMI without thrombus	14	12.7 (2.1–75.1)	**0.005**	39.8 (4.9–324.4)	**0.001**
Men ≥ 53 years					
SCD	194	1.6 (0.6–4.5)	0.39	1.5 (0.5–4.0)	0.46
AMI	56	1.5 (0.4–5.0)	0.51	1.6 (0.5–5.3)	0.41
AMI with thrombus	26	2.3 (0.3–20.5)	0.47	3.0 (0.4–26.1)	0.31
AMI without thrombus	30	1.8 (0.5–7.1)	0.39	2.2 (0.6–8.3)	0.23

Statistics: OR (95% CI) were calculated in multivariate analysis with age, BMI, smoking, alcohol consumption, diabetes and hypertension as covariates.
Abbreviations: AMI, acute myocardial infarction; CI, confidence interval; OR, odds ratio; SCD, sudden cardiac death.

(Table 3). This was mainly due to the victims of AMI without thrombus, among whom TT homozygotes had an OR of 39.8 ($P = 0.001$) when compared to CC homozygotes (Table 3).

4. Discussion

The results of this study, based on a large cohort of Finnish men who had died suddenly pre-hospital, suggest that the homozygous TT genotype of the HL C-480T polymorphism is a genetic risk factor for SCD and particularly for AMI caused by severe CAD without plaque rupture and resulting thrombosis. Moreover, this association was more clearly seen among younger men. To our knowledge, this study is the first autopsy study showing the association between a common C-480T polymorphism in the promoter region of the HL gene and SCD. Our results also suggest that this association may be due to the presence of more severe coronary stenosis among TT genotype carriers, developing as early as in early middle age.

It has been shown previously that there is a high incidence of coronary heart disease in patients with HL gene mutations, leading to complete HL deficiency [26]. Patients with HL deficiency have often been shown to have premature atherosclerosis despite having increased HDL_2 cholesterol levels [27,28]. HL deficiency in humans is also associated with hypercholesterolemia and hypertriglyceridemia [27,28]. Previous studies have reported that cholesterol and triglyceride concentrations in American [29] and Japanese [30] cases of SCD are significantly higher than in cases of non-SCD. In a study conducted in the Finnish region of North Karelia, it was found that the strongest SCD risk factor in this population was serum cholesterol [2]. Elevated cholesterol may contribute to the development of fat-containing, vulnerable rupture-prone plaques [31]. The −480T allele of the HL gene has been reported to associate with reduced HL activity, increased HDL cholesterol levels, and, in some studies, higher triglycerides or total cholesterol level [10,14,18,32,33]. Low HL activity has been reported in patients with clinically overt CAD [9,34,35]. The effect of the HL C-480T polymorphism on CAD has been sought in several studies with conflicting results [9,10,19,20,36–39]. Furthermore, in a family-based study on 1012 patients from 386 families, the pedigree disequilibrium test demonstrated significant excess transmission to affected patients of the HL −480 T allele, which suggests that this may constitute a novel disease-susceptibility locus [21].

Our finding may be related to the HL activity either in the liver or in artery wall macrophages. The HL gene polymorphism, by affecting HL synthesis and activity, may significantly contribute to the process of reverse cholesterol transport modulating HDL catabolism. Interestingly, dietary fat intake has recently been shown to significantly modify the association between the polymorphism and HDL cholesterol concentrations. The T allele was correlated with higher HDL cholesterol concentrations and, thus, a lower risk of CAD only in individuals who usually adhered to a low-fat diet. In contrast, the TT genotype was associated with lower HDL cholesterol levels, with a possibly higher risk of CAD in individuals whose diet usually contained high amounts of fat [40,41]. TT subjects may have an impaired ability to adapt to diets high in animal fats, which might result in increased cardiovascular risk. An impaired triglyceride-rich lipoprotein remnant catabolism may also play a role here. The −480T allele was also associated with higher plasma triglycerides and total cholesterol in some studies [14,42]. von Eckardstein et al. [43] have suggested that the coincidence of hypertriglyceridemia and elevated HDL cholesterol increases the risk of MI. On the other hand, HL is expressed in the macrophages of mice and humans [7,8]. Macrophage HL expression in the arterial wall enhanced early lesion forma-

tion in mice without modification of plasma lipoprotein lipids or HL activities. It has been speculated that HL might have a direct role in the pathogenesis of atherosclerosis through a pathway that does not involve changes in plasma lipoprotein metabolism [7,8]. The C-480T polymorphism may, therefore, confer atherosclerosis susceptibility in people with different risk factors for CAD through multiple and possibly synergistic mechanisms.

This is a post hoc analysis of HSDS, a study not prospectively designed to assess the impact of the HL C-480T polymorphism on SCD. The limitations of our study are related to the nature of autopsy studies: since the subjects had died suddenly and most subjects had not seen a doctor nor had any blood samples taken prior to their death, we could not measure the HL activity. We also did not measure the cholesterol and apolipoprotein levels in postmortem samples. Another possible limitation is the fact that the methods of determining risk factors were limited to information obtained from relatives.

According to our results, the HL C-480T polymorphism seems to affect the SCD risk especially among younger men. This is in agreement with a Swedish twin study [44] showing that important genetic factors in the pathogenesis of atherosclerosis exert their strongest effect in youth or in the early middle age. However, due to the fact that 46.6% of the men in our series carried at least one copy of the T allele, this genetic marker alone cannot be used to predict future SCD events at population level.

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Hepatic Lipase C-480T Genotype-Dependent Benefit from Long-Term Hormone Replacement Therapy for Atherosclerosis Progression in Postmenopausal Women

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Hepatic lipase (HL) is a lipolytic enzyme that hydrolyzes triglycerides and phospholipids in almost all major classes of lipoproteins. The HL gene has a functional promoter polymorphism at position −480, which affects transcription and leads to CC, CT, and TT genotypes. We investigated the effect of long-term hormone replacement therapy (HRT) on the progression of atherosclerosis in a 5-yr follow-up observational study of 88 postmenopausal women with different HL genotypes (CC, n = 49; CT, n = 34; TT, n = 5). These women, aged 45–71 yr, were divided into three groups based on the use of HRT. The HRT-EVP group (n = 26) used sequential estradiol valerate (EV) plus progestin (levonorgestrel), the HRT-EV group used EV alone (n = 32), and the control group (n = 30) used no HRT. The HRT-EV and HRT-EVP groups started estrogen at menopause for estrogen-deficiency symptoms, whereas the control group took no estrogen due to either the absence of such symptoms or a dislike of estrogen therapy. In addition to serum lipid concentration and HL genotype, the atherosclerosis severity score (ASC) for the abdominal aorta and carotid arteries was determined by ultrasonography. There was a significant interaction between HRT therapy and HL genotypes on the increase in ASC ($P = 0.046$) after adjustment for age, body mass index, changes in high-density lipoprotein cholesterol and baseline ASC. In subjects with the T allele, the progression of ASC was significantly faster in the control group than the HRT group ($P = 0.0006$), whereas in the CC genotype, there were no significant differences in ASC progression between the control and HRT groups. Our results suggest that the beneficial effect of HRT on atherosclerosis progression was restricted to women with the T allele, in whom the progression of ASC was slower by half. These results may help us understand in greater detail the benefits and possible risks associated with HRT in atherosclerotic diseases. **(*J Clin Endocrinol Metab* 90: 3786–3792, 2005)**

CORONARY ATHEROSCLEROSIS IS an underlying cause of morbidity and mortality among women in the industrialized world. A number of observational studies have suggested that hormone replacement therapy (HRT) reduces the risk of atherosclerosis and coronary events in postmenopausal women (1–4). However, recently published results from randomized clinical trials of HRT indicate that the therapy does not slow the progression of coronary atherosclerosis in postmenopausal women (5–7). As to why not all patients benefit and some are even harmed by such therapy, our understanding is limited. However, it is still possible that a genetically determined subgroup of the population could benefit from this therapy. Previous studies have demonstrated that subgroups with apolipoprotein (apo) E4 (−) (8), myeloperoxidase promoter −463 GG (9), or estrogen receptor 1 *Pvu*II P/P genotype (10) are propitious for HRT. As part of an ongoing study of the genetic risk factors underlying variation in response to HRT, we focused on identifying genes predisposing to these differences.

One genetic candidate is a gene for hepatic lipase (HL), which is a lipolytic enzyme synthesized mostly by the liver (11). It has also recently been reported that HL is synthesized by macrophages (12). HL hydrolyzes triglycerides and phospholipids in almost all major classes of lipoproteins. It has also been held to act as a ligand that mediates the binding and uptake of lipoproteins via proteoglycans and/or receptor pathways (13). The HL gene has a functional promoter polymorphism at position C-480T (or 514) affecting transcription and leading to three genotypes (CC, CT, and TT). Previous studies have indicated that the presence of the T allele at position −480 is significantly consistently associated with low HL activity (14–17). On the other hand, the significant association of this allele with elevated high-density lipoprotein (HDL) cholesterol levels is at odds with this (14, 15, 18, 19). HL activity appears to be regulated by several factors, including intraabdominal fat (20), sex steroid hormones (21, 22), and HL C-480T polymorphism (17, 23). HRT has been found to reduce postheparin plasma HL activity (24), which activity is inversely associated with endogenous estrogen levels (25) and is higher in postmenopausal compared with premenopausal women (26). Given the wide spectrum of the effects HL exerts on lipoprotein metabolism and the significance of the promoter variant, it is reasonable to hypothesize

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Abbreviations: ANCOVA, Analysis of covariance; apo, apolipoprotein; ASC, atherosclerosis severity score; BMI, body mass index; CHD, coronary heart disease; EV, estradiol valerate; EVP, EV and progestin; HDL, high-density lipoprotein; HL, hepatic lipase; HRT, hormone replacement therapy; LDL, low-density lipoprotein; NAP, total number of plaques; RANOVA, ANOVA for repeated measures.

that genetic variation at this locus may also be involved in the variability in response to HRT treatment.

Two previous studies have investigated the relationship between the HL C-480T polymorphism and lipid profiles in postmenopausal women (27, 28). However, no previous study has reported on the relationship between the HL −480T polymorphism and progression of atherosclerosis severity in postmenopausal women during long-term HRT.

To elucidate the genetic variants that modulate HRT effects, we now assessed the association between the HL C-480T polymorphism and the effect of HRT in response of atherosclerosis severity among postmenopausal women. The purpose was to establish whether, as hypothesized, the HL C-480T polymorphism modifies the effect of HRT on the development of atherosclerosis.

Subjects and Methods

Subjects

In 1993 women attending a private outpatient clinic in Tampere for annual routine gynecological examinations were invited to participate. For the cross-sectional baseline study in 1993 (3), 120 nonsmoking, nondiabetic postmenopausal women aged 45–71 yr were enrolled. Eighty-eight of these women participated in this 5-yr follow-up study from 1993 to 1998. They had no clinically evident cardiovascular diseases or hypertension and were classified into three groups based on the use of HRT. The HRT-EVP group (n = 26) used estradiol valerate (EV) at 2 mg/d for 11 d, followed by EV continued with progestin (P) (levonorgestrel, 0.25 mg/d) for 10 d. In the HRT-EV group (n = 32), the treatment was EV at 2 mg/d continuously; the control group (n = 30) had never used HRT. In the HRT-EVP and HRT-EV groups, there was a pause in therapy for 7 d after each 21-d cycle. None of these women discontinued the therapy during follow-up. HRT, when used, was started at the time of menopause for climacteric symptoms. In the control group the main reasons for nonuse of HRT was the absence of vasomotor and other climacteric symptoms and dislike of HRT. In the HRT-EV, HRT-EVP, and control groups, 24, four, and six women had undergone hysterectomy, respectively. At baseline, the mean duration of EV and EVP treatment was 9.2 ± 3.7 and 10.9 ± 2.5 yr, respectively. The mean time from menopause in the control group was 11.9 ± 4.0 yr. The mean ages in the HRT-EVP, HRT-EV, and control groups were 59.7 ± 5.5, 60.4 ± 4.8, and 61.5 ± 5.8 yr, respectively ($P = 0.441$, by ANOVA). The mean body mass index (BMI) was similar in all study groups ($P = 0.953$). At baseline, all women were clinically healthy and used no lipid-lowering or other chronic medication. Ultrasonography and blood sampling took place in the University Hospital of Tampere. The study was approved by the ethics committee of the hospital. All participants signed an informed consent document.

Blood samples

Blood samples for serum lipid and genotype analyses were taken after overnight fasting. Sampling took place within 3 wk from ultrasonography and for HRT-EVP users during the third week of the hormone regimen. After separation of serum by low-speed centrifugation, the sera were divided into aliquots and stored at −70 C until analyzed.

Ultrasonography

Ultrasonography at baseline and follow-up were performed with Sonolayer V SSA 100 equipment (Toshiba Corp., Tokyo, Japan), as reported in detail elsewhere (3, 10). In brief, transverse and longitudinal scans of the extracranial carotid arteries were made bilaterally at four different segments of the carotid. Only fibrous and calcified atherosclerotic lesions were taken into consideration and were defined as plaques when distinct areas of mineralization and/or focal protrusion into the lumen were identified. An intimal-media far-wall thickness equal to or more than 1.3 mm at any carotid artery segment was defined as an atherosclerotic plaque (29) and the total number of plaques (NAP) was calculated. All carotid artery examinations were made with a 5.0-MHz convex transducer probe.

Longitudinal ultrasonographs of the abdominal aorta were obtained at 1-cm intervals and transverse scans at 2-cm intervals in the area of three aortic segments. Significant aortic plaques were defined as an intima-media far-wall thickness equal to or more than 3.0 mm (29). All aortic examinations were performed with a 3.75-MHz convex transducer probe. The reproducibility of our ultrasonographic protocol for significant aortic and carotid plaques was also examined: 1 month after the first assessment, 20 randomly selected subjects were invited to attend for a repeat examination. The repeatability of NAP between the first and second examination was 90% for the carotid artery segment areas and 100% for the aortic segments. All ultrasonographies were performed in blinded manner by one experienced ultrasonographer and radiologist (P.D.).

The atherosclerosis severity score (ASC) was constructed by dividing the atherosclerosis in the abdominal aorta and carotid arteries into three severity classes, *i.e.* 1 = slight (1.3–2 mm), 2 = moderate (2–3 mm), and 3 = severe (more than 3 mm). The ASC was then calculated as the sum of the severity classes in aorta and carotid artery. The total NAP was calculated (in the baseline study only because 5-yr data were not available) according to the NAP (criteria for plaques as above). Scoring was done by one person (P.D.) in blinded manner, without knowledge of HRT and HL genotype status.

HL genotyping

DNA was isolated from white blood cells using a commercial kit (QIAGEN Inc., Valencia, CA). As described previously (30), determination of the HL promoter C-480T genotype was carried out by PCR amplification using the primers 5′-AAGAAGTGTGTTTACTCTAGGATCA-3′ and 5′-GGTGGCTTCCACGTGGCTGCCTAAG-3′. Thermal cycling conditions were initial denaturation at 96 C for 3 min, followed by 33 cycles of amplification at 96 C for 1 min, annealing at 65.5 C for 1 min, and extension at 72 C for 1 min, with a final extension at 72 C for 5 min. The amplified DNA fragments were digested with the restriction enzyme *Nla*III (New England Biolabs, Beverly, MA) followed by electrophoresis on 3% agarose gel.

Other laboratory analyses

Lipid measurements were made at baseline and after the 5-yr follow-up. Serum total cholesterol and triglycerides were determined by a commercial method (Kodak Echtachem 700 XR; Eastman Kodak Co., Clinical Products Division, Rochester, NY). Serum HDL cholesterol and its subfractions (HDL_2 and HDL_3) were separated by a dextran-sulfate-magnesium precipitation procedure, and the cholesterol content was analyzed with a Monarch 2000 analyzer (Instrumentation Laboratory, Lexington, KY), using the cholesterinoxidase-peroxidase/antiperoxidase cholesterol reagent (catalog no. 237574; Roche, Mannheim, Germany) and a primary cholesterol standard (catalog no. 530, Orion Diagnostics, Helsinki, Finland). Low-density lipoprotein (LDL) cholesterol content was calculated according to the Friedewald formula (31). Apolipoproteins A1 and B were determined on a Monarch analyzer by an immunoturbidimetric method (32) (catalog no. 67265 and 67249, Orion Diagnostics). In the follow-up study, all lipid analyses were performed with a Cobas Integra 700 automatic analyzer with the reagents and calibrators recommended by the manufacturer (Roche Diagnostics, Basel, Switzerland).

Statistical analyses

Differences in clinical characteristics and concentrations of lipids and lipoproteins between the HL C-480T subgroups within the HRT and control groups were measured by one-way analysis of covariance (ANCOVA), using age and BMI as covariates when appropriate. Because the triglycerides concentrations had a skewed distribution, the statistical analyses were based on log-transformed data. The triglycerides concentrations in the tables are nevertheless given as crude values. Changes in lipid concentrations, lipoprotein concentrations, and atherosclerosis severity during follow-up were expressed as mean (± SD), and differences within the treatment groups were tested by ANCOVA, with age and BMI as covariates.

Interaction between the HL genotype and treatment (control or HRT) was tested by two-way ANCOVA. We tested whether the interaction between genotype and treatment group was independent of changes in HDL cholesterol during the trial, baseline ASC, age, and BMI, using these as covariates in two-way ANCOVA.

Longitudinal follow-up data were analyzed by ANOVA for repeated measures (RANOVA) to find interactions between treatment groups (HRT *vs.* control) and time points. RANOVA was performed separately within the HL genotype groups using the least significant differences *post hoc* test to determine the significance of differences between treatment groups in ASC and NAP at baseline. A similar analysis was made in ASC at follow-up. All analyses were performed with the Statistica for Windows version 5.1 software package (Statsoft, Inc., Tulsa, OK). The level of significance was set at $P < 0.05$.

Results

In the total cohort, the C and T alleles were found at frequencies of 0.75 and 0.25, respectively; 49 (55.7%) had the CC genotype, 34 (38.6%) the CT, and 5 (5.7%) the TT genotype. In the HRT-EV group, the CC genotype was found in 16 subjects (50%), CT in 14 subjects (43.8%), and TT genotype in two subjects (6.3%). In the HRT-EVP group, the CC genotype was observed in 16 subjects (61.5%) and the CT genotype was observed in 10 subjects (38.5%). In the control group, the corresponding data for CC, CT, and TT genotype were 17 (56.7%), 10 (33.3%), and three (10%). These frequencies did not differ significantly among the three groups. Because there were no TT homozygous subjects in the HRT-EVP group and there was no statistically significant difference between the CT and TT genotype groups in any baseline characteristics, the T allele carriers were combined into one group, and this was compared with subjects with the CC homozygous genotype.

The baseline distribution of known characteristics among postmenopausal women with respect to treatment and HL C-480T genotype status is presented in Table 1. Among the total, CC genotype carriers were older than women with the T allele ($P = 0.01$). There was an association of this allele with high levels of HDL_3 cholesterol in all subjects ($P = 0.04$). When the subjects were classified according to their C-480T genotype, there were no statistically significant differences between groups at baseline in total cholesterol, triglycerides, LDL cholesterol, apo AI, apo B, HDL, HDL_2, and HDL_3 cholesterol (Table 1). In the HRT-EV group, the CC genotype carriers were older than women with the T allele ($P < 0.001$).

At baseline, ASCs in the HRT-EV, HRT-EVP, and controls were 1.13, 1.31, and 1.59, respectively, in subjects with the CC genotype. There was no significant treatment effect on ASC (ANCOVA for trend, $P = 0.282$, adjusted by age and BMI; Fig. 1A). After the 5-yr follow-up, the corresponding differences between the HRT-EV and HRT-EVP groups and controls were 2.94, 3.31, and 3.71, respectively (ANCOVA for trend, $P = 0.266$, adjusted by age and BMI; Fig. 1B). At baseline, CC genotype carriers in HRT-EV had a mean of 45.9% (1.75 *vs.* 3.24 in controls, $P = 0.006$) lower total NAP. There was, however, no significant treatment effect on NAP between HRT-EVP and controls (2.44 *vs.* 3.24, $P = 0.131$) in CC carriers (ANCOVA for trend, $P = 0.021$, adjusted by age and BMI).

At baseline, subjects with the T allele in HRT-EV tended to have an average 45.8% lower ASC (1.13 *vs.* 2.08 in controls, $P = 0.005$), and those in HRT-EVP had a 37.4% lower ASC (1.30 *vs.* 2.08 in controls, $P = 0.037$) than the controls (ANCOVA for trend, $P = 0.014$, adjusted by age and BMI; Fig. 1C). After the 5-yr follow-up, the corresponding differences between the HRT-EV and HRT-EVP groups and controls were 43.3% (2.88 *vs.* 5.08, $P = 0.0003$) and 40.9% (3.00 *vs.* 5.08, $P = 0.002$; ANCOVA for trend, $P = 0.001$, adjusted for age and BMI; Fig. 1D). At baseline, subjects with the T allele in HRT-EV had a mean 60.7% (1.88 *vs.* 4.77 in controls, $P = 0.0003$) lower total NAP, and subjects in HRT-EVP a 43.4% (2.70 *vs.* 4.77 in controls, $P = 0.015$) lower total NAP than the controls (ANCOVA for trend, $P = 0.002$, adjusted by age and BMI).

In subsequent analyses, we combined the data on use of EV and EVP because the results for the two therapies were similar. The HL C-480T polymorphism had no effect on changes in total cholesterol, LDL cholesterol, triglycerides, and HDL cholesterol concentrations in the two study groups. HRT reduced total cholesterol and LDL cholesterol and increased HDL cholesterol and triglycerides to a similar extent in the two genotype groups (Table 2). There were no statistically significant differences between HL genotypes in response of the studied lipid traits (HL genotype group by time point interaction not significant for all lipids within study groups).

A significant interaction between the HL C-480T genotype and HRT in respect of increase in ASC was observed ($P = 0.046$) after adjustment for age, BMI, changes in HDL cho-

TABLE 1. Serum lipids and apolipoproteins in postmenopausal women at baseline by HRT and HL genotype status

	HRT-EV		HRT-EVP		Controls		All	
	CC	CT, TT	CC	CT, TT	CC	CT, TT	CC	CT, TT
No. of subjects	16	16	16	10	17	13	49	39
Age (yr)	63.3 ± 3.9	57.5 ± 3.8[a]	59.8 ± 5.0	59.7 ± 6.4	62.6 ± 5.0	60.2 ± 6.6	61.9 ± 4.8	58.9 ± 5.6[a]
BMI (kg/m^2)	26.1 ± 2.4	25.4 ± 3.4	25.9 ± 2.9	25.3 ± 3.7	26.6 ± 3.1	24.9 ± 3.1	26.2 ± 2.8	25.2 ± 3.3
Total cholesterol (mmol/liter)	6.47 ± 0.91	6.63 ± 1.02	5.77 ± 0.90	5.83 ± 0.87	6.62 ± 1.12	6.65 ± 1.13	6.29 ± 1.04	6.43 ± 1.06
LDL cholesterol (mmol/liter)	4.19 ± 0.96	4.29 ± 0.81	4.00 ± 0.86	3.88 ± 0.96	4.57 ± 1.03	4.54 ± 1.14	4.26 ± 0.97	4.27 ± 0.98
Triglycerides (mmol/liter)	1.52 ± 0.76	1.35 ± 0.59	0.73 ± 0.19	0.88 ± 0.27	1.27 ± 0.60	1.23 ± 0.67	1.18 ± 0.65	1.19 ± 0.58
Apolipoprotein A1 (g/liter)	1.55 ± 0.23	1.60 ± 0.29	1.33 ± 0.16	1.38 ± 0.12	1.42 ± 0.19	1.42 ± 0.16	1.43 ± 0.21	1.48 ± 0.23
Apolipoprotein B (g/liter)	0.98 ± 0.15	0.96 ± 0.20	0.82 ± 0.15	0.82 ± 0.18	0.96 ± 0.23	0.94 ± 0.21	0.92 ± 0.19	0.92 ± 0.20
HDL cholesterol (mmol/liter)	1.59 ± 0.36	1.73 ± 0.38	1.44 ± 0.23	1.56 ± 0.27	1.49 ± 0.32	1.56 ± 0.28	1.51 ± 0.31	1.63 ± 0.32
HDL_2 cholesterol (mmol/liter)	0.53 ± 0.26	0.57 ± 0.27	0.42 ± 0.18	0.48 ± 0.26	0.47 ± 0.22	0.49 ± 0.20	0.47 ± 0.22	0.52 ± 0.24
HDL_3 cholesterol (mmol/liter)	1.07 ± 0.21	1.15 ± 0.20	1.02 ± 0.12	1.08 ± 0.15	1.01 ± 0.16	1.07 ± 0.11	1.03 ± 0.16	1.11 ± 0.16[a]

Values are mean ± SD. EV, 2.0 mg/d; P, levonorgestrel, 0.25 mg/d.
[a] $P < 0.05$ ANCOVA among HL genotypes, age, and BMI as covariate when appropriate.

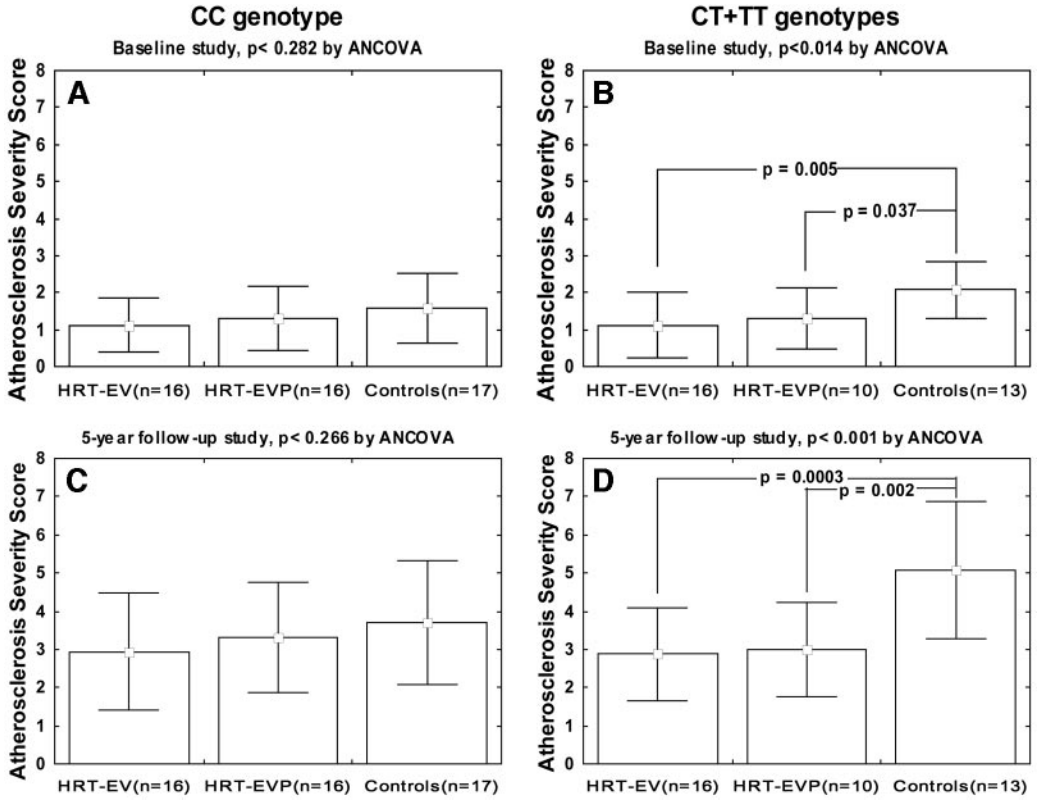

FIG. 1. A–D, ASC in postmenopausal women by HRT group and HL genotype status (CC and CT, TT). A and B, Results from the baseline study. C and D, Results from the cross-sectional study after 5-yr follow-up. The *P* values for the mean (± SD, whiskers) differences between the HRT groups and controls shown in the figure were obtained by ANCOVA, with least significance test as *post hoc* test. Results are adjusted for age and BMI.

lesterol during the trial, and ASC at baseline. A larger increase in ASC indicates greater progression of atherosclerosis. The benefit of HRT was restricted to women with the T allele. Among subjects with the CC genotype, the progression rate of ASC in HRT users and controls did not differ statistically significantly (treatment group by time point interaction in RANOVA, $P = 0.618$, Fig. 2A). Among the T allele carriers, the ASC progression rate differed significantly between HRT users and controls (treatment group by time point interaction in RANOVA, $P = 0.0006$, Fig. 2B).

TABLE 2. Influence of HL genotypes on the baseline and follow-up lipid levels and ASC in the HRT and control groups

	HRT (n = 58)			Controls (n = 30)		
	CC (n = 32)	CT, TT (n = 26)	*P* value[a]	CC (n = 17)	CT, TT (n = 13)	*P* value[a]
Total cholesterol (mmol/liter)						
Baseline	6.12 ± 0.96	6.32 ± 1.02		6.62 ± 1.12	6.65 ± 1.13	
Follow-up	5.81 ± 0.88	6.00 ± 0.85		6.21 ± 1.09	6.27 ± 0.83	
Decrease in total cholesterol	−0.31 ± 0.67	−0.33 ± 0.63	0.603	−0.41 ± 0.74	−0.39 ± 0.74	0.974
LDL cholesterol (mmol/liter)						
Baseline	4.09 ± 0.90	4.13 ± 0.87		4.56 ± 1.03	4.54 ± 1.14	
Follow-up	3.52 ± 0.83	3.58 ± 0.82		3.94 ± 0.95	3.86 ± 0.83	
Decrease in LDL cholesterol	−0.56 ± 0.71	−0.54 ± 0.66	0.518	−0.62 ± 0.70	−0.67 ± 0.65	0.639
HDL cholesterol (mmol/liter)						
Baseline	1.52 ± 0.30	1.66 ± 0.34		1.49 ± 0.32	1.56 ± 0.28	
Follow-up	1.77 ± 0.34	1.78 ± 0.48		1.62 ± 0.43	1.75 ± 0.39	
Increase in HDL cholesterol	0.25 ± 0.24	0.12 ± 0.28	0.391	0.14 ± 0.27	0.19 ± 0.25	0.246
Triglycerides (mmol/liter)						
Baseline	1.13 ± 0.68	1.17 ± 0.54		1.27 ± 0.60	1.23 ± 0.67	
Follow-up	1.15 ± 0.44	1.39 ± 0.59		1.43 ± 0.69	1.45 ± 0.63	
Increase in triglycerides	0.02 ± 0.59	0.21 ± 0.50	0.349	0.15 ± 0.63	0.22 ± 0.34	0.969
ASC						
Baseline	1.22 ± 0.79	1.19 ± 0.85		1.59 ± 0.94	2.08 ± 0.76	
Follow-up	3.13 ± 1.48	2.92 ± 1.20		3.71 ± 1.61	5.08 ± 1.80	
Increase in ASC	1.91 ± 1.42	1.73 ± 0.87	0.933	2.12 ± 1.36	3.00 ± 1.22	0.04

Values are mean ± SD.
[a] ANCOVA among HL genotypes, age, and BMI as covariate when appropriate.

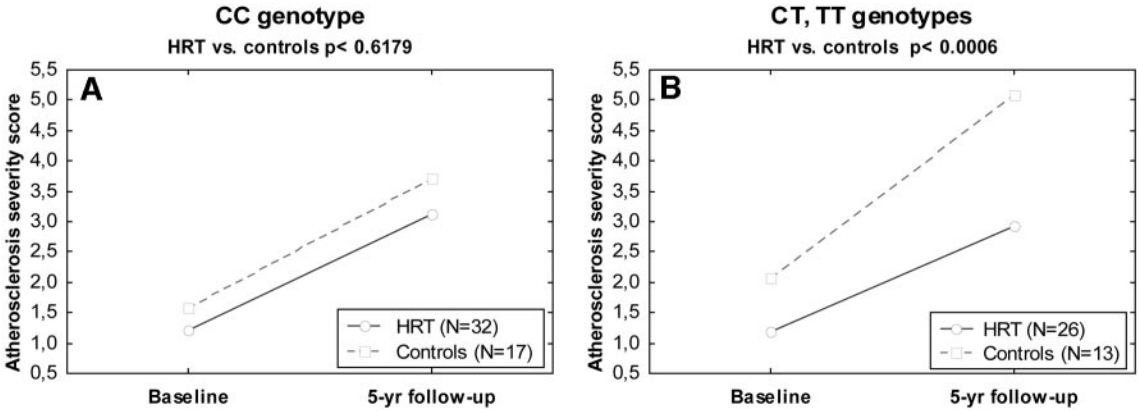

FIG. 2. A and B, The effect of HRT on the progression of atherosclerosis, as measured by ASC in postmenopausal women with CC genotype (A) and CT or TT genotype (B), compared with the progression in controls with the same HL genotype and time elapsed from menopause but without HRT. The *P* values shown in the figure are from two-way ANOVA (grouping HRT/control) for repeated measures.

The corresponding changes in serum lipid and lipoproteins and changes in ASC during follow-up, according to HL genotype and treatment status, are shown in Table 2. There were no statistically significant differences between HL genotypes in changes in lipid and lipoprotein concentrations. Among control women, the T allele was associated with greater changes in ASC during follow-up than the CC genotype ($P = 0.04$) (Table 2). The association of the T allele with more substantial progression of atherosclerosis, as observed in the control group, was influenced by the use of HRT. In fact, the T allele appeared to be associated with lesser progression in women who were receiving HRT. In these women, the progression of atherosclerosis severity was slower by half in those receiving HRT, whereas no difference was observed in women carrying the CC genotype.

Discussion

The present study assessed the role of the HL C-480T polymorphism and long-term HRT in atherosclerosis severity in postmenopausal women. The T allele was found to be associated with greater changes in ASC during follow-up than the CC genotype among control women. However, T allele carriers were seen to reflect beneficial effects on atherosclerosis progression during long-term HRT. The effect of the T allele on ASC with HRT during follow-up was independent of HDL cholesterol. No positive effect of HRT was seen in the CC genotype group, in which the progression of atherosclerosis tended to be similar among HRT users and controls during the 5-yr follow-up. Moreover, we found a significant interaction between the HL C-480T polymorphism and HRT in respect of atherosclerosis progression. We have thus revealed a genetic predisposition for response to HRT.

In this study, all subjects were nonsmokers; did not have diabetes; were without clinically evident cardiovascular disease, hypertension, or chronic medication; and were otherwise clinically healthy. In addition, dietary analysis revealed no substantial differences in the use of saturated fat or dietary cholesterol between the HRT groups (3); our results thus are unlikely to have been influenced by differences in dietary habits between the HRT and control groups. Because some other factors possibly differing between users and nonusers (*e.g.* socioeconomic status) were not accounted for, it is conceivable that some unknown factors may have biased our results.

Several observational studies have suggested that HRT reduces the risk of atherosclerosis and coronary events in postmenopausal women (1–4). However, an adverse effect appears in the first year of HRT use, decreasing over time (6). It has been reported that HRT has beneficial effects in the early stages of atherosclerosis progression but is of little or no benefit in advanced stages of the disease (33). In our study, the plaques detected by ultrasonography were mostly uncalcified, constituting less advanced atherosclerotic lesions. Recent studies from our group (8–10) have shown that the effect of HRT varies among individuals and that this variation is partly genetically determined by functional variations in candidate genes. Together with our finding, it is becoming increasingly clear that the individual response to treatment is related to a great extent to genetically defined differences.

Zambon *et al.* (34) found HL genotype differences in effect on coronary stenosis progression in the HL genotype order of CC < CT < TT during intensive lipid medication. However, the design in the study in question differed from that here. In our study we also included a control group without medication in addition to the HRT treatment group, which allowed us first to test HL genotype-by-treatment group (control/HRT) interaction, which was statistically significant, and thereafter compare the effects in treated *vs.* nontreated groups on ASC progression in different genotype groups. Zambon *et al.* did not include a control group in their statistical analysis, and it is thus difficult to know which part of the changes during intervention including extensive lipid-lowering therapy was due to medication or alternatively other lifestyle changes during the same period. Thus, the differences in study design and medications used make comparison of the results from these two studies very difficult.

In our study, the HL promoter polymorphism had no effect on the lipid response to HRT in postmenopausal women, as previously reported (27, 28). Because HL is expressed within the macrophages of the atherosclerosis

plaque (12, 35), it has been postulated that HL might have a direct role in the pathogenesis of atherosclerosis through a pathway not involving changes in plasma lipoprotein metabolism (12, 35). The C-480T polymorphism is a key determinant of HL activity, accounting for up to 38% of its variability (36). HL activity is also hormonally affected. Estrogen replacement therapy in postmenopausal women acutely reduces HL activity (24, 37, 38). Tikkanen *et al.* (25) have shown that HL activity falls significantly in healthy premenopausal women during the luteal phase of the menstrual cycle, when endogenous estradiol levels are highest. HL activity decreases progressively throughout pregnancy as estradiol levels increase (39). The fall in endogenous estrogen with menopause, again, is associated with a rise in HL activity (26, 40). The effect of the HL C-480T polymorphism on coronary heart disease (CHD) has been sought in several studies. The T allele is associated with endothelial dysfunction in young healthy men (30). This allele is also associated with coronary artery calcification (41), the extent of coronary stenosis in patients with CHD (42), and prevalent CHD case-control status in men (14, 43). On the other hand, Rundek *et al.* (44) reported that the CC genotype is associated with greater intimal-media thickness than in T allele carriers. A group under Faggin (45) showed the CC genotype to be associated with an abundance of macrophages in patients with severe carotid artery stenosis undergoing carotid endarterectomy. Thus, the relationship between the HL C-480T polymorphism and CHD is not consistent. Moreover, there is evidence for an interaction of this polymorphism with dietary fat intake (46), lipid-lowering medications (34), physical activity (47), and HDL cholesterol level (48). Our finding may be related to the HL activity either in the liver or alternatively in artery wall macrophages (12, 35). The exact mechanism remains to be established.

Ultrasonographic methods permit evaluation of atherosclerosis in asymptomatic subjects (49, 50). In our study ASC represents the severity of the plaques found in the large arteries, *i.e.* carotids and aorta. NAP represents only the number of total plaques found in the above-mentioned regions. The number of plaques may be substantial but if they are not large enough to cause stenosis of the artery, they will not induce severe clinical symptoms. Because ASC represents the size and severity of plaques, we feel that ASC is a better marker of the severity of atherosclerosis. Because individuals with more advanced atherosclerotic disease, characterized by stenotic or occlusive lesions, were not included here, the association of the HL genotype with atherosclerosis severity status may have been modified. Likewise, because we did not study the cardiovascular end process, we cannot rule out selection bias related to HRT or certain other noncausal explanations for our findings.

Our results suggest that the C-480T polymorphism confers a genotype-specific responsiveness to HRT in atherosclerosis progression in postmenopausal women. The findings may help us understand in greater detail the benefit and possible risk of HRT in atherosclerotic diseases. In the future, identification and characterization of the genotype might help patients and physicians assess the risks and benefits of HRT and lead to new insights into HRT action.

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