Lipoprotein lipase S447X: from beneficial gene variant to gene therapy

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FROM BENEFICIAL GENE VARIANT TO GENE THERAPY
LIPOPROTEIN LIPASE S447X
FROM BENEFICIAL GENE VARIANT TO GENE THERAPY

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Faculteit der Geneeskunde
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CHAPTER 1

GENERAL INTRODUCTION

Melchior C. Nierman

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General introduction

Cardiovascular disease plays a major role in our Western society and cardiovascular death and irrespective of successful drug intervention remains the number one killer. Traditional risk factors such as lipids, smoking, blood pressure, diabetes, bodyweight and lifestyle account for most of the risk of cardiovascular disease worldwide. In addition, increased triglyceride (TG) levels significantly contribute to increased risk of cardiovascular disease, whereas severe hypertriglyceridemia (HTG) (fasting TG > 10 mmol/l) is also associated with increased risk of pancreatitis.

TG metabolism

TG are an essential source of energy in humans. TG constitute of a glycerol core with three esters of fatty acids. They can be hydrolyzed by various lipases, which results in the generation of free fatty acids (FFA) and glycerol. Packaged in lipoproteins, TG enter the circulation via the 'endogenous pathway' (predominantly hepatic production) and the 'exogenous pathway' (absorption via the intestine). In the endogenous pathway, very low-density lipoproteins (VLDL), produced by the liver, is secreted into the circulation where it is converted into smaller lipoproteins that all have apoB100 as their main structural protein: very low-density lipoproteins (VLDL = VLDL1 and VLDL2), intermediate density lipoproteins (IDL) and low-density lipoproteins (LDL) (see figure 1). These apoB100 TRL differ in size and in TG content: VLDL1 is largest and carries most TG. In the circulation, the conversion of the triglyceride-rich lipoprotein (TRL) or apoB100 turnover in the so called 'dilapidation cascade' is predominantly the result of TG hydrolysis via LPL. Of note, apoB100 lipoproteins play a crucial role in the development of atherosclerosis. In the 'exogenous pathway', TG are absorbed in the intestine which repackages the TG into the largest of all lipoproteins, i.e. chylomicrons. These lipoproteins have a truncated form of apoB100, i.e. apoB48 as structural protein. Upon secretion into the blood stream, chylomicrons are converted to chylomicron remnants by LPL-mediated lipolysis as shown in figure 1. Thus, both apoB100 and apoB48-containing TRL are hydrolysed by lipoprotein lipase (LPL) as discussed below.
Elevated plasma TG
TG levels can be elevated by genetic factors, but also by deleterious conditions like diabetes mellitus, excessive alcohol consumption, hypothyroidism and use of drugs like or estrogens and atenolol. Increased plasma TG levels can be caused by either increased hepatic TRL synthesis, decreased TRL removal or both. Since apoB100 is the structural apolipoprotein of only hepatic TRL, this protein is essential in analysing the hepatic VLDL production. Hepatic overproduction of VLDL ultimately results in high apoB100 concentrations and a shift towards small dense low-density lipoprotein (LDL) particles. This condition ultimately leads to an increased concentration of apoB particles and since these particles have been shown to play a role in atherogenesis, this condition is associated with increased risk for cardiovascular disease. Focusing on increased TG levels due to impaired degradation of large TRL, including VLDL and chylomicrons, the role of lipoprotein lipase (LPL) is pivotal.

Lipoprotein lipase (LPL)
Several different cell types can produce LPL, but in humans, this is primarily restricted to cardiac muscle cells, skeletal muscle cells, and adipocytes. After production in these parenchymal tissues, LPL is transported to the vascular endothelium where it binds to heparan sulphate-containing proteoglycans. It finds its cofactor apolipoprotein CII (apoCII), necessary for LPL’s catalytic activity,
on its lipoprotein substrates. LPL is active as a dimer and its main function is hydrolyzing TG for production of free fatty acids (FFA) for energy production in the muscle and for use or for storage in adipocytes (LPL catalytic function). With a short-half lifetime, LPL disintegrates into monomers, thereby losing its catalytic activity. Monomeric LPL facilitates lipoprotein interaction with various cell surface receptors and plays a key role in the removal of (atherogenic) lipoproteins from the circulation (LPL ligand function). LPL is also produced by macrophages, where in contrast it has been shown to be atherogenic.

LPL testing

Thus, the two most important functions of LPL are TG hydrolysis (LPL catalytic function) and enhancing the uptake of lipoproteins by the liver (LPL ligand function). LPL hydrolyses TG from preferably the largest TRL, thus allowing principal hydrolysis to take place in those particles richest in TG. The dominant form of LPL in the circulating blood, (i.e. before heparin challenge) is the inactive monomeric form of LPL. In vitro, monomeric LPL has a 6000-fold lower affinity for heparan sulphate (and for heparin) than catalytically active dimeric LPL. Therefore, monomeric LPL is predominantly found in pre-heparin plasma. Assessment of LPL activity in humans is generally performed after an intravenous injection of heparin. Heparin competes with LPL for binding to the heparan sulphate-containing proteoglycans that causes the release of endothelium-bound LPL into the circulation, but these post-heparin values do not reflect the true in vivo LPL-mediated lipolytic capacity. Even with sensitive enzyme-linked immuno sorbent assays (ELISA’s) and other tests for catalytic activities at hand it is difficult to assess the true LPL function in humans. This is mainly because LPL functions at the endothelial surface in the blood vessels of the LPL producing tissues and at the same time serves as a ligand for the uptake of lipoproteins. Assessment of the turnover rate of apoB TRL as mediated by TRL conversion via LPL-mediated TG hydrolysis may best reflects the in vivo LPL catalytic function. Next to hydrolysis, LPL also facilitates lipoprotein clearance, i.e. disappearance of lipoproteins from the circulation, either via hepatic uptake or via uptake by fat or muscle tissue. Notably, functional LPL activity is the result of activity of bound LPL protein to lipoproteins. Binding of LPL to lipoproteins has been published previously and Olivecrona et al. showed that that circulating LPL is predominantly present in the apoB fraction.
LPL gene variants

Virtually every mutation in the LPL gene is associated with both reduced LPL function and increased CVD risk.\(^1\) One frequent found loss-of-function mutation with attenuated LPL activity, denoted as LPLN291S, is present in 5% of the general population and has been shown to result in impaired VLDL handling.\(^13,14\) However, another naturally occurring LPL variant lacking the terminal serine and glycine residues from the carboxy-terminal end of the protein, denoted as LPLS447X, (present in approximately 20% of the general population) is considered to constitute a gain of function mutation. This variant is associated with moderately elevated high-density lipoprotein cholesterol (HDL-C) levels and lower TG levels.\(^13,25-27\) In line with the beneficial lipid profile, LPLS447X is also clearly associated with a lower incidence of both cardiovascular\(^25,28-30\) and cerebrovascular events\(^31,32\) compared to non-carriers. To date, the exact mechanisms contributing to an anti-atherogenic lipid profile as well as cardiovascular protection are unknown.

LPL deficiency

Genetic LPL deficiency is a rare autosomal recessive disorder caused by homozygous or compound heterozygous LPL gene mutations. Such mutations result in loss of catalytically active LPL and cause a condition that is known as hyperchylomicronemia. This syndrome is characterized by the body’s inability to reduce circulating triglyceride-rich chylomicrons resulting in extremely high concentrations of plasma TG. The clinical syndrome of hyperchylomicronemia was first described by Bürger and Grütz in 1932 and 56 years later, the first LPL mutation responsible for such a condition was revealed.\(^33\) LPL deficiency typically manifests itself in early childhood with a presentation of symptoms including severe abdominal pain, repetitive colicky pain, acute pancreatitis and “failure-to-thrive”.\(^34,35\) Also, eruptive xanthomas, lipaemia retinalis and hepatosplenomegaly can be present. This combination of symptoms is often not recognized or thought to be directly related to the hyperchylomicronemia syndrome and the diagnosis often becomes clear only after the occurrence of pancreatitis.\(^36\) Plasma is lactescent showing increased TG and reduced HDL-C levels. The increased TG concentration is thought to be mainly responsible for the increased risk of pancreatitis,\(^37\) which can occur at TG concentrations > 10 mmol/L.\(^4\) Exact data on the prevalence of LPL deficiency are not available, but estimations vary between 1:1,000,000\(^38\) and 1:5,000 in French Quebec (caused by a so called “founder effect”).\(^32,39\) Based on extensive efforts to track down all LPL deficient patients in the Netherlands, we estimate a prevalence of approximately 1:500,000.
The main health risk of LPL deficient patients is pancreatitis. The exact etiology of pancreatitis in hypertriglyceridemia is unclear; however the high concentration of chylomicrons in the pancreatic microcirculation can lead to increased 'free radical' activity which in turn can lead to episodes of pancreatic ischemia. Under normal conditions, a small amount of lipase is present in the pancreatic microcirculation. Disruption of the microcirculation, caused by hyperchylomicronemia, leads to damage of pancreatic cells followed by an increase release of lipase. This lipase activity causes local hydrolysis of chylomicrons with a resulting strong increase in local FFA, resulting in increased local pancreatic inflammation. This cascade of more free radical production and inflammation by FFA can eventually lead to pancreatitis. It is unclear whether LPL deficiency in the long run is associated with an increased incidence of cardiovascular disease (CVD). Although two publications show premature atherosclerosis in LPL deficient patients, the general idea is that LPL deficiency is not associated with increased risk for CVD. The observed lack of atherosclerosis is possibly related to the low concentration of LDL-C in LPL deficiency, illustrated by a case of a homozygote LPL deficient patient suffering from familial hypercholesterolemia (FH). FH is normally associated with marked increases of plasma LDL-C levels, but in this LPL deficient patient the LDL-C was even lower than his unaffected family members. Another explanation for the lack of premature atherosclerosis is the proposed inability of chylomicrons to penetrate the vascular wall to induce an atherogenic effect. Also, the lack of production of highly atherogenic particles such as chylomicron-remnants and VLDL remnants may play a role in this context. Finally, LPL deficiency has been described to be associated with the accumulation of TRL in macrophages inside the vascular wall is reduced possibly leading to reduced atherosclerosis.

The primary target for treatment of LPL deficiency is reducing the risk of recurrent pancreatitis by reducing plasma TG levels. However, current medication has shown to be unsuccessful. The only option is a reduction of the exogenous TG production pathway via harsh dietary interventions; the intake of dietary fats has to be severely reduced to provide less than 10% of the total caloric intake. In Western societies with a dietary fat intake of approximately 120 grams per day, compliance to such a diet is difficult. This is exemplified by persisting hypertriglyceridemia and hospitalization of some of these patients. Omega-3 fatty acids can be added to the diet and medium-chain triglyceride (MCT) oils can be an additional source for energy. In some cases, antioxidant therapy through dietary supplements has been able to alleviate some of the symptoms of LPL deficiency, however, these observations were based on case reports and are therefore very
difficult to interpret and for that reason, no hard conclusions can be drawn whether such interventions actually reduce hypertriglyceridemia and safely reduce the risk for recurrent pancreatitis in these patients. Due to the lack of effective pharmacological interventions and poor diet compliance, the prevention of pancreatitis is unsuccessful in LPL deficiency and effective therapeutic modalities are therefore urgently requested.
Outline of this thesis

The naturally occurring LPL variant LPLS447X is present in approximately 20% of the general population and is associated with lower triglyceride levels as well as reduced CVD risk. However, the responsible mechanisms remain to be elucidated.

In the first part of this thesis, beneficial effects of LPLS447X on lipids are described. Specifically, the LPLS447X effects on TRL metabolism are investigated. An overview of current literature regarding this LPL variant is given and potential pathways for the beneficial effects that are associated with LPLS447X are discussed.

In the second part of this thesis, several beneficial effects of the LPLS447X variant on various phenotypes with increased CVD risk are discussed. The association of LPLS447X with future cardiovascular disease and survival in subjects with (micro) albuminuria are investigated. In addition, the effects of this LPL variant on survival and outcome in subjects that required a coronary intervention are reported. Also, the effects of LPL concentration on cardiovascular outcome are studied.

Finally, in the third part of this thesis, absence of LPL activity due to mutations in the LPL gene, known as LPL deficiency is discussed. Symptoms and signs of this condition are described in detail and this thesis ends with the work in progress regarding LPL gene therapy. Overall, our aim is to investigate the possible mechanisms behind the beneficial effects of LPLS447X on both lipids and lipoproteins but also on cardiovascular disease in general.

Chapter two reviews the current literature on LPLS447X and also discusses in part the studies in chapters three, four and five. In addition to the effects on lipoproteins, and cardiovascular disease, the beneficial effects on traditional risk factors (overweight, hypertension), Alzheimer disease and cancer are discussed. Finally, potential mechanisms responsible for the beneficial effects of this LPL variant are discussed.

Chapter three describes the effects of LPLS447X on postprandial apoB48 clearance in heterozygous LPLS447X carriers compared to matched controls.
Chapter four addresses the enzymatic and non-enzymatic consequences of this LPL variant on apoB100 TRL metabolism during feeding via infusion of stable isotope L-[1-13C]-valine in LPLS447X homozygotes and matched controls.

Chapter five confirms the beneficial effects of LPLS447X on TRL apoB48 metabolism, during feeding via infusion of stable isotope L-[1-13C]-valine and via analyzed by SAAMII modeling in LPLS447X homozygotes and matched controls.

In chapter six, the impact of LPL gene polymorphisms on restenosis as defined by target vessel revascularization is examined in a large patient-population undergoing percutaneous coronary intervention using data from the GENDER (GENetic DEterminants of Restenosis) study.

In chapter seven, the effect of LPLS447X on cardiovascular risk is investigated in subjects with different levels of albuminuria without known cardiovascular disease using data from the PREVEND (Prevention of REnal and Vascular ENdstage Disease) study.

In chapter eight, the relation between circulating LPL protein and future cardiovascular disease is studied in a large prospective cohort using data from the EPIC-Norfolk Prospective Population Study.

Chapter nine describes the full clinical, biochemical and molecular analyses of severe hypertri-glyceridemic individuals in one Turkish and three Chinese families. In addition, a novel discovered LPL gene mutation that is associated with complete absence of LPL protein is presented.

In chapter ten, the clinical presentation of LPL deficiency is described and preclinical studies on LPL gene therapy in animal models is summarized. Also, the rationale to develop gene therapy for this monogenetic disorder of lipid metabolism in humans is discussed.

In chapter eleven, the proof of principle or preclinical investigations of LPL gene therapy in animal models is described in more detail. This study also includes the results from toxicology and biodistribution studies. In addition, the first in vitro LPL gene therapy results are presented as well as detailed characterization of the patients that are aimed to include in this future study, which was actually started at August 2005. The LPL gene therapy trial is currently in its final stage and is expected to be completed in the first quarter of 2007. Hence, the final data of this study cannot be included in this thesis.
Reference List


Chapter 1


HOW LIPOPROTEIN LIPASE S447X EXERTS ITS BENEFICIAL EFFECTS
LIPOPROTEIN LIPASE S447X,
A NATURALLY OCCURRING GAIN-OF-FUNCTION MUTATION

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Abstract

Lipoprotein lipase (LPL) hydrolyzes triglycerides in the circulation and promotes the hepatic uptake of remnant lipoproteins. Since the gene was cloned in 1989, more than a hundred LPL gene mutations have been identified, the vast majority of which cause loss of enzymatic function. In contrast to this, the naturally occurring LPLS447X variant is associated with increased lipolytic function and an anti-atherogenic lipid profile, and can therefore be regarded as a gain-of-function mutation. This notion combined with the facts that 20% of the general population carries this prematurely truncated LPL, and that it may protect against cardiovascular disease, has led to extensive clinical and basic research into this frequent LPL mutant. It is only until recently that we begin to understand the molecular mechanisms that underlie the beneficial effects associated with LPLS447X. This review summarizes the current literature on this interesting LPL variant.
Introduction

Lipoprotein lipase (LPL) plays a central role in human lipid homeostasis and energy metabolism. The main function of this enzyme is the hydrolysis of plasma triglycerides (TG) that are packaged in apolipoprotein (apo) B containing lipoproteins. It furthermore mediates the clearance of atherogenic remnant lipoproteins from the circulation. The gene encoding for LPL is located on chromosome 8, and is expressed mainly in skeletal muscle, adipose tissue and heart muscle. Homozygosity or compound heterozygosity for either missense, non-sense mutations, deletion or insertions in the LPL gene, resulting in complete loss of enzyme function, cause the accumulation of chylomicrons in the circulation, a phenotype known as Type I hyperlipoproteinemia. This rare autosomal recessive disorder can be lethal due to (recurrent) hemorrhagic pancreatitis.

The LPL gene locus is highly polymorphic and many single nucleotide polymorphisms (SNP) in both coding and non-coding regions have been used to study associations with lipids, lipoproteins, and risk for atherosclerosis. Most of these SNP’s have only mild detrimental effects on LPL function or are mere markers for genetic variation elsewhere in the genome. Two SNP in the coding DNA (cSNPs) that have been studied extensively concerning point mutations in exon 2 and 6, causing the substitution of an aspartic acid to an asparagin residue at position 9 (D9N), and an aspartic acid to serine at position 291 (N291S), respectively. These mutations occur at high frequencies in the general population (up to 5%) and are associated with elevated TG, decreased high-density lipoprotein (HDL) cholesterol levels and concomitantly with a higher incidence of cardiovascular disease (CVD), compared to non-carriers. Several in vivo and in vitro studies have shown that both LPLD9N and LPLN291S have decreased lipolytic activity compared to LPL WT. For LPLD9N this was reported to relate to decreased cellular secretion while LPLN291S was shown to be less stable compared to LPL WT. In a more recent study, Fisher et al. showed that LPLD9N causes enhanced LDL binding and monocyte adhesion compared to LPL WT and was thus suggested to enhance foam cell formation in the vascular wall.

A third frequently occurring cSNP concerns a C to G mutation in exon 9 at position 1595. This nucleotide change introduces a premature stop codon at position 447, resulting in a mature protein that lacks the C-terminal serine and glycine, from now on denoted as LPLS447X. In contrast to all other LPL variants, this mutation is associated with beneficial effects on lipid homeostasis and atheroprotection. Such gain-of-function as the result of a mutation in genomic DNA has
rarely been reported in the literature, but interestingly, most are associated with protection against CVD. These mutations may be especially favourable in modern times now that people live longer and are subject to a much higher risk to develop CVD due to a poor life-style. The molecular event that underlies the appearance of LPLS447X occurred before the Indo-German division, taken that the mutation is found in both individuals of Caucasian and Asian descent. With carrier frequencies around 20% in both populations (with slightly lower frequencies in Afro-Americans), it concerns a highly frequent variant which will be the subject of this review.

**Plasma lipids and lipoproteins**

Table 1 provides an overview of all studies on LPLS447X and their main findings that have been published thus far. Focusing on lipid metabolism, several studies have shown significantly lower plasma TG levels and higher plasma HDL cholesterol levels in carriers of the LPLS447X variant compared with non-carriers. In some reports, a clear allele dosage effect was observed, indicative of a biological relationship these parameters. In addition, most investigators reported that carriers of the mutation did not exhibit changes in total cholesterol and low-density lipoprotein (LDL) cholesterol levels compared to non-carriers.

Interestingly, the mutation appears to especially lower plasma TG levels in smoking and drinking females, in obese subjects, in carriers of deleterious apoCIII polymorphisms and in subjects with the apoE4 allele. Thus, it appears that LPLS447X moderates the effects of risk factors for CVD but the mechanisms that underlies these observations are unclear.

The lipid measurements in the majority of studies have been performed in the fasted condition. However, LPL action is especially required under postprandial conditions where dietary lipids transported in chylomicrons need to be catabolized to enable uptake of free fatty acids (FFA) by skeletal/heart muscle and adipose tissue. Five studies have thus far addressed the question whether LPLS447X has an impact on postprandial TG metabolism. In an initial report, Humphries et al. showed in 332 offspring of fathers with premature myocardial infarction and 342 age- and sex-matched controls, that carriers of the LPLS447X variant have lower postprandial TG levels compared to non-carriers after a standardized meal. In a second report, others did not observe significant differences in TG clearance after infusion of chylomicron-like emulsions in a small mixed population of 7 male and 5 female heterozygotes vs. 6 male and 7 female controls. In a third study, it was found that healthy male heterozygotes (n=15) had an increased postprandial
clearance of triglyceride-rich lipoproteins (TRL) compared to non-carriers (n=36). In a recent study by our group, 15 healthy male volunteers, heterozygous for LPLS447X variant, showed an increased postprandial apoB48 clearance compared to non-carriers after a standardized oral fat load, when compared to controls matched for gender, age, alcohol use, BMI, and smoking. We also found that carriers of the mutation have a higher LPL concentration in preheparin serum (further discussed below). With these findings, we set out to test the hypothesis that LPLS447X enhances apoB100 catabolism. In short, five healthy male homozygotes for LPLS447X variant and five male controls were continuously fed and received continuous infusion of the stable isotope. Compared to controls, carriers presented with a 2-fold enhanced conversion of TRL in addition to an enhanced LDL removal. In conclusion, four out of five studies indicate that carriers of the LPLS447X variant mutation have an enhanced capacity to lower postprandial TG levels when compared to non-carriers.

Cardiovascular disease, blood pressure, Alzheimer disease and cancer

Cardiovascular disease
A considerable number of studies have suggested that carriers of the LPLS447X variant have a lower CVD risk, but this was not confirmed by other investigators. Wittrup et al. were the first to conduct a meta-analysis on the associations between several LPL gene variants and risk of ischemic heart disease (using eight of the above studies), and calculated a 17% decreased risk in carriers of LPLS447X. In a second meta-analysis, the same investigators noted that the protective effect was gender-specific, providing benefit only to males with 18% reduced risk of future CVD. In a review, Hokanson et al., however, reported a 19% risk reduction in both sexes. Taken together, it appears that LPLS447X is associated with protection against CVD in accordance with the beneficial changes it confers to the lipid profile.
Table 1: Publications in which the associations between LPLS447X with plasma lipid levels and/or cardiovascular disease was investigated.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Plasma TG +/- and -/-</th>
<th>Plasma HDL-C +/- and -/-</th>
<th>Cardiovascular disease +/- and -/-</th>
<th>Subjects Male/Female (Carriers/Non-carriers)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nierman et al, 2005 57</td>
<td>No diff</td>
<td>No diff</td>
<td></td>
<td>M (6/-/6) Netherlands</td>
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<tr>
<td>Nierman et al, 2005 54</td>
<td>14% No diff</td>
<td>N.S. No diff</td>
<td></td>
<td>M (15/-/-/15) Netherlands</td>
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<tr>
<td>Goodarzi et al, 2005 29</td>
<td>↑12% No S</td>
<td>↑2.8%</td>
<td>&lt;0.05</td>
<td>M • F (44/353) Mexican-Americans</td>
</tr>
<tr>
<td>Lopez-Miranda et al, 2004 56</td>
<td>No diff</td>
<td>No diff</td>
<td></td>
<td>M (26/25) Spain</td>
</tr>
<tr>
<td>Lee et al, 2004 46</td>
<td>↑10.6% 0.057</td>
<td>↑5% ** 0.001</td>
<td>↑7% ** 0.001</td>
<td>M (390/1491) • F(413/1763) Singapore</td>
</tr>
<tr>
<td>Almeida et al, 2003 55</td>
<td>No diff</td>
<td>No diff</td>
<td></td>
<td>M • F (13/12) Brazil</td>
</tr>
<tr>
<td>Corella et al, 2002 44</td>
<td>↑1.66%</td>
<td>↑74%</td>
<td>&lt;0.05</td>
<td>M (74/303) North European, 50 years old</td>
</tr>
<tr>
<td>Morabia et al, 2003 45</td>
<td>ND ND</td>
<td>ND ND</td>
<td></td>
<td>M+F (185 non-atherogenic controls, 186 atherogenic cases)</td>
</tr>
<tr>
<td>Wittrup et al, 2002 30</td>
<td>↑6.5% +/- 0.001</td>
<td>↑4.2% ** 0.001</td>
<td>↑2% ** 0.99</td>
<td>M (42/-, 627/-, 2887) • F(56/-, 837/-, 3508) Denmark</td>
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<tr>
<td>Corella et al, 2002 44</td>
<td>↑18%</td>
<td>↑11%</td>
<td>↑4.2%</td>
<td>↑2%</td>
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<tr>
<td>Ukkola et al, 2001 38</td>
<td>↑7%</td>
<td>↑5%</td>
<td>0.03</td>
<td>↑7%</td>
</tr>
<tr>
<td>Shimo-Nakanishi et al, 2001 37</td>
<td>↑10% No S</td>
<td>↑3%</td>
<td>N.S.</td>
<td>Protected against CVD (OR 0.68; P&lt;0.03) and atherothrombotic infarction (OR 0.42; P&lt;0.04)</td>
</tr>
<tr>
<td>McLaggery et al, 2001 42</td>
<td>↑111% No S</td>
<td>↑77%</td>
<td>0.055</td>
<td>M+F (102/300) Chinese Canadians</td>
</tr>
<tr>
<td>Clee et al, 2001 38</td>
<td>↑20.4%</td>
<td>No diff</td>
<td>0.001</td>
<td>Trend of reduced vascular disease (OR: 0.61; P=0.10)</td>
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<tr>
<td>Chen et al, 2001 37</td>
<td>↑18.7%</td>
<td>↑2.8%</td>
<td>0.05</td>
<td>M+F (120/709) Bogalusa Heart Study</td>
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<tr>
<td>Gorenc et al, 2000 34</td>
<td>↑21.8% ** 0.01</td>
<td>↑4.4% ** 0.01</td>
<td>↑7% ** 0.06</td>
<td>M(40/188) • F(43/204) HERITAGE family study</td>
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<td>Arca et al, 2000 32</td>
<td>No diff</td>
<td>↑5%</td>
<td>0.05</td>
<td>M+F (167/555) Italy, 6.32 CAD patients 191 controls</td>
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<tr>
<td>Sass et al, 2000 34</td>
<td>↑13.3% ** 0.01</td>
<td>↑4% ** 0.01</td>
<td>↑1% ** 0.01</td>
<td>M+F, France, Stanislas cohort</td>
</tr>
</tbody>
</table>

M, males; F, females; +/-, heterozygous S447X carriers; -/-, homozygous S447X carriers; ND, not determined; NS, non-significant
Table 1: Publications in which the associations between LPLS447X with plasma lipid levels and/or cardiovascular disease was investigated.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Plasma TG +/- and -/-</th>
<th>Plasma HDL-C +/- and -/-</th>
<th>Cardiovascular disease +/- and -/-</th>
<th>Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sass et al, 2000</td>
<td>▼13.3%</td>
<td>▼4%</td>
<td>N.S.</td>
<td>M+F, France, Stanislas cohort</td>
</tr>
<tr>
<td>Gagne et al, 1999</td>
<td>▼14%</td>
<td>▼5.3%</td>
<td>0.01</td>
<td>M (173/935) + F (200/944) Framingham Offspring Study</td>
</tr>
<tr>
<td>Hallman et al, 1999</td>
<td>▼15%</td>
<td>▼3.7%</td>
<td>&lt;0.05</td>
<td>M (112/396) REGRESS study</td>
</tr>
<tr>
<td>Sass et al, 1998</td>
<td>▼23.6%</td>
<td>▼N.S.</td>
<td></td>
<td>M+F (39/111) France</td>
</tr>
<tr>
<td>Humphries et al, 1998</td>
<td>▼5.4%</td>
<td>▼0.01</td>
<td>Protect against MI (OR: 0.71)</td>
<td>M+F (302/1143) Europe, EARS I</td>
</tr>
<tr>
<td>Kuivenhoven et al, 1997</td>
<td>▼8%</td>
<td>▼4.4%</td>
<td>0.044</td>
<td>M (50/191) Netherlands, high, medium and low HDL groups</td>
</tr>
<tr>
<td>Groenemeijer et al, 1997</td>
<td>▼19%</td>
<td>▼4.4%</td>
<td>0.013</td>
<td>M (149/662) REGRESS study, CAD patients</td>
</tr>
<tr>
<td>Salah et al, 1997</td>
<td>No diff</td>
<td>No diff</td>
<td>Protect against CAD (OR 0.73, P=0.05)</td>
<td>M (211/997) Caerphilly Prospective Heart Disease Study</td>
</tr>
<tr>
<td>Peacock et al, 1997</td>
<td>No diff</td>
<td>No diff</td>
<td>Protect against hyperlipidemia (OR 0.27, P=0.037)</td>
<td>M+F (45/717) Canada, Hutterite population</td>
</tr>
<tr>
<td>Stokke et al, 1997</td>
<td>No diff</td>
<td>No diff</td>
<td>Protect against hyperlipidemia (OR 0.27, P=0.037)</td>
<td>M+F (31/121) hyperTG patients and controls</td>
</tr>
<tr>
<td>Mattu et al, 1994</td>
<td>▼3%</td>
<td>▼3%</td>
<td>No difference for heart disease (OR 0.85, P=N.S.)</td>
<td>M (165/556) France, Ireland, ECTIM study</td>
</tr>
<tr>
<td>Peacock et al, 1992</td>
<td>▼N.S.</td>
<td>▼4.8%</td>
<td>No difference for heart disease (OR 0.89, P=N.S.)</td>
<td>M (22/101) Welsh, CAD patients and controls</td>
</tr>
<tr>
<td>Stocks et al, 1992</td>
<td>▼N.S.</td>
<td>▼4.5%</td>
<td></td>
<td>M (18/155) Sweden, MI survivors</td>
</tr>
<tr>
<td>Hata et al, 1990</td>
<td>▼N.S.</td>
<td>▼4.5%</td>
<td>Protect against hyperlipidemia (OR 0.27, P=0.037)</td>
<td>M+F (31/121) hyperTG patients and controls</td>
</tr>
<tr>
<td>Meta-Analyses</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wittrup et al, 2002</td>
<td>▼10%m</td>
<td>▼4%m</td>
<td>&lt;0.001</td>
<td>M+F (31/121) hyperTG patients and controls</td>
</tr>
<tr>
<td>Hokanson et al, 1999</td>
<td>▼8%</td>
<td>▼4.4%</td>
<td>Protect against CHD (OR 0.8)</td>
<td></td>
</tr>
</tbody>
</table>

M, males; F, females; +/-, heterozygous S447X carriers; -/-, homozygous S447X carriers; ND, not determined; NS, non-significant
Blood pressure, Alzheimer disease, Cancer

This paragraph summarizes a small number of reports on the relation between LPLS447X and blood pressure, Alzheimer disease and cancer.

The association between the LPLS447X variant and hypertension was assessed in highly diverse study cohorts. In healthy volunteers (n=696), the LPLS447X variant was associated with decreased systolic and diastolic blood pressure levels, but only in women (n=337). In individuals with familial hypercholesterolemia, a decreased diastolic blood pressure and a trend towards decreased systolic blood pressure was found in 128 both male and female LPLS447X carriers compared to 488 controls. In contrast, in dyslipidemic Chinese patients with essential hypertension, carriers were shown to exhibit moderately increased blood pressure. In contrast, haplotype analysis in 501 normotensive and 497 hypertensive Chinese subjects showed that the mutation was more frequent in the normotensive group, in fact suggesting a protective effect of LPLS447X.

LPL also plays a central role in cholesterol metabolism in the brain. The highest LPL activity is found in the hippocampus and the presence of LPL is thought to have a favorable effect on the survival and regeneration of neurons. LPL could therefore putatively affect the development of Alzheimer’s disease. Supporting this line of thought, a lower incidence of Alzheimer’s disease in carriers of the LPLS447X variant was recently shown in three studies. In contrast, two other studies could not show a relationship between LPLS447X and Alzheimer disease.

Since prostate cancer is associated with increased dietary fat intake, genetic factors that influence lipid metabolism may also be linked to the development of prostate cancer. A possible role of LPL in the development of prostate cancer was shown in only one study with 273 Japanese prostate cancer patients, 205 benign prostatic hyperplasia patients, and 230 male controls. In this study, LPLS447X was found associated with an increased risk for prostate cancer which was attributed to an increased availability of free fatty acids, released by LPL activity. Unequivocal data regarding the association between LPLS447X, blood pressure, and Alzheimer’s disease are likely hampered by small sample size, differences in genetic background, and different inclusion/exclusion criteria urging for careful interpretation. In general, genetic associations studies to study biological relationships need the use of very large population samples as recently reviewed and commented by Hattersley et al. and Cordell et al.
Mechanism underlying the beneficial effects of the S447X variant

The LPLS447X variant is thus associated with changes in lipid and lipoprotein metabolism and cardiovascular protection, but what molecular mechanisms are responsible for these beneficial effects? This question is not easily answered when one considers that the effects of this mutant LPL are only appreciated when studied in large groups of individuals indicating that the effects are mild in nature. Second, it may be realized that LPL function is regulated by numerous pathways and it is therefore likely that the beneficial effects of the LPLS447X variant are in fact the resultant of many (maybe hardly detectable) changes. In the next paragraphs, we will restrict to specific aspects of LPL biology that may be altered if LPL’s monomers lack the two C-terminal amino acids. We will focus on LPL activity and LPL concentration in the circulation, on the stability of LPL and its binding to heparin sulphate (HS) containing proteoglycans, on the LPL-meditated clearance of (remnant) lipoproteins by the liver, and finally on the expression of LPL and uptake of lipoproteins by macrophages (figure 1).

LPL activity and LPL concentration

Catalytic activity

Increased LPL activity results in lower plasma TG levels and higher HDL cholesterol levels. Since such a lipid profile is characteristic of carriers of the LPLS447X variant, one may hypothesize that LPLS447X simply has enhanced lipolytic capacity compared to wild-type LPL. Reviewing the literature on this topic, however, reveals unequivocal results. In direct comparisons (in vitro) with LPLWT, LPLS447X has been reported to exert increased (+85%), unchanged and even reduced catalytic activity (-30%). These discrepancies may relate to the type of cells used, how the culture media was harvested (in presence or absence of heparin) and handled. Irrespective of these equivocal results, data on LPL activity in carriers of the mutation suggest that overall, LPLS447X has increased lipolytic potential over LPLWT.
Postheparin LPL activity has been measured in at least 8 studies, summarized in table 2. In two initial studies in Swedish myocardial infarction survivors (n=173) and in hypertriglyceridemic patients (n=174) from Finland, post-heparin LPL activity was shown to be similar in patients that did or did not have the mutation. Using larger population samples two studies (475 and 397 subjects, respectively), however, showed significant 18% to 36% increases in post-heparin LPL activity in carriers compared to non-carriers. Our group previously genotyped and assessed post-heparin LPL activity levels in 804 males with established coronary atherosclerosis. In this cohort, we identified an overrepresentation of carriers of the LPLS447X variant in the highest quartile of LPL activity compared with the lowest quartile (18.3% vs. 11.5%; P<0.006).

Unpublished thus far, table 3 presents that post-heparin LPL activity levels were significantly higher in heterozygote carriers (n=118) but not in the small number of homozygote carriers (n=6) of the LPLS447X variant compared to non-carriers (n=539). In two subsequent studies
Table 2 List of studies in which the plasma LPL concentration and/or activity of carriers of the LPLS447X variant was assessed and compared with non-carriers

<table>
<thead>
<tr>
<th>Reference</th>
<th>LPL activity and concentration</th>
<th>Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peacock et al, 1992</td>
<td>Post-heparin LPL activity not different</td>
<td>M (18/155) Sweden, MI survivors</td>
</tr>
<tr>
<td>Knudsen et al, 1997</td>
<td>Post-heparin LPL activity not different</td>
<td>M-F Finland, 99 HyperTG + 75 controls</td>
</tr>
<tr>
<td>Garenc et al, 2000</td>
<td>†Post-heparin LPL activity (+18.8%, P&lt;0.05) in men only</td>
<td>M(40/188)-F(43/204) HERITAGE family study</td>
</tr>
<tr>
<td>Goodarzi et al, 2005</td>
<td>†Post-heparin LPL activity (+35.9%, P&lt;0.05)</td>
<td>M + F (44/353) Mexican-Americans</td>
</tr>
<tr>
<td>Henderson et al, 1999</td>
<td>†Post-heparin LPL activity (P&lt;0.05)</td>
<td>M (118/613) REGRESS study</td>
</tr>
<tr>
<td>Nierman et al, 2005</td>
<td>†Pre-heparin LPL concentration (5-fold, P&lt;0.01) Post-heparin LPL activity and concentration not different</td>
<td>M (6/-/-6) Netherlands</td>
</tr>
<tr>
<td>Nierman et al, 2005</td>
<td>†Pre-heparin LPL concentration (2.4-fold, P&lt;0.0001) Posts-heparin LPL activity and concentration not different</td>
<td>M (15/-/-15) Netherlands</td>
</tr>
<tr>
<td>Skoglund-Andersson et al, 2003</td>
<td>†Pre-heparin LPL activity (+58.8%; P&lt;0.001)</td>
<td>M (74/303) North European, 50 years old</td>
</tr>
</tbody>
</table>

* M, male; F, female

Concerning only 15 heterozygotes (compared to 15 controls) and 6 homozygotes (compared to 6 controls), we did not find differences in post-heparin LPL activity likely due to the very small sample sizes. Taken together, the published literature suggests enhanced post-heparin LPL activity in carriers of the LPLS447X variant compared to controls but large numbers of individuals are required to unmask this effect. In all of the above studies, heparin was used to release LPL from the endothelium to run the usual assays for LPL activity.

But to what extent does this methodology reflect the actual LPL-mediated TG hydrolysis in vivo? Some investigators have shown that it is also possible to measure LPL activity levels in non-heparinized plasma, though the activity levels are very low. Using a very sensitive activity assay, Skoglund-Andersson et al. identified a 60% increase in preheparin LPL activity in 18 carriers of
These investigators postulated that this increase could indeed be responsible for the slightly decreased TG levels and increased HDL cholesterol levels. Further indirect supporting evidence that LPLS447X has superior lipolytic activity over wild-type LPL was given by the higher apoB100 turnover rates of TRL in carriers as already discussed above. Since TRL conversion in plasma is almost entirely attributable to LPL-mediated TG hydrolysis, this suggests increased lipolytic activity of the mutant enzyme. Furthermore, a recent study in LPL knock-out mice showed 2-fold higher LPL activity after adenoviral gene transfer of cDNA encoding for LPLS447X compared to transfer of the wild-type LPL cDNA. This study also demonstrated that expression of the LPLS447X variant is a more potent triglyceride-lowering strategy than a similar one using wild-type LPL.

**LPL concentration**

Assessment of LPL concentration by enzyme linked immunosorbent assays (ELISA) either before or after heparinisation, is another frequently used biochemical means to assess LPL function in man. Using a commercially available ELISA, we recently showed that in postheparin plasma, LPL concentration is identical in carriers of the LPLS447X variant and wild-type controls. Interestingly, however, LPL concentration in non-heparinized serum was found approximately 2-fold increased in heterozygotes and 4-fold increased in the homozygotes for this mutation. Not bound to the endothelium, it is likely that this preheparin LPL concerns primarily catalytically inactive monomers, representing a catabolic product of catalytically active dimeric LPL bound to heparan sulphate (HS)-containing proteoglycans as indicated by the group of Olivecrona in 1993. This parameter may be a marker for the amount of systemically available (catalytically) active LPL. In fact, we recently showed that preheparin LPL concentration

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**Table 3 Post-heparin LPL activity levels and heterozygosity and homozygosity for the LPLS447X variant in males with established coronary atherosclerosis from the Regress study.**

<table>
<thead>
<tr>
<th></th>
<th>non-carriers</th>
<th>Heterozygotes for LPLS447X</th>
<th>Homozygotes for LPLS447X</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>539</td>
<td>118</td>
<td>6</td>
</tr>
<tr>
<td>LPL activity (mU/ml)</td>
<td>107 ± 43</td>
<td>121 ± 54*</td>
<td>108 ± 32†</td>
</tr>
</tbody>
</table>

Values were presented as mean ± SD. *p=0.01 vs. -/- group, †p=0.9 vs. -/- group, †p=0.5 vs. +/- group, all adjusted for BMI, age, NYHA class, systolic blood pressure and medication.
is inversely correlated with the risk of future CAD using the prospective 'European Prospective 
Investigation into Cancer and Nutrition' Norfolk cohort. The 1006 CAD cases and 1980 
matched controls studied here are, however, not yet genotyped for the SNP underlying 
LPLS447X, but these results are anticipated soon.

In summary, the published literature gives strong support for the notion that the LPLS447X 
variant exerts higher lipolytic potential compared to LPL and is present at higher concentrations 
in preheparin plasma. These findings may explain the beneficial effects of LPLS447X on lipid 
profiles and CVD.

Stability of LPL, binding to heparan sulphate containing proteoglycans and lipoproteins 
In the circulation, LPL is normally bound to HS-containing proteoglycans at the endothelium and 
primarily active as a dimer (monomeric LPL has also been reported to have residual catalytic 
activity). The affinity of the dimers for HS is higher compared with (inactive) monomeric LPL 
and, moreover, LPL dimers are stabilized by HS binding. Thus, the differences found in 
pre-heparin plasma LPL concentration and activity, and postheparin LPL activity may derive 
from differences in LPL dimer stability (or the stability of chimaeric heterodimers in heterozygotes). 
Zhang et al. showed, however, that LPLWT and LPLS447X as produced by transiently transfected 
COS cells had similar stabilities as tested by measuring catalytic activities after incubations at 
37 degrees Celsius. We recently confirmed this by measuring catalytic activities of recombinant 
LPLWT and LPLS447X after prolonged incubations at 37 degrees Celsius and in the presence of 
0-0.5 mM guanidine chloride. On the other hand, the increased concentration of LPLS447X in 
pre-heparin plasma may also be caused by decreased affinity of LPLS447X for HS-proteoglycans 
compared to LPLWT. Zhang et al. tested this for the two variants using heparin sepharose 
columns but found similar affinities for both (monomers and dimers). It could also be hypothesized 
that LPLS447X has higher affinity for lipoproteins in the circulation compared to LPLWT. Some 
evidence for this idea comes from a recent study by our group showing a higher concentration of 
LPL on apoB-containing lipoproteins in carriers of the mutation compared to controls (further 
discussed below).

In summary, the biochemical analyses performed to date have been unable to provide a convincing 
explanation for the increased LPL activity and LPL concentrations (in pre-heparin plasma) 
observed in carriers of the mutation.
Clearance of lipoproteins by the liver

It is already mentioned that LPL promotes the uptake of atherogenic lipoproteins by the liver via the VLDL and LDL receptors through acting as a ligand and/or a molecular bridge. It may thus be hypothesized that a better clearance of atherogenic remnant lipoproteins in carriers of the LPLS447X variant underlies the observed reduced risk of atherosclerosis. However, Salinelli et al. showed that the binding, uptake, and degradation of VLDL in LPLS447X producing COS cells was not different from LPLWT producing cells. Also, the hepatic clearance of a radioactive labeled chylomicron-like emulsion in a small number of carriers of the LPLS447X variant was found comparable to controls. However, we recently showed that homozygotes and heterozygotes for LPLS447X variant have enhanced LDL and apoB48 clearance rates, respectively, supporting the idea of an increased bridging function for the LPLS447X variant when considering increased levels of freely circulation LPL in these subjects compared to controls.

Uptake of lipoproteins by macrophages

It has been generally acknowledged that LPL in addition to skeletal, heart and adipose tissue, is also produced by monocyte-derived cells in the subendothelial space and that this leads to foam cell formation, a key event in atherogenesis. Clee et al. provided evidence that LPL in the vascular wall was indeed a proatherogenic factor, albeit in a mouse model for atherosclerosis. This hypothesis finds support in studies of LPL overexpression in macrophages leading to increased atherosclerosis in the aorta of rabbits. Thus, it could be hypothesized that the atheroprotective effects of LPLS447X may derive from reduced expression of LPL by macrophages but more likely by reduced uptake of (modified) LDL in subendothelial macrophages in carriers of the mutation. Such an effect would provide a straightforward explanation of the anti-atherogenic effects that are associated with LPLS447X.

Conclusions

The bulk of evidence summarized shows that carriers of the LPLS447X variant mutation have lower TG levels, and increased HDL cholesterol levels with a concomitant lower incidence of CVD compared to non-carriers. These findings support the notion that it concerns a gain-of-function mutation, the very reason for the use of LPLS447X in the development of gene therapy for human LPL deficiency. The unraveling of the molecular mechanisms responsible for these beneficial effects has, however, proven difficult. Most studies in humans indicate that the beneficial effects are associated with enhanced TG lowering capacity mainly attributed to increased lipolytic
function. However, the noted differences were rather small and as a result mainly identified in studies with larger groups of individuals. The idea that LPL WT and LPLS447X are only slightly different and likely impact simultaneously numerous aspects of LPL biology (with cumulative, synergistic or opposing effects) in vivo, may underlie the fact that many molecular (in vitro) studies did not identify differences between LPL WT and LPLS447X regarding catalytic activity, stability of the protein, affinity for heparin sepharose, or capacity to mediate uptake of lipoproteins.\textsuperscript{17,80,85}

**Future research**

Additional insight into the molecular mechanisms how LPLS447X exerts its beneficial effects may come from studies on the affinity of this mutant for circulating lipoproteins\textsuperscript{97}. Also a comparison of LPL WT and LPLS447X in the processes of foam cell formation, intracellular trafficking, cellular secretion, and translocation (over the endothelium) may be warranted but chances to find marked differences may be slim for the reasons indicated above. The need for heparin injections to assess LPL function in humans, which likely kept many investigators from studying LPL in their clinical studies, has unfortunately limited our knowledge on how LPL is related to (patho)physiological conditions. Maybe the use of sensitive ELISA’s\textsuperscript{83,98} or the use of minor amounts of catalytically active LPL on circulating lipoproteins\textsuperscript{84} may bring relief for future studies on LPL and its natural variants.

Furthermore, studies on the interactions of both LPL variants with its activators apoCII\textsuperscript{99} and apoAV\textsuperscript{100,101} and with negative regulators such as apoCIII, angptl2, and angptl3\textsuperscript{102-104} have not been published thus far. Adding to the complexity, Karpe et al. have furthermore provided evidence for differential regulation of the secretion (and uptake) of active and inactive LPL in adipose tissue and skeletal muscle in humans, which may be explained by local differences in LPL affinity for endothelial cells.\textsuperscript{105} These intriguing and poorly understood aspects of LPL biology may also need to be accounted for when comparing the actions of LPL and its natural mutants.

**Acknowledgement**

Part of this work was enabled by a grant of the Netherlands Heart Foundation (2000T039).
Reference List


CARRIERS OF THE FREQUENT LIPOPROTEIN LIPASE S447X VARIANT EXHIBIT ENHANCED POSTPRANDIAL APOPROTEIN B-48 CLEARANCE

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³ Department of Epidemiology and Biostatistics, Academic Medical Center, University of Amsterdam, the Netherlands.
⁴ Department of Internal Medicine and Molecular Science, Osaka University Graduate School of Medicine, Japan.

Abstract

**Objective:** The frequent lipoprotein lipase S447X variant (LPLS447X) is firmly associated with a lower incidence of cardiovascular disease (CVD), the mechanisms for which remain to be established. To further unravel these beneficial effects, we studied the consequences of LPLS447X heterozygosity on LPL mass and activity, as well as on the postprandial lipoprotein profile.

**Methods:** 15 male heterozygous LPLS447X carriers and 15 matched control subjects received an oral fat load (30 grams/m²). Lipid parameters were evaluated at baseline and 2, 3, 4 and 6 hours after fat loading. LPL concentration and activity were analyzed and endothelial function was evaluated non-invasively as flow mediated dilation (FMD) of the brachial artery.

**Results:** Whereas baseline apoB48 levels were similar, the rise in apoB48 was attenuated in LPLS447X carriers with 25% lower peak values compared to controls (p=0.04). In conjunction, LPLS447X carriers were characterized by a 2.4 fold increase in pre-heparin LPL mass (p<0.0001). The decrease in postprandial FMD was comparable in both groups.

**Conclusion:** LPLS447X carriers exhibit enhanced apoB48 clearance with concomitant increase in pre-heparin LPL mass, without changes in LPL activity. This combination might suggest a role for increased ligand action of LPL in LPLS447X carriers contributing to the cardiovascular protection in carriers of this mutation.
Introduction

Lipoprotein lipase (LPL) is a principal determinant in the metabolism of triglyceride-rich lipoproteins (TRLs). Whereas the enzymatic activity of LPL mediates hydrolysis of triglycerides (TG) from fasting (VLDL) and postprandial TRLs, LPL also exhibits a ligand function that mediates hepatic clearance of TRLs. Increased LPL activity in the circulation has been associated with a less atherogenic lipid profile. Conversely, LPL located within the vessel wall gives rise to local release of pro-atherogenic substrates (free fatty acids (FFA), remnant particles) and may facilitate lipoprotein trapping within the subendothelial matrix, resulting in stimulated foam cell formation. The presence of functional variants in the LPL protein has facilitated assessment of the role of LPL in the development of atherosclerotic vascular disease.

The LPLS447X variant, present in 18-22% of individuals in the general population, has been associated with increased post-heparin LPL mass, whereas the LPL activity associated with this variant has created contrasting data. The LPLS447X variant is associated with beneficial lipid profile changes, notably elevated high-density lipoprotein (HDL) cholesterol and lower TG levels as well as a lower prevalence of cardiovascular disease (CVD). The mechanism for this cardiovascular protection is unknown. Enhanced ‘postprandial’ lipoprotein clearance as well as a direct effect on the arterial wall have been put forward as potential mechanisms contributing to the anti-atherogenic effects of LPLS447X. In the present study we evaluated the consequences of LPLS447X heterozygosity on LPL mass and activity, as well as on postprandial lipoprotein profile.

Methods

Participants

Fifteen male heterozygous LPLS447X carriers were selected from a database of the Research Lipid Clinic of the Academic Medical Center Amsterdam. The control group (n=15), selected from the same database, was matched for gender, age, body mass index (BMI), smoking habits and alcohol use. Subjects had no signs of overt CVD. All medication was stopped two weeks prior to the investigation. The study protocol was approved by the Institutional Review Board (IRB) of the AMC and all participants gave written informed consent. The investigation conforms to the principles outlined in the Declaration of Helsinki.
Genotyping
LPL and apoE genotyping were performed as previously described. \textsuperscript{13,14}

Study design
All participants refrained from alcoholic beverages and smoking 24 hours prior to investigation. At the day of the study at 08:00 a.m., fasting blood samples were drawn. Subsequently, all participants ingested an oral fat load (T=0), consisting of 30 grams of fat per square meter body surface area\textsuperscript{15}, administered as cream (35g fat per 100 mL). Blood sampling was repeated at t=2, 3, 4 and 6 hours after fat load ingestion.

Biochemical measurements
Blood for lipid analysis was drawn in EDTA-coated tubes and plasma was isolated by centrifugation at 3000 RPM at 4º C for 15 minutes and aliquoted for storage at -80º C. Baseline total cholesterol (TC) and triglycerides (TG) were measured by standard enzymatic methods (CHOD-PAP and GPO-PAP, Roche Diagnostics, Mannheim, Germany). HDL-C was measured in the supernatant fraction after precipitation of apolipoprotein (apo) B-containing lipoproteins with dextran sulphate and magnesium chloride. Low-density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald formula\textsuperscript{16}. Baseline and postprandial TGs were measured using a commercially available kit (Triglyceride GPO-trinder, Sigma diagnostics, Mannheim, Germany). Plasma apoB48 levels were measured by a sandwich ELISA using anti-human apoB48 monoclonal antibodies as reported previously with minor modification.\textsuperscript{17} Blood for pre- and post-heparin LPL concentration and activity measurements was collected in heparin-containing tubes before and 15 minutes after an intravenous injection of heparin (50 IU/kg body weight). LPL activity was analyzed as previously published\textsuperscript{18}. LPL concentrations were measured using a commercially available kit (Markit-M LPL, Dainippon Pharmaceutical Co, Osaka, Japan).

Endothelial function
Endothelial function was assessed as Flow Mediated Dilation (FMD) of the brachial artery as described previously\textsuperscript{19} at baseline, two and six hours after the fat load. Endothelium-independent vasodilatation was assessed at baseline, two and six hours after the fat load, which was evoked by administrating 0.3 mg nitroglycerine (NTG) sublingually.
Statistical Analysis
Continuous (baseline) variables were compared between heterozygous LPLS447X carriers and control subjects using the Mann-Whitney test. Chi-square test was applied for comparing distribution of dichotomous data. Longitudinal changes in TG and apoB48 between the carriers and the controls were tested by analysis of repeated measures, using linear mixed models. In this model, first-order auto-regressive was used to specify the covariance structure for the residuals. The correlations coefficients were calculated using Spearman correlation. P-values < 0.05 were considered statistically significant.

Results

Baseline characteristics
Baseline characteristics of heterozygous LPLS447X carriers and controls are shown in table 1. Baseline TC, HDL cholesterol and LDL cholesterol and TG were not significantly different between carriers and controls (p=0.94, p=0.97, p=0.65 and p=0.31, respectively).

Lipoprotein Lipase Mass and Activity
In the carrier group, pre-heparin LPL mass was increased 2.4-fold compared to the matched controls (22.4 ± 8.3 vs. 52.9 ± 20.8 ng/mL, p<0.0001). In contrast, post-heparin LPL mass (403.5 ± 97.2 vs. 404.4 ± 155.3 ng/mL) and post-heparin LPL activity (496.6 ± 126.5 vs. 474.7 ± 102.9 mU/mL) were similar in controls and carriers (p=0.87 and p=0.62, respectively).

Lipid metabolism after an oral fat load
In both groups, oral fat loading resulted in a significant increases in mean TG levels (p<0.001) reaching maximum values three hours after the fat load (figure 1A). Although mean TG levels were not significantly different between the groups (p=0.93), peak TG levels tended to be lower in carriers (-14.8%, p=0.09). The area under the triglyceride curve (AUC-TG) was 13% lower in carriers compared to matched controls, however without significance (p=0.16). Since postprandial TGs are transported in apoB48-containing chylomicrons (CM), we subsequently focused on postprandial apoB48 levels. As expected, mean apoB48 levels increased significantly after fat loading in both groups (p<0.001; figure 1B). The mean increase in apoB48 levels was approximately 25% lower in carriers compared to controls (p=0.04). Notably, correction for baseline TG
levels resulted in further increase of statistical significance (p=0.034). In addition, after the fat load apoB48 reached its maximum in the carrier group one hour earlier compared to the controls. Six hours after the fat load, apoB48 levels returned towards baseline in both groups.

<table>
<thead>
<tr>
<th>Table 1 Baseline Characteristics of Controls and Carriers.</th>
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<tr>
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<tr>
<td>LDL Cholesterol (mmol/L)</td>
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<td>Triglycerides (mmol/L)</td>
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</table>

Carriers: Heterozygous Lipoprotein Lipase S447X carriers; SBP: systolic blood pressure, DBP: diastolic blood pressure. All data are presented as means and standard deviations or as percentage.

Endothelial function after an oral fat load

In both cohorts, fat loading induced a significant FMD impairment (p<0.001) reaching a maximum two hours after fat loading. The degree of impairment in FMD response was not significantly different between the two groups (p=0.57). At T=6 hours, FMD had returned towards baseline levels in both groups. Baseline NTG-induced vasodilatation was not significantly different between the two groups (p=0.25). Fat loading had no effect on the NTG-induced vasodilatation in any group (p=ns).
Discussion

We show that heterozygous LPLS447X carriers exhibit an attenuated increase in apoB48 levels after ingestion of an oral fat load, whereas this enhanced clearance is not accompanied by aggravation of postprandial endothelial dysfunction. Concomitantly, carriers present with a significantly increased pre-heparin LPL mass. These data imply that enhanced apoB48 clearance in LPLS447X carriers may contribute to cardiovascular protection.

Since the major role of LPL is predominantly confined to the postprandial phase, we focused on postprandial lipid handling. Peak TG levels tended to be lower in carriers compared to controls,
which falls in line with findings from the European Atherosclerosis Research Study (EARS).\textsuperscript{20} Subsequently, we also assessed CM removal by measuring apoB\textsubscript{48} levels. The peak apoB\textsubscript{48} levels in carriers were 25\% lower compared to controls, whereas peak levels were also reached earlier in carriers. These findings corroborates recent data, showing that the LPLS\textsubscript{447X} variant was associated with enhanced postprandial clearance of TRLs.\textsuperscript{21}

To date, measurement of LPL in vivo has focussed on post-heparin values. More recently, the measurement of pre-heparin LPL data has been optimized. In our present investigation, we find a 2.4-fold increase in pre-heparin LPL mass in carriers. These data corroborate in vitro findings, in which LPLS\textsubscript{447X} was shown to be associated with increased production of LPL-monomers.\textsuperscript{5} In vivo, LPL monomers are also present in the circulation,\textsuperscript{22} based on lower binding affinity of monomeric LPL to endothelial proteoglycans compared to dimeric LPL.\textsuperscript{23} The LPLS\textsubscript{447X} carriers exhibited a 2-fold increase in pre-heparin LPL concentration compared to controls, whereas predominantly pre-heparin LPL concentration has been shown to reflect monomeric LPL proteins.\textsuperscript{24} The finding of increased pre-heparin LPL mass in carriers corresponds to previously reported associations between increased pre-heparin LPL mass and HDL-increase and TG-decrease, which is a hallmark in LPLS\textsubscript{447X} carrier.\textsuperscript{22,25,26} Interestingly, pre-heparin LPL mass also has been reported to be inversely correlated to CVD and its progression.\textsuperscript{27}

Inactive LPL has also been shown to mediate TRL removal from plasma in vivo.\textsuperscript{1,28} With regard to the postprandial state, inactive LPL was shown to bind to chylomicrons in vitro,\textsuperscript{29} whereas binding of inactive LPL to CM was comparable to that of catalytically active LPL.\textsuperscript{29} Consequently, in LPLS\textsubscript{447X} carriers, the increased pool of ‘enzymatically-inactive’ LPL might contribute to enhanced hepatic apoB\textsubscript{48} clearance.

We have previously demonstrated that the site of enzymatic LPL activity determines its potential impact on vascular physiology. Whereas vessel wall LPL has predominantly pro-atherogenic consequences, circulating LPL appears to exert anti-atherogenic effects.\textsuperscript{30} Vessel wall LPL has been suggested to increase local exposure of pro-atherogenic substrates to the endothelial lining. In view of the predictive value of endothelial dysfunction for future cardiovascular disease, increased clearance of TRL due to TRL entrapment in the vascular wall has been suggested lead to enhanced endothelial dysfunction. Whereas we show clear attenuation of FMD upon fat loading in the present study, we do not find an aggravation of endothelial dysfunction in LPLS\textsubscript{447X} carriers in spite of the increased apoB\textsubscript{48} removal rate. Hypothetically, these data may indicate increased removal of TRL particles through a mechanism other than TRL hydrolysis near the vascular endothelium in LPLS\textsubscript{447X} carriers.
Study limitations
Some aspects of our study deserve closer attention. The first aspect is, that most studies have demonstrated that the LPLS447X variant is associated with an anti-atherogenic lipid profile consisting of decreased TG levels and increased HDL-C levels. The fact that we could not show significance in baseline TG levels has two explanations. Since our primary aim was to evaluate changes upon oral fat loading, we excluded subjects with obesity and carefully matched carriers and controls for gender, age, BMI, smoking habits and alcohol use. Moreover, in view of the primary aim, we used a limited sample size. Reviewing the usually modest TG differences in carriers compared to controls, differences in TG levels in carriers of the frequent LPL variant have been reported in much larger numbers of carriers (n=118-413). The second aspect is, that with regard to LPL activity, pre-heparin LPL activities were all below the detection limit. Whereas post-heparin LPL activities were not different between carriers and controls, it should be noted that the latter reflects the activity of the total LPL pool, of which only a part is physiologically active. Thus, based on data from the current study, we cannot exclude effect of this frequent LPL variant on enzymatic activity of LPL.

Summary
We show attenuated increase in apoB48 levels upon fat loading in LPLS447X carriers. Concomitantly, carriers exhibited increased pre-heparin LPL mass. These findings of increased apoB48 clearance combined with increased circulating LPL mass might suggest a role for increased ligand action of LPL in LPLS447X carriers contributing to the lower prevalence of CVD. Additional kinetic studies are required to confirm these findings in LPLS447X carriers.

Reference List


CHAPTER 4

ENHANCED CONVERSION OF TRIGLYCERIDE-RICH LIPROTEINS AND INCREASED LOW-DENSITY LIPROTEIN REMOVAL IN LIPROTEIN LIPASE S447X CARRIERS

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Abstract

Objective LPL exerts two principal actions, comprising enzymatic hydrolysis of triglyceride-rich lipoproteins (TRL) and non-enzymatic ligand capacity for enhancing lipoprotein removal. The common LPLS447X variant has been associated with cardiovascular protection, for which the mechanism is unknown. We therefore evaluated enzymatic and non-enzymatic consequences of this LPL variant on TRL metabolism.

Methods and Results TRL apoB100 metabolism was determined in 5 homozygous LPLS447X carriers and 5 controls. Subjects were continuously fed and received infusion of stable isotope L-[1-13C]-valine. Results were analyzed by SAAM II modeling. Also, pre- and post-heparin LPL concentration and activity were measured. Compared to controls, carriers presented increased VLDL1 to VLDL2 apoB100 flux (p=0.04), increased VLDL2 to IDL apoB100 flux (p=0.02), increased IDL to LDL apoB100 flux (p=0.049) as well as an increased LDL clearance (p=0.04). Additionally, IDL apoB100 synthesis was attenuated (p=0.05). Pre-heparin LPL concentration was 4-fold higher compared to controls (p=0.01) and a correlation was observed between pre-heparin LPL concentration and LDL clearance (r^2=0.92, p=0.01).

Conclusions Enhanced TRL conversion and enhanced LDL removal combined with increased pre-heparin LPL concentration suggest increased enzymatic consequences as well as increased non-enzymatic consequences of LPL in LPLS447X carriers, that might both contribute to the cardiovascular benefit of this LPL variant.
Introduction

Evidence has accumulated to show that triglyceride-rich lipoproteins (TRL) contribute to atherogenesis. The TRL pool ranges from very large particles consisting of chylomicrons (CM) and very-low-density lipoprotein (VLDL₁) to smaller particles like VLDL₂ and intermediate-density lipoproteins (IDL). VLDL₁ and VLDL₂ contribute to lipid accumulation in human macrophages and promote foam cell formation. Lipoprotein lipase (LPL) plays a crucial role in TRL delipidation as this enzyme largely determines the conversion of large TRL (VLDL₁) via smaller TG-depleted TRL (VLDL₂, IDL) towards LDL. In addition, LPL exerts a ligand function, facilitating non-enzymatic removal of lipoprotein particles.

Most LPL gene variants have been associated with an increased cardiovascular disease (CVD) risk and result in partial loss of function. In contrast, the frequent LPLS447X variant, present in 20% of the population, is associated with decreased TG and increased HDL-C. Data on the LPL concentration and activity in this variant have been conflicting. Overall, this LPL variant is associated with a lower incidence of CVD. However, the precise mechanisms remain to be determined.

Increased turnover of large VLDL to LDL, with ensuing enhanced clearance of LDL, could be a potential mechanism for cardiovascular protection in LPLS447X carriers. To test this hypothesis, we evaluated apoB100 kinetics in homozygous LPLS447X carriers in the fed state by infusion of the stable isotope L-[1-¹³C]-valine.

Materials and Methods

Participants
Five male homozygous LPLS447X carriers were selected from a database at the Academic Medical Center in Amsterdam and matched to controls with respect to age, body mass index (BMI), smoking habits, lipid levels and use of alcohol. None of the subjects had signs of CVD, nor did they exhibited E2/E2 or E4/E4 genotype. All participants gave written informed consent. The study protocol was approved by the Institutional Review Board of the AMC. The study conforms to the principles outlined in the Declaration of Helsinki.
Genotyping

LPL genotyping was performed as described previously. Out of 2000 DNA samples, six males were found to be homozygous carriers of the LPLS447X variant, five of whom were willing to participate.

Experimental protocol

The protocol for infusion of labeled valine has been described previously. All participants entered the research ward on the evening prior to the study. At 03:00 a.m. (t=0), baseline blood sampling was performed. Subsequently, a shake, equivalent to 1/20th of their daily food intake, was ingested every hour until 09:00 p.m. This shake consisted of 14% of calories as protein, 44% as carbohydrates, 42% fat (17% saturated, 17% monounsaturated, 8% polyunsaturated) and 90 mg cholesterol per 1000 kcal. Five hours after baseline blood sampling, intravenous catheters were placed in both forearms and a priming dose of 17 µmol/kg L-[1-13C]-valine was given intravenously, followed by continuous infusion of 15 µmol/kg/hour for 13 hours. Blood samples were obtained from the contralateral arm at baseline and after 5, 5 1/2, 6, 7, 8, 9, 10, 11, 12, 14, 16, 18 hours.

Isotope and chemicals

L-[1-13C]-valine (isotope mole fraction > 0.99; MassTrace, Woburn, MA, USA) was dissolved in sterile 0.9% saline and sterilized through a 0.22 µm filter. Density solutions were made with KBr in 0.9% NaCl.

Biochemical measurements

Blood for lipid analysis was drawn in EDTA-coated tubes. Plasma was isolated by centrifugation and stored at -80º C. Baseline total cholesterol (TC) was measured by standard enzymatic methods (CHOD-PAP, Roche Diagnostics, GmbH, Mannheim, Germany). Baseline HDL-C was measured in the supernatant fraction after precipitation of apoB-containing lipoproteins with dextran sulphate and magnesium chloride. TG and free fatty acids (FFA) were measured using commercially available kits (Triglyceride GPO-trinder, Sigma Diagnostics Inc., St. Louis, MO, USA and NEFA-C, Wako Chemicals GmbH, Neuss, Germany). LDL-C was calculated using the Friedewald formula.
Measurement of LPL concentration and activity
Blood for pre- and post-heparin LPL concentration and activity was collected in heparin-containing tubes before and 15 minutes after an intravenous injection of heparin (50 IU/kg body weight) at least one month prior to the study. LPL activity was analyzed as previously published. LPL concentrations were measured using a commercially available kit (Markit-M LPL, Dainippon Pharmaceutical Co, Osaka, Japan).

LPL analysis in apoB and non-apoB fractions
ApoB containing lipoproteins were precipitated with a solution of magnesium chloride, dextran sulfate and magnetic beads (Polymedco, Cortlandt Manor, NY). Pre-heparin plasma (200 L) was mixed with 40 µL of the beads solution. The supernatant was collected and the pellet was resuspended in 150 µL LPL stabilizer solution from a LPL ELISA kit (Markit-M) after which the beads were separated using a magnet. LPL concentration was subsequently measured in both the supernatant and in the resuspended apoB containing pellet.

Isolation and analysis of apoB100 containing lipoproteins
Isolation of apoB100 containing lipoproteins was performed as previously described. In short, CM, VLDL₁, VLDL₂, IDL, and LDL were isolated using a discontinuous salt gradient by cumulative ultracentrifugation (UC) (Beckman Ultracentrifuge Sw41 Ti rotor, Beckman Instruments, Fullerton, CA, USA). After three subsequent UC spins, the CM fraction (32 min, 40700 rpm, 4ºC, acceleration 5, brake 5), the VLDL₁ fraction (3:28 h) and the VLDL₂, the IDL, the LDL and the bottom fraction (HDL + lipoprotein deficient plasma) were collected after 17 hours. After sample collection of the first and the second UC spin, the UC tubes were refilled using 1 ml D=1.006 g/mL KBr. All fractions were stored at -20 ºC. Fractional ApoB100 concentrations were determined using a nephelometric assay (Dade Behring, Marburg, Germany).

ApoB100 isolation from lipoproteins and determination of 13C-valine enrichment
ApoB100 was isolated from lipoproteins and determination of 13C-valine enrichment was performed as described previously.

ApoB100 kinetics
ApoB100 production was measured as the rate of incorporation of 13C-enriched valine into circulating VLDL₁, VLDL₂, IDL, and LDL apoB100 and best data fit in a six compartment model.
was determined using the SAAM II software (Simulation Analysis and Modelling, version 1.1.1., SAAM Institute, Seattle, WA, USA) as described previously and shown in figure 1. Plasma volumes were calculated from body surface area. The plasma pool was calculated by multiplying the plasma volume by the plasma apoB concentration. It was assumed that during the study each subject remains in steady state with respect to apoB100 metabolism, during which fractional catabolic rate (FCR) equals fractional synthetic rate (FSR). The direct catabolism in VLDL₁ apoB100 was assumed to be identical to the direct catabolism of VLDL₂ apoB100 in order to reduce the number of unknown variables.

Figure 1: Multi-compartmental model for ApoB 100 metabolism.

Compartment 1 represents plasma valine into which the valine tracer was injected. Compartment 2 represents a delay compartment. Valine is incorporated in VLDL₁ apoB100, VLDL₂ apoB100, IDL apoB100, and LDL apoB100 via compartment 3, 4, 5, and 6. The k values represent the rate constants.

Statistical Analysis
All data are presented as mean values±SD. All data were analyzed with SPSS (version 12.0.1, SPSS Inc., Chicago, Illinois, USA) and were compared using an independent samples t-Test. Correlations were performed by linear regression. Statistical significance was accepted at a level of $P < 0.05$. 
Results

Baseline Characteristics

Baseline characteristics of carriers and controls are shown in table 1. Age, BMI, blood pressure and waist/hip-ratio and lipids were not significantly different. Also, fasting apoB100, glucose, insulin and calculated plasma volumes were comparable. Non-fasting lipids, plasma apoB and apoB100 in the different lipoprotein fractions and FFA were not significantly different between groups (data not shown). During feeding, plasma TG increased significantly compared to baseline (p<0.01) without differences between groups (data not shown).

<table>
<thead>
<tr>
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<th>Controls (n=5)</th>
<th>LPLS447X (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
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<td>BMI, kg/m²</td>
<td>23.6±0.9</td>
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<td>Waist/Hip ratio</td>
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<td>SBP (mmHg)</td>
<td>130.8±9.7</td>
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<tr>
<td>DBP (mmHg)</td>
<td>80.0±94</td>
<td>80.0±98</td>
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<tr>
<td>Total Cholesterol (mmol/L)</td>
<td>5.23±0.88</td>
<td>6.00±1.27</td>
</tr>
<tr>
<td>HDL Cholesterol (mmol/L)</td>
<td>1.65±0.28</td>
<td>1.62±0.27</td>
</tr>
<tr>
<td>LDL Cholesterol (mmol/L)</td>
<td>3.21±0.55</td>
<td>3.99±1.05</td>
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<tr>
<td>Triglycerides (mmol/L)</td>
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<tr>
<td>ApoB100 (mg/L)</td>
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<tr>
<td>Glucose (mmol/L)</td>
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<tr>
<td>Plasma Volume (mL)</td>
<td>2692±298</td>
<td>2713±307</td>
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</table>

SBP: systolic blood pressure; DBP: diastolic blood pressure
Kinetics

Rate constants are listed in table 6.

**VLDL\(_1\) apoB100 metabolism**

VLDL\(_1\) apoB100 poolsize was not significantly different between groups (table 2). VLDL\(_1\) apoB100 synthesis, VLDL\(_1\) apoB100 production rate and transfer of VLDL\(_1\) apoB100 were not significantly different between both groups. Interestingly, the flux of VLDL\(_1\) to VLDL\(_2\) apoB100 was 2.4-fold increased in carriers (p=0.04). No significant differences were found for direct catabolism, total FCR and direct clearance of VLDL\(_1\) apoB100.

**VLDL\(_2\) apoB100 metabolism**

VLDL\(_2\) apoB100 poolsize, VLDL\(_2\) apoB100 synthesis, VLDL\(_2\) apoB100 production rate and transfer of VLDL\(_2\) apoB100 were similar in both groups (table 3). The flux of VLDL\(_2\) to IDL apoB100 was 1.7-fold higher in carriers (p=0.02). No significant differences were found in direct catabolism, total FCR and direct clearance of VLDL\(_2\) apoB100.

**IDL apoB100 metabolism**

IDL apoB100 poolsize, IDL apoB100 production rate, transfer of IDL apoB100 to LDL apoB100, direct catabolism, total FCR and direct clearance were not significantly different (table 4). The flux of IDL to LDL apoB100 was 1.6-fold higher in carriers (p=0.049). IDL apoB100 synthesis was lower in carriers (p=0.05).

**LDL apoB100 metabolism**

No significant differences were found for LDL apoB100 pool size, synthesis, production rate or total FCR between both groups (table 5). Interestingly, carriers exhibited a 1.4-fold increase in direct clearance of LDL apoB100 (p=0.03).
Legend for tables 2-5

VLDL₁ apoB100 (mg): VLDL₁ apoB100 poolsize in mg
VLDL₂ apoB100 (mg): VLDL₂ apoB100 poolsize in mg
IDL apoB100 (mg): IDL apoB100 poolsize in mg
LDL apoB100 (mg): LDL apoB100 poolsize in mg
Synthesis (mg/day): synthesis of apoB100 in the lipoprotein fraction from the liver in mg per day
Production Rate (mg/kg/day): production rate of apoB100 in the lipoprotein fraction from the liver in mg per kg bodyweight per day
Transfer (pools/day): transfer of apoB100 from the lipoprotein fraction in pools per day
Flux (mg/day) total transfer of apoB100 from the lipoprotein fraction in mg per day
Direct Catabolism (pools/day): direct catabolism of apoB100 from the lipoprotein fraction in pools per day
Total FCR (pools/day): Total fractional catabolic rate of apoB100 from the lipoprotein fraction in pools per day
Direct Clearance (mg): total clearance of apoB100 from the lipoprotein fraction in mg per day

Table 2 VLDL₁ ApoB100 (Sf 60-400) Metabolism in LPLS447X Carriers and Controls

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<tr>
<th></th>
<th>VLDL₁ apoB100</th>
<th>Synthesis</th>
<th>Production</th>
<th>Transfer</th>
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| p  | 0.08 | 0.06 | 0.10 | 0.34 | 0.04 | 0.35 | 0.85 | 0.51 |
Enhanced Conversion of Triglyceride-Rich Lipoproteins And Increased Low-Density Lipoprotein Removal in Lipoprotein Lipase S447X Carriers

Table 3 VLDL2 ApoB100 (Sf 20-60) Metabolism in LPL S447X Carriers and Controls

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<th>VLDL2 apoB100 Synthesis</th>
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<tr>
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<td>71</td>
<td>1042</td>
<td>14</td>
<td>7.2</td>
<td>506</td>
</tr>
<tr>
<td>2</td>
<td>92</td>
<td>594</td>
<td>7</td>
<td>13.0</td>
<td>1196</td>
</tr>
<tr>
<td>3</td>
<td>113</td>
<td>888</td>
<td>13</td>
<td>8.6</td>
<td>972</td>
</tr>
<tr>
<td>4</td>
<td>45</td>
<td>401</td>
<td>4</td>
<td>13.7</td>
<td>610</td>
</tr>
<tr>
<td>Controls</td>
<td>77 ± 27</td>
<td>679 ± 275</td>
<td>9 ± 4</td>
<td>10.8 ± 2.8</td>
<td>802 ± 281</td>
</tr>
<tr>
<td>S447X</td>
<td>111 ± 26</td>
<td>637 ± 357</td>
<td>8 ± 5</td>
<td>12.4 ± 1.7</td>
<td>1365 ± 323</td>
</tr>
<tr>
<td>6</td>
<td>119</td>
<td>1198</td>
<td>16</td>
<td>13.0</td>
<td>1551</td>
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<tr>
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<td>143</td>
<td>688</td>
<td>7</td>
<td>11.9</td>
<td>1704</td>
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<tr>
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<td>83</td>
<td>637</td>
<td>8</td>
<td>10.4</td>
<td>861</td>
</tr>
<tr>
<td>9</td>
<td>123</td>
<td>379</td>
<td>5</td>
<td>11.7</td>
<td>1432</td>
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<tr>
<td>10</td>
<td>86</td>
<td>280</td>
<td>4</td>
<td>14.9</td>
<td>1278</td>
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| p | 0.07 | 0.84 | 0.74 | 0.31 | 0.02 | 0.35 | 0.16 | 0.31 |

LPL
In carriers, pre-heparin LPL concentration was increased 4-fold (carriers: 104.6 ± 38.3 vs. controls: 25.4 ± 9.3 ng/mL, p=0.01). Pre-heparin LPL activity was below the detection limit in both groups. Post-heparin LPL concentration (carriers: 367 ± 90 vs. controls: 369 ± 150 ng/mL) as well as post-heparin LPL activity (carriers: 235.2 ± 45.0 vs. controls: 196.3 ± 76.1 mU/mL) were similar between groups (p=0.97 and p=0.29 respectively).

Correlations
In carriers, pre-heparin LPL concentration correlated with direct LDL apoB100 clearance (r²=0.92, p=0.01). No correlation was found between pre-heparin LPL and LDL clearance in the controls (p=0.27).
To verify whether pre-heparin LPL was associated with apoB-containing lipoproteins, we quantified LPL in whole plasma, in apoB-depleted plasma and in a resuspended pellet which resulted from precipitated apoB-containing lipoproteins. In LPLS447X carriers and controls, 71±7% and 60±15% of the total serum LPL was found in the apoB-containing fraction. Compared to controls, the LPLS447X carriers presented an absolute higher amount of LPL (from 200 μL plasma) in the apoB containing fraction (carriers: 8.9±2.5 vs. controls: 2.8±0.6 ng; p<0.01) as well as in the non-apoB fraction (carriers: 3.8±1.6 vs. controls: 2.3±2.1 ng; p=0.25) (figure 2). In separate control experiments, we confirmed the presence of LPL in the apoB fraction using an alternative methods to separate non-apoB and apoB containing lipoproteins (MgCl₂ and phosphotungstic acid; data not shown).

| Table 4 IDL ApoB100 (Sf 12-20) Metabolism in LPLS447X Carriers and Controls |
|-----------------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| IDL apoB100 Synthesis Rate | Production Transfer Flux Direct Total Catabolism Direct Clearance |
| (mg) | (mg/day) | (mg/kg/day) | (pools/day) | (mg/day) | (pools/day) | (mg/kg/day) | (pools/day) | (mg/day) | (pools/day) |
| 1 | 77 | 88 | 1 | 10.5 | 812 | 0.0 | 10.5 | 0.0 |
| 2 | 113 | 475 | 6 | 6.5 | 733 | 2.2 | 8.7 | 247 |
| 3 | 91 | 58 | 0 | 9.9 | 896 | 4.0 | 13.9 | 358 |
| 4 | 91 | 133 | 2 | 7.7 | 692 | 4.6 | 12.2 | 413 |
| 5 | 114 | 289 | 3 | 7.3 | 832 | 0.6 | 7.9 | 66 |
| Controls | 97±16 | 209±173 | 3±2 | 84±1.7 | 793±81 | 2.3±2.0 | 10.6±2.5 | 218±180 |
| S447X | 111±46 | 27±32 | 0±0 | 12.4±5.1 | 1230±414 | 1.2±2.7 | 13.7±3.6 | 162±362 |
| 6 | 132 | 1 | 0 | 5.6 | 744 | 6.1 | 11.7 | 809 |
| 7 | 166 | 73 | 1 | 10.7 | 1777 | 0.0 | 10.7 | 0 |
| 8 | 48 | 47 | 1 | 191 | 908 | 0.0 | 191 | 0 |
| 9 | 128 | 4 | 0 | 11.2 | 1436 | 0.0 | 11.2 | 0 |
| 10 | 83 | 9 | 0 | 15.6 | 1288 | 0.0 | 15.6 | 0 |
| p | 0.53 | 0.050 | 0.08 | 0.13 | 0.049 | 0.52 | 0.16 | 0.77 |

Pre-heparin LPL and lipoproteins

To verify whether pre-heparin LPL was associated with apoB-containing lipoproteins, we quantified LPL in whole plasma, in apoB-depleted plasma and in a resuspended pellet which resulted from precipitated apoB-containing lipoproteins. In LPLS447X carriers and controls, 71±7% and 60±15% of the total serum LPL was found in the apoB-containing fraction. Compared to controls, the LPLS447X carriers presented an absolute higher amount of LPL (from 200 μL plasma) in the apoB containing fraction (carriers: 8.9±2.5 vs. controls: 2.8±0.6 ng; p<0.01) as well as in the non-apoB fraction (carriers: 3.8±1.6 vs. controls: 2.3±2.1 ng; p=0.25) (figure 2). In separate control experiments, we confirmed the presence of LPL in the apoB fraction using an alternative methods to separate non-apoB and apoB containing lipoproteins (MgCl₂ and phosphotungstic acid; data not shown).
Table 5 LDL ApoB100 (Sf 0-12) Metabolism in LPLS447X Carriers and Controls

<table>
<thead>
<tr>
<th></th>
<th>LDL apoB100</th>
<th>Synthesis</th>
<th>Production Rate</th>
<th>Total FCR</th>
<th>Direct Clearance</th>
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<tr>
<td></td>
<td>(mg)</td>
<td>(mg/day)</td>
<td>(mg/kg/day)</td>
<td>(pools/day)</td>
<td>(mg/day)</td>
</tr>
<tr>
<td>1</td>
<td>1341</td>
<td>98.0</td>
<td>1</td>
<td>0.6</td>
<td>910</td>
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<tr>
<td>2</td>
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<td>988</td>
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<td>1123</td>
<td>54.8</td>
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<td>888</td>
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<tr>
<td>Controls</td>
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<td>125.2 ± 778</td>
<td>2 ± 1</td>
<td>0.6 ± 0.2</td>
<td>918 ± 97</td>
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<table>
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<tr>
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<th>2138 ± 495</th>
<th>94.9 ± 143.4</th>
<th>1 ± 2</th>
<th>0.6 ± 0.1</th>
<th>1320 ± 323</th>
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<td>0.7</td>
<td>1319</td>
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| p     | 0.09       | 0.69         | 0.71          | 0.95      | 0.03           |

Table 6 Rate constants of LPLS447X Carriers and Controls

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<th>k_2</th>
<th>k_3</th>
<th>k_4</th>
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<table>
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Chapter 4

LPL in both apoB and apoB-depleted fractions from 200 µL of pre-heparin plasma. In both groups, LPL was mainly recovered in the apoB containing fraction (71±7% and 60±15% in carriers and controls, respectively). The absolute higher concentrations of LPL in the LPLS447X carriers as shown here relates to the initial 4-fold higher pre-heparin LPL concentrations in these individuals compared to controls.

Discussion

In the present study, we show for the first time that LPLS447X carriers exhibit enhanced TRL conversion and increased LDL apoB100 clearance. Also, carriers exhibited a 4-fold increase in pre-heparin LPL concentration, which was strongly associated with LDL apoB100 clearance. These data suggest enhanced hydrolytic activity as well as increased ligand capacity of LPL in carriers of the LPLS447X variant.

The delipidation cascade reflects TRL conversion due to LPL mediated TG hydrolysis. To date, analysis of TRL hydrolysis capacity has concentrated on measurement of post-heparin LPL activity. Carriers in the present study exhibited identical post-heparin LPL activity compared to controls, all of which were comparable to previously reported activities. Of note, post-heparin testing only reflects activity of the total LPL pool, of which only part is physiologically active. Hence, post-heparin values do not reflect the actual in vivo LPL-mediated lipolytic capacity. In the present study, pre-heparin LPL activity was below the detection limit. Following a previous report showing impaired VLDL handling in a LPL variant characterized by attenuated LPL...
activity, we now show enhanced conversion of TRL in LPLS447X carriers implying increased lipolytic capacity. Such an increase in LPLS447X carriers can be explained by increased lipolytic activity of the LPL dimer or by enhanced LPL-TRL binding mediating facilitated enzymatic conversion in carriers. The LPLS447X protein lacks two amino acids at the terminal carboxyl part, which preserves the binding capacity of the LPL protein to lipoproteins and also to heparan sulphate proteoglycans.

Besides increased TRL conversion, the carriers also exhibited enhanced LDL apoB100 clearance. This LPL-mediated LDL removal has been shown to be largely LDL receptor reliant, whereas only a minor portion occurs receptor-independent. Carriers were characterized by a 4-fold increase in pre-heparin LPL concentration. Since LPL has been previously been reported to be present on LDL, the increased pre-heparin LPL concentration in carriers might contribute to the enhanced clearance of LDL. The latter is underscored by the correlation between pre-heparin LPL concentration and LDL apoB100 clearance in the current study. Subsequently, we sought for evidence that LPL in serum is indeed associated with apoB-containing lipoproteins. To this purpose, we measured LPL concentration in apoB and non-apoB plasma fractions prepared from plasma of both LPLS447X carriers and controls. Our results show that a large portion of plasma LPL can be traced in the apoB fraction. These findings concur with those reported by Olivecrona who showed that pre-heparin LPL is predominantly present in the apoB fraction. In line with the four-fold increase of LPL concentration in plasma of LPLS447X carriers, a 3-4-fold increase in LPL content was also observed in the apoB fraction in LPLS447X carriers. Collectively, these data lend further support to a potential role for LPL in mediating increased LDL removal in LPLS447X carriers.

Several aspects of our study deserve closer attention. First, we have based our conclusions on data derived from a small group of homozygous carriers (n=5). Still, for kinetic studies a small sample size is not uncommon, even ranging from 2-5 subjects in total. Second, the increased VLDL₁ to VLDL₂ flux in carriers may have been affected by high values for direct catabolism resulting in low values for transfer in subject 2. This outlying value is a consequence of the concept that no parameter should be artificially fixed in the model. However, upon reanalysis of the data by changing \( k_1 \) and \( k_3 \) in subject 2 (\( k_1 = k_3 = 0 \)), we observed no changes to our initial conclusions. Third, the variation in direct catabolism (variation 0.0-79 pools/day) as well as the variation in LDL apoB100 synthesis (0-348 mg/day) in our study appears quite large. However,
such large variations are in line with other apoB100 kinetic studies and are therefore likely to reflect biological reality.\textsuperscript{26,27} Finally, whereas increased turnover of TRL is generally associated with increased HDL and reduced TG levels,\textsuperscript{8} lipid profiles in the present study were not significantly different between carriers and controls. This apparent discrepancy has several likely explanations, including baseline matching for lipid levels between carriers and controls as well as the limited study size.\textsuperscript{28-30}

Conclusions
In the present study we show that homozygous LPLS447X carriers exhibit enhanced TRL conversion as well as increased LDL removal. Combined with increased concentration of LPL in the pre-heparin plasma, our data suggest increased in vivo enzymatic consequences as well as increased non-enzymatic consequences of LPL in LPLS447X carriers. Both mechanisms might contribute to the cardiovascular protection that is associated with the LPLS447X variant.

Acknowledgements
We thank Mariëtte Ackermans (Laboratory of Endocrinology and Radiochemistry, Department of Clinical Chemistry, Academic Medical Center, Amsterdam, The Netherlands) for measuring the valine enrichment in the LDL fractions. Jose de Boer was thanked for measurement of the valine enrichment in VLDL\textsubscript{1} apoB100, VLDL\textsubscript{2} apoB100 and IDL apoB100. We also thank Frans Hoek (Department of Clinical Chemistry, Academic Medical Center, Amsterdam, The Netherlands) for extensive measurement of the apoB100 analyses. Florianne de Ruijter is thanked for her contributions to the design of the study as well as laboratory methods.

Reference List


CHAPTER

ENHANCED APOB48 METABOLISM IN LIPOPROTEIN LIPASE X447 HOMOZYGOTES

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Chapter 5
Abstract

**Rationale** Lipoprotein lipase (LPL) X447 homozygotes are characterized by enhanced conversion of TRL apoB100. Here, we set out to investigate whether this LPL variant is also associated with enhanced apoB48 clearance. Therefore, we evaluated apoB48 kinetics in X447 homozygotes in the fed state by infusion of isotope L-[1-13C]-valine and subsequent compartmental modeling.

**Methods and Results** ApoB48 metabolism was assessed in five X447 homozygotes (X/X genotype) and five S447 homozygotes (S/S genotype). Subjects were continuously fed and received infusion of stable isotope L-[1-13C]-valine. Results were analyzed by SAAM II modeling. Fasting (2.4-fold, p=0.02) as well as non-fasting (1.6-fold, p=0.09) apoB48 concentration was increased in the X447 homozygotes compared to S447 homozygotes. In addition, the X447 homozygotes exhibited a 1.7-fold higher apoB48 poolsize (p=0.04). Interestingly, apoB48 fractional catabolic rate was 1.9-fold higher (p=0.007) and apoB48 synthesis was more than 2-fold higher (p=0.006) in the X447 homozygotes compared to S447 homozygotes.

**Conclusion** In the present study, we show that X447 homozygotes exhibit enhanced apoB48 clearance. Previously, these homozygotes were shown to present with enhanced apoB100 TRL conversion. Combined, this LPLS447X gain of function variant affects apoB48 as well as apoB100 TRL metabolism.
Introduction

The negative consequences of increased levels of triglyceride (TG)-rich lipoproteins (TRL) on atherogenesis have generally been acknowledged. The enzyme lipoprotein lipase (LPL) drives, to a large extend, the processing of these TRL. LPL hydrolyses TG in chylomicrons (CM), as well as in VLDL. These lipoproteins have separate structural apolipoproteins: apoB48 and apoB100, respectively.

The X447 variant in the LPL gene is associated with decreased TG and increased HDL-C as well as a lower incidence of cardiovascular disease (CVD). Even though the precise mechanism for this apparent cardiovascular protection remains to be determined, X447 homozygotes were recently shown to exhibit enhanced apoB100 TRL conversion. This enhanced conversion was the first in vivo evidence for the gain of function associated with this particular LPL gene variant. In addition, X447 has also been reported to have a direct effect on postprandial apoB48 levels.

We hypothesized that the effect of X447 might also extend towards increased apoB48 clearance. To test this hypothesis, we evaluated kinetics of apoB48 in X447 homozygotes in the fed state by infusion of isotope L-[1-13C]-valine and subsequent compartmental modeling.

Materials and Methods

Participants

Five male X447 homozygotes (X/X genotype) were selected from the genetic database of the department of Vascular Medicine of the Academic Medical Center in Amsterdam, as reported previously. The control group, i.e. S447 homozygotes (S/S genotype), was described elsewhere and consists of five healthy male individuals that were selected from the same genetic database. The five controls were selected to match the X447 homozygotes with respect to age, body mass index (BMI), smoking habits, lipid levels, and use of alcohol. In addition, none of the subjects had signs of cardiovascular disease or used medication. The presence of natural variants in apolipoprotein E (apoE) gene, apoE2 or apoE4, have been shown to affect lipid metabolism. ApoE genotyping was performed in the participants and the subjects did not have E2/E2 or E4/E4 genotype. All participants gave written informed consent. They were asked to refrain from alcohol-containing beverages for at least three days before the start of the study. The study protocol was
approved by the Institutional Review Board of the AMC. The study conforms to the principles outlined in the Declaration of Helsinki.

**Genotyping**

We performed LPL gene analyses in more than 2000 DNA samples from healthy male subjects from the genetic database of the department of Vascular Medicine and found only five males to be X447 homozygotes and were willing to participate. The LPL gene analysis was as follows as described by Kuivenhoven et al. In short, we amplified the target sequence of the LPL gene (terminal part of exon 9) by using 5'-TACACTAGCAATGTCTAGGTGA-3' as upstream primer and 5'-TCAGCTTTAGCCCAGAATGC-3' as downstream primer. The amplification reactions were performed in 10 mmol/L Tris-HCl (pH 9.0), 50 mmol/L KCl, 0.1% wt/vol gelatin, 1.5 mmol/L MgCl₂, 1% Triton X-100, and 0.2 mg/mL bovine serum albumin containing 0.1 to 0.5 µg genomic DNA and final concentrations of 200 µmol/L dNTPs and 0.5 µmol/L primers in a total volume of 50 µL. After initial denaturation (10 minutes, 95°C), 1.0 U thermostable DNA polymerase (Supertaq; HT Biotechnology Ltd) was added, followed by 30 amplification cycles of 95°C (1 minute), 60°C (1 minute), and 72°C (1 minute) with a final extension step of 10 minutes at 72°C. Twenty percent of the PCR reaction product was used for digestion with 3 U Mnl I according to the instructions of the manufacturer (New England Biolabs) in a total volume of 20 µL for 2 hours at 37°C. After electrophoresis of the PCR product in 3% agarose containing ethidium bromide, DNA restriction fragments were visualized and analyzed on a transilluminator.

**Experimental protocol**

The protocol for infusion of labeled valine has been described in detail. After baseline blood sampling (t=0), a hospital-made food drink was ingested hourly. This food drink, equivalent to one twentieth of subjects daily food intake, was ingested every hour and consisted of 14% of calories as protein, 44% as carbohydrates, 42% fat (17% saturated, 17% monounsaturated, and 8% polyunsaturated) and 90 mg cholesterol per 1000 kcal as previously published. Five hours after baseline sampling, a priming dose of 17 µmol/kg L-[1-¹³C]-valine was given intravenously, followed by a continuous infusion of 15 µmol/kg/hour for 13 hours. Blood samples were obtained from the contralateral arm at baseline and after 5, 5 1/2, 6, 7, 8, 9, 10, 11, 12, 14, 16, 18 hours.

**Isotope and chemicals**

L-[1-¹³C]-valine (isotope mole fraction = 0.99; MassTrace, Woburn, MA, USA) was dissolved in
sterile 0.9% saline and sterilized through a 0.22 µm filter. Density solutions were made with KBr in 0.9% NaCl.

**Biochemical measurements**

Blood for lipid analysis was drawn in EDTA-coated tubes. Analyses of baseline lipids (TC, HDL-C, LDL-C and TG) and apoB100 are described in detail in a previous report. Plasma apoAI concentration was determined by immunonephelometry (Dade Behring). Plasma apoB48 concentration was measured by a sandwich ELISA using anti-human apoB48 monoclonal antibodies as reported previously.

**Isolation of free amino acids, apoB48 from lipoproteins**

The isolation of free amino acids from plasma was performed as described in detail elsewhere. Isolation of apoB containing lipoproteins was performed as described below. In short, CM, VLDL1, VLDL2, IDL and LDL were isolated using a discontinuous salt gradient by cumulative ultracentrifugation (UC; Beckman Ultracentrifuge Sw41 Ti rotor; Beckman Instruments). After 3 subsequent UC spins, chylomicron (CM) fraction (32 min, 40700 rpm, 4°C, acceleration 5, brake 5), VLDL1 fraction (3:28 h, 40700 rpm, 4°C, acceleration 5, brake 5) and VLDL2, IDL, LDL and bottom fraction (HDL + lipoprotein-deficient plasma) were collected after 17 hours. After sample collection of the first and the second UC spin, the UC tubes were refilled using 1 mL d=1.006 g/mL KBr. All fractions were aliquoted and stored at -20°C. ApoB from all isolated lipoprotein fractions was precipitated and delipidated followed by separation of apoB48 and apoB100 protein by preparative SDS-PAGE. From each lipoprotein fraction, apoB48 bands were excised from the gels, followed by hydrolysis in 0.5 mL 6M HCl at 110°C for 24 hours, cation-exchange chromatography (AG-50W-X8, Bio-Rad Laboratories, Hercules, CA, USA) and finally the samples were dried under N2. ApoB48 in the different lipoprotein fractions was pooled per time-point and analysed.

**Determination of 13C-valine enrichment**

Determination of 13C-valine enrichment was performed as previously described. Derivatives of plasma-free amino acids and of pooled apoB48 were analyzed by gas chromatography coupled to mass spectrometry (GC-MS).
**ApoB48 kinetics**

ApoB48 fractional synthesis rate (FSR) was measured as the rate of incorporation of $^{13}$C-enriched valine into circulating apoB48 and best data fit in a three compartment model was determined using the SAAM II software (Simulation Analysis and Modeling, version 1.1.1., SAAM Institute, Seattle, WA, USA). The used model is shown in figure 1. Plasma volumes were calculated from body surface area. The apoB48 plasma pool was calculated by multiplying the plasma volume by the plasma apoB48 concentration. It was assumed that during the study each subject remains in steady state with respect to apoB48 metabolism, during which fractional catabolic rate (FCR) equals FSR. The FCR was defined as the disappearance of the apoB48 protein from the plasma. The absolute synthesis rate (ASR) is the amount of apoB48 protein synthesized per day, is expressed as mg protein/day and is calculated as follows: ASR apoB48 = FSR apoB48 * apoB48 poolsize. Production rate is calculated by dividing ASR by body weight.

**Figure 1: Compartmental model for apoB48 metabolism.**

![Compartmental model for apoB48 metabolism](image)

*Compartment 1 represents plasma valine into which the valine tracer was injected. Compartment 2 represents a delay compartment and valine is incorporated in apoB48 protein via compartment 3. The k value represents the rate constant. The equation used for calculation of FCR/day = k1 * 24 * 60; 24 * 60: conversion of pools/min to pools/day.*

**Statistical Analysis**

All data are presented as mean values ± SD. All data were analyzed with SPSS (version 12.0.1, SPSS Inc., Chicago, Illinois, USA) using an independent samples t-Test. A paired-samples t-Test was used to compare the means of apoB100 FCR and apoB48 FCR in both groups. Correlations were performed by linear regression as previously shown. A p-value < 0.05 was considered to be statistically significant.
Results

Baseline characteristics
Baseline characteristics of the X447 homozygotes and S447 homozygotes have been described in detail previously. Briefly, age, BMI, waist/hip-ratio, blood pressure, lipids and fasting apoB100 were not significantly different. Whereas fasting apoA1 was not significantly different between both groups (X447: 1472 ± 178 mg/L vs. S447: 1352 ± 212 mg/L; p=0.36), the X447 homozygotes presented a significantly higher fasting apoB48 concentration (X447: 3.92 ± 1.38 vs. S447: 1.62 ± 0.22 mg/L; p=0.02). Calculated plasma volumes were similar for both groups. Plasma apoB48 was measured at all time points. Mean non-fasting plasma apoB48 concentration tended to be higher in the X447 homozygotes (9.29 ± 1.89 vs. 5.98 ± 3.26 mg/L; p=0.09).

| Table 1 ApoB48 Metabolism in X447 and S447 Homozygotes |
|-----------------|-----------------|-----------------|-----------------|
| ApoB48          | Synthesis       | Production Rate | Total FCR       |
| (mg)            | (mg/day)        | (mg/kg/day)     | (pools/day)     |
| 1.4             | 19              | 0.3             | 1.4             |
| 1.2             | 15              | 0.2             | 1.3             |
| 2.5             | 59              | 0.6             | 2.3             |
| 4.4             | 65              | 0.9             | 2.7             |
| 7.7             | 8               | 0.1             | 1.0             |
| S447            | 16 ± 8          | 33 ± 27         | 0.4 ± 0.3       | 1.7 ± 0.7 |
| X447            | 27 ± 7          | 87 ± 19         | 11 ± 0.2        | 3.2 ± 0.6 |
| 6.2             | 25              | 103             | 1.4             | 4.1 |
| 7.3             | 33              | 83              | 0.8             | 2.5 |
| 8.4             | 27              | 88              | 1.1             | 3.3 |
| 9.5             | 34              | 103             | 1.3             | 3.0 |
| 10.6            | 18              | 57              | 0.9             | 3.2 |
| p*              | 0.04            | 0.006           | 0.008           | 0.007 |

ApoB48 (mg): apoB48 poolsize in mg; Synthesis (mg/day): synthesis of apoB48 in mg per day; Production Rate (mg/kg/day): production rate of apoB48 in mg per kg bodyweight per day; Total FCR (pools/day): Total fractional catabolic rate of apoB48 in pools per day
Valine enrichment and SAAM II modeling
Mean rate of incorporation of $^{13}$C-enriched valine in plasma was not significantly different between both groups as illustrated in figure 2. After 5 hours of tracer infusion a plateau for apoB48 $^{13}$C-valine is reached in all subjects. Since apoB48 kinetics was analyzed using a linear model, we only used the first 4 hours in this kinetic model to study the effect of LPLS447X on apoB48 kinetics. In addition, Welty et al. determined apoB48 kinetics in 19 healthy subjects and they also observed a plateau for apoB48 $^{13}$C-leucine enrichment after a period of 5 to 6 hours after start of the infusion, suggesting that subjects indeed reach a $^{13}$C-enrichment apoB48 plateau after such a period of 5 to 6 hours of tracer infusion.

*Figure 2: Plasma and apoB48 valine tracer/tracee ratios.*

ApoB48 metabolism
ApoB48 metabolism in the X447 and S447 homozygotes is shown in table 1. We used individual apoB48 poolsizes for the time points (0 - 240 minutes) to calculate a mean apoB48 pool size per subject for determination of apoB48 kinetics (table 2). The X447 homozygotes presented a 1.7-fold higher apoB48 poolsize ($p=0.04$) and an increased apoB48 synthesis compared to S447 homozygotes (either expressed as mg/day: 2.6-fold; $p=0.006$ or mg/kg/day: 2.8-fold; $p=0.008$). In addition, the X447 homozygotes exhibited a 1.9-fold increase in FCR as compared to S447 homozygotes ($p=0.007$).
Correlations

ApoB48 FCR did not correlate with apoB100 FCRs of VLDL_1, VLDL_2, IDL or LDL in either group (data are adapted from 4).

Discussion

In the present study, apoB48 metabolism was examined in five male X447 homozygotes and five matched S447 homozygotes by infusion of isotope L-[1-13C]-valine and subsequent compartmental modeling. ApoB48 FCR was increased in the X447 homozygotes, that were previously shown to present enhanced TRL apoB100 conversion. In addition, no correlations between apoB48 FCR and TRL apoB100 FCR became obvious. Overall, both increased apoB48 FCR as well as the enhanced apoB100 conversion are suggestive for gain of LPL function of the 447X variant of the LPL gene, which could possibly explain the beneficial lipid profile as well as the observed cardiovascular protection that are both associated with this frequent LPL variant.

In the present study, we show enhanced apoB48 clearance in the X447 homozygotes. Recently, also heterozygous X447 carriers were shown to present increased postprandial apoB48 TRL

| Table 2 ApoB48 Poolsize (mg) in X447 and S447 Homozygotes (0 - 240 minutes) |
|-----------------|-----|-----|-----|------|-----|-----|
|                 | S447| X447| 0   | 30   | 60  | 120 | 180 | 240 |
| 1               | 15.5| 30.4| 16.2| 13.0 | 13.8 | 13.5| 12.6|
| 2               | 11.0| 38.1| 12.5| 13.9 | 13.9 | 12.7| 10.4|
| 3               | 30.8| 26.8| 28.7| 31.4 | 19.5 | 15.7| 23.5|
| 4               | 23.6| 33.4| 25.5| 24.0 | 24.8 | 22.6| 23.1|
| 5               | 7.3 | 22.3| 6.7 | 6.7  | 8.2  | 76  | 70  |

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In the present study, we show enhanced apoB48 clearance in the X447 homozygotes. Recently, also heterozygous X447 carriers were shown to present increased postprandial apoB48 TRL
clearance compared to non-carriers. Accordingly, others also found that male heterozygous X447 carriers have lower postprandial apoB48 TRL compared to male non-carriers. In contrast, Almeida et al. could not demonstrate an effect of X447 on postprandial fat loading. They found similar CM FCR estimated by radio-active labeling of oleate-esters in heterozygous LPLS447X carriers and controls. Since these subjects still have a LPL wild type allele, it might be possible that no differences in outcome are found due to the presence of this allele (normal LPL protein and function). Therefore, we only studied homozygous X447 subjects. In addition, a potential gender bias may be involved. Whereas in some studies X447 associated lipid changes were modest in females compared to males, other studies were unable to show any consequences of this variant on lipids in females at all. In this respect, Almeida et al. included both male and female carriers, which may have minimized a potential difference in CM FCR between carriers and controls.

13C-valine enrichment in apoB48 protein was measured per time-point in pooled apoB48 containing lipoprotein fractions. Therefore, we are unable to demonstrate whether enhanced apoB48 FCR in the X447 homozygotes is the result of enhanced apoB48 conversion through enhanced lipolysis or enhanced receptor mediated uptake. In addition, a potential role for altered clearance due to differences in apoB48 particle size can also not be excluded. However, Zheng et al. recently showed that LPL-bound lipoproteins were cleared much faster than same sized lipoproteins lacking LPL. They concluded that the effect of enhanced clearance was therefore not particle size driven, suggesting that the last possibility for enhanced clearance in X447 homozygotes is not very likely. In addition, the X447 homozygotes were previously shown to present an increased TRL apoB100 conversion and an increased LPL-mediated LDL apoB100 clearance. Both observations argue for a role of enhanced LPL function. Next, the X447 homozygotes exhibited a higher amount of LPL bound to apoB particles. Collectively, these observations suggest that increased apoB48 FCR in the X447 homozygotes is most likely the result of enhanced LPL function rather than to differences in particle size.

ApoB48 and apoB100 metabolism are known to share the same LPL mediated pathway. Interestingly, we did not observe a correlation between apoB48 FCR and TRL apoB100 FCR. This observation supports a previous report showing that the metabolic routing of apoB100 and apoB48 TRL are in fact independently regulated. Specifically, the authors reported an inverse correlation between apoB48 FCR and apoA1 FCR (r² = 0.40, p < 0.05). This relation has been
attributed to an increased postprandial chylomicron clearance, which results in decreased flux of TG from TRL to HDL via Cholesteryl Ester Transfer Protein (CETP). Decreased CETP activity leads to TG-poor HDL particles, known to be associated with a reduced FCR of apoAI.24

Some aspects of this study require closer attention. First, the reason for the increased apoB48 concentration and production rate in X447 homozygotes is unknown. ApoB48 consists of the N-terminal 2152 amino acids of apoB100 and is necessary for synthesis, assembly and secretion of TG-enriched CM. In the first step, apoB48 undergoes a MTP dependent co-translational lipidation process to form a lipoprotein of HDL-size and HDL-density (apoB48 HDL). In a second step, these particles are converted to large TG-rich apoB100 containing VLDL/CM-particles.25,26 This conversion is highly dependent on TG-biosynthesis. Since the TG-pool and HDL-TG in LPLS447X is low, an increased apoB48 production rate is therefore not expected. However, it has also been suggested that apoB48 can be recruited for posttranslational lipoprotein assembly from the ER membrane.26 In addition, since plasma apoB48 concentrations have been shown to vary greatly in the general population (1 - 24 mg/L) as shown previously,27 mean apoB48 levels in small number of subjects is very difficult or perhaps even impossible to interpret and as a direct consequence, apoB48 PRs are also very difficult or perhaps even impossible to interpret. Thus, differences in apoB48 PR in our current study can possibly be due to low number of subjects. In addition, the increased apoB48 concentrations at baseline may result from an increased synthesis and/or decreased catabolism. Therefore, additional studies with larger number of X447 homozygotes are warranted to clarify whether apoB48 levels and consequently apoB48 production are truly affected in X447 homozygotes. The reason for the increased apoB48 levels in X447 homozygotes is unknown.

Second, the apoB48 FCR values in our present study are lower compared to previous apoB48 kinetic studies (FCR: 4.2 ± 2.2 - 5.0 ± 2.2) even though similar compartmental models were used.13,14,24,28 In these studies however, VLDL enrichment at plateau was used to fit the data, whereas in this study, VLDL1 apoB100 and VLDL2 apoB100 were both isolated instead of total VLDL apoB100.4 We used plasma valine enrichment at plateau as a surrogate for the amino-acyl-tRNA pool in the liver to fit apoB48 kinetic data from the X447 and S447 homozygotes. The latter has been reported to contribute to underestimation of the true values for apoB48 kinetics, which can explain the discrepancies of the lower apoB48 kinetic values. However, after reanalysis of apoB48 kinetics using total VLDL apoB100 (by adding VLDL1 apoB100 and VLDL2 apoB100,
data adapted from 4) enrichment at plateau, the FCR in the X447 homozygotes remained approximately 2-fold increased when compared to S447 homozygotes. Finally, apoB48 is present on a wide spectrum of particles ranging from very large chylomicron-size particles down to LDL-size particles. Unfortunately, we are unable to provide the data on individual types of apoB48 lipoproteins, since we analysed apoB48 using a three compartmental model. The conversion of larger apoB48 towards smaller apoB48 is a very interesting concept, since such multicompartmental analysis would likely reflect natural biology better. Within this respect, several practical issues limit use of a multicompartmental study on apoB48 metabolism as described for apoB100 in 4. In the first place, in some lipoprotein fractions, apoB48 concentrations were below the detection limit. In the second place, 13C-valine enrichment analyses was too low for reliable quantification and in the third place, large amount of blood would be needed to obtain enough apoB48 protein for reliable analysis of 13C-valine enrichment.

Conclusion

We showed an increased apoB48 FCR in the X447 homozygotes that were previously shown to present enhanced TRL apoB100 conversion. 4 Taken together, increased apoB48 FCR as well as increased apoB100 TRL conversion are suggestive for a gain of function of the X447 protein that might be responsible for the beneficial effects on the lipid profile associated with frequent found LPL gene variant.

Acknowledgements

We thank Jose de Boer for measurement of 13C-valine enrichment in free amino acids from plasma and in the isolated apoB48 containing lipoprotein fractions. We also thank Birgitte Karstenkov for laboratory support. Florianne de Ruijter is acknowledged for her contributions to the design of the study as well as laboratory methods. We are grateful to the X447 homozygotes and to the S447 homozygotes for participating in the kinetic studies.
Reference List


Enhanced ApoB48 Metabolism in Lipoprotein Lipase X447 Homozygotes
LPLS447X RELATES TO VARIOUS BENEFICIAL PHENOTYPES
LIPOPROTEIN LIPASE GENE POLYMORPHISMS AND THE RISK OF TARGET VESSEL REVASCULARIZATION AFTER PERCUTANEOUS CORONARY INTERVENTION


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Abstract

Objectives: To identify polymorphisms in genes that predispose to restenosis.

Background: Variations in the lipoprotein lipase (LPL)-gene have been implicated in a number of pathophysiological conditions associated with coronary heart disease. The present study examines the impact of polymorphisms in the LPL-gene on restenosis (defined by target vessel revascularization, TVR) in a large patient-population undergoing percutaneous coronary intervention (PCI). A mouse model for restenosis was used to further investigate LPL’s role in restenosis.

Methods: The GENetic DEterminants of Restenosis (GENDER) project is a multicenter prospective study design that enrolled 3,104 consecutive patients after successful PCI. These patients were genotyped for four different LPL-gene polymorphisms. In ApoE*3-Leiden transgenic mice, arterial mRNA was used to assess LPL-expression during a cuff-induced restenotic process.

Results: Using multivariable analysis, carriers of the 447Ter allele of the LPL-enzyme showed a lower risk of TVR compared to 447Ser homozygotes (p=0.005). In the mouse model, LPL-mRNA levels were increased 40-fold compared to control arteries at 6 hours post cuff-placement.

Conclusions: The LPL C/G polymorphism (Ser447Ter) resulting, in a truncation of the two C-terminal amino acids of the mature LPL-protein, appears to be an important protective factor for TVR in man. LPL’s role in this process was further established in a mouse model, where LPL-expression was very strongly upregulated in the target arterial wall, suggesting a contribution of this lipolytic enzyme to restenosis. Possibly, LPL Ser447Ter genotyping may lead to better risk stratification and tailored therapy in the prevention of restenosis after PCI.
Introduction

Lipoprotein lipase (LPL) is the rate-limiting enzyme in the lipolysis of plasma triglyceride-rich lipoproteins in the circulation. In adulthood, it is synthesized in parenchymal cells of adipose tissue as well as in skeletal and cardiac muscle, followed by transfer to heparin sulphate binding sites at the vascular side of the endothelium. The hydrolytic function of LPL is essential for the processing of triglyceride-rich chylomicrons and very-low density lipoproteins (VLDL) to remnant particles and also for the transfer of phospholipids and apolipoproteins to HDL. Furthermore, LPL plays a key role in the receptor-mediated removal of lipoproteins from the circulation. The gene coding for LPL, located on chromosome 8p22, encompasses 10 exons and is rather polymorphic. Abnormal LPL-function has been reported to be associated with a number of pathophysiological conditions which underlie coronary heart disease. In line, changes in LPL-gene expression, or amino acid substitutions as a result of point mutation in the LPL-gene affect triglyceride and HDL cholesterol levels, which in turn are implicated in atherosclerotic risk.

Percutaneous coronary intervention (PCI), an important treatment for patients with atherosclerosis, is limited by the development restenosis, despite the advent of drug-eluting stents. There is increasing evidence that inherited factors may explain in part the excessive risk of restenosis in certain patients. Identifying such patients may improve stratification of patients to a more individually tailored treatment. To our knowledge, the role of LPL-polymorphisms in restenosis, has thus far not been investigated. Therefore, the purpose of the current study was to evaluate in a large consecutive study population whether four different well-known variants in the LPL-gene, denoted as -93T/G, Asp9Asn, Asn291Ser and the Ser447Ter, have predictive value towards the risk of restenosis (defined by target vessel revascularization, TVR) after PCI. To further establish the role of LPL in restenosis, we quantified LPL-mRNA expression in the restenotic vessel in an established mouse model for restenosis.

Methods

Study design

The present study population has been described previously. In brief, the GENetic DEterminants of Restenosis project (GENDERN) was designed to study the association between
genetic polymorphisms and clinical restenosis. Patients were eligible for inclusion if they were successfully treated for stable angina, non-ST-elevation acute coronary syndromes or silent ischemia by PCI. Patients treated for acute ST elevation myocardial infarction were excluded. All patients were treated in four of the 13-referral centers for interventional cardiology in the Netherlands. The overall inclusion period lasted from March 1999 until June 2001. The study protocol conforms to the Declaration of Helsinki and was approved by the Medical Ethics Committees of each participating institution. Written informed consent was obtained from each participant before the PCI procedure.

**List of Abbreviations**

- **BMI**: Body mass index
- **CABG**: Coronary artery bypass grafting
- **CI**: Confidence interval
- **LAD**: Left anterior descending coronary artery
- **LPL**: Lipoprotein lipase
- **MI**: Myocardial infarction
- **PCI**: Percutaneous coronary intervention
- **RCX**: Circumflex branch of the left coronary
- **RR**: Relative risk
- **TVR**: Target vessel revascularization

**PCI procedure**

Standard angioplasty and stent placement were performed by experienced operators using a radial or femoral approach. Before the procedure, patients received aspirin 300 mg and heparin 7500 IU. The use of intracoronary stents and additional medication, such as glycoprotein IIb/IIIa inhibitors, was at the discretion of the operator. In case of stent implantation, patients received either ticlopidin or clopidogrel for at least one month following the procedure depending on local practice. During the study, no drug-eluting stents were used.

**Follow-up and study endpoints**

Follow-up lasted at least nine months, except when a coronary event occurred. Patients were either seen in the outpatient clinic or contacted by telephone. TVR, either by PCI or coronary
artery bypass grafting (CABG), was considered as restenosis and was our primary endpoint. An independent clinical events committee adjudicated the clinical events. Events occurring within the first month were excluded from the analysis, since these events were attributable mainly to sub-acute stent thrombosis or occluding dissections, and less likely to restenosis. Data were collected with standardized case-report forms that were completed by the research coordinator at each site, who was blinded to the genotype of the patients. Representatives from the data-coordinating center monitored all sites.

Genotyping
Blood was collected in EDTA tubes at baseline and DNA was extracted following standard procedures. The LPL G/A, A/G and the C/G polymorphisms in exon 2, 6 and 9, respectively, resulting in the following amino acid substitutions; Asp9Asn, Asn291Ser and the Ser447Ter respectively, were determined by validated multilocus genotyping assay (Roche Molecular Systems). A similar method was used to detect the LPL -93T/G promoter polymorphism. All four polymorphisms were selected on the basis of their previously described relation to CAD and/or their influence on LPL-activity. In short, each DNA sample was amplified in a multiple polymerase chain reaction (PCR) using biotinylated primers. The PCR product pool was then hybridized to a matching panel of sequence-specific oligonucleotide probes, immobilized in a linear array on nylon membrane strips. A colorimetric detection method based on incubation with streptavidin-horseradish peroxidase conjugate, using hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine as substrates, was used. Operators blinded to restenosis status performed genotyping. To confirm genotype assignments, the PCR-procedure was performed in replicate on 10% of the samples. Two independent observers carried out scoring. Disagreements (<1%) were resolved by further joint reading, and when necessary, genotyping was repeated.

Lipid analysis
To study the effect of the different LPL-variants on lipid levels, we measured plasma triglycerides, total cholesterol and HDL-cholesterol in a subpopulation of patients. Cholesterol and triglyceride concentrations in serum were measured with a fully automated Hitachi 747 (Hitachi, Tokyo, Japan). HDL-C was determined with a turbidimetric assay on a Hitachi 911. LDL-C was calculated according to the equation of Friedewald et al. Blood was drawn before the PCI-procedure. Two of the four participating centers (Leiden University Medical Center and Academic Hospital Maastricht) collected systematically extra blood samples to perform additional laboratory measurements to examine other predictors of restenosis.
**Mouse model of restenosis**

We further studied LPL-gene expression during the development of restenosis in an established mouse model for diet-induced atherosclerosis. Specifically, we analyzed LPL-mRNA levels in the vessel wall of ApoE*3-Leiden transgenic mice after cuff-placement. Prior cuff-placement, the mice were fed a western-type diet containing 1% cholesterol and 0.05% cholate (Hope Farms, Woerden, the Netherlands) 3 weeks prior to surgery and continued after surgery in order to obtain stable plasma cholesterol levels. This diet results in a human-like lipoprotein profile. Femoral arteries, either cuffed or non-cuffed sham operated, were pooled (two arteries per sample, two samples per time point) and total RNA was isolated per time point using the Trizol protocol (Invitrogen). Subsequently, cDNA synthesis of all RNA samples was achieved using Ready-To-Go RT-PCR beads (Amersham Biosciences, Uppsala, Sweden). All experimental procedures in mice were approved by the Animal Welfare Committee of TNO-PG, Leiden, the Netherlands.

Intron-spanning primers (forward: 5’GTGGCCGAGAGCGAGAAC 3’, reverse: 5’TCCACCTCCGT-GTAATCAAGA 3’) and probe (5’TTCCTTTCCACTGGCCGAGGT 3’) for mouse LPL-gene were designed using Primer Express™ 1.5 software (Perkin-Elmer Applied Biosystems, Foster City, California, USA). The housekeeping genes, HPRT, Cyclophilin and GAPDH were used as controls. Real time (RT)-PCR was performed on an ABI Prism™ 7700-sequence detection system (Perkin Elmer Biosystems, Boston, Massachusetts, USA). Cycle conditions were: 50°C for 2 min, followed by 10 min on 95°C, amplification phase of 45 cycles of 15 s at 95°C, followed by 1 min at 60°C. RT-PCR analysis was performed using RT-PCR mastermix (Eurogentec, Seraing, Belgium). Aqua-dest was incorporated as a negative control.

**Statistical methodology**

Deviations of the genotype distribution from that expected for a population in Hardy-Weinberg equilibrium (HWE) was tested using the Chi-squared test with one degree of freedom. Allele frequencies were determined by counting; the 95% confidence intervals of the allele frequencies were calculated from sample allele frequencies, based on the approximation of the binomial and normal distributions in large sample sizes. Polymorphisms not in HWE were excluded from further analysis. In the first stage, the association between each LPL-polymorphism and TVR was assessed using a Cox proportional regression model under a co-dominant genetic model. No adjustment for covariates was performed at this stage to allow for the assessment of their possible
involvement in the causal pathway. If less than 10 patients were homozygous for a particular allele, two groups were formed with the absence or presence of that allele as group variable. All polymorphisms were also assessed using dominant and recessive models and the model with the lowest Akaike information criterion was used in multivariable regression analysis. The LPL-polymorphisms were combined into haplotypes and the effect of haplotypes on restenosis risk was estimated according to the methods developed by Tanck et al. Multivariable regression analysis of the TVR risk was performed on all polymorphisms using a stepwise backward selection algorithm. In the final step clinical variables associated with TVR or associated with genotype were entered into the regression model. The Kruskal-Wallis test was used to examine the association of the different genotypes with concentrations of HDL-cholesterol, LDL-cholesterol and triglyceride levels. Animal data are presented as mean ± SEM. Data were analysed using the Mann-Whitney U-test. P-values < 0.05 were considered statistically significant. Statistical analysis was carried out using SPSS 11.5.

Results

Patient characteristics
A total of 3,146 patients had complete follow-up (99.3%) with a median duration of 9.6 months (interquartile range 3.9). A total number of 42 patients experienced an event in the first 30 days and were therefore excluded from further analysis, according to the protocol. In the remaining 3,104 patients, we assessed the frequencies of the following LPL-polymorphisms: -93T/G, Asp9Asn (G/A), Asn291Ser (A/G) and the Ser447Ter (C/G) polymorphism. Successful DNA-genotyping was possible in 3028, 3031, 3021 and 3054 patients, respectively. Results of the remaining patients were missing due to lack of DNA or inconclusive genotyping. The frequencies of the rare -93G, 9Asn, 291Ser and the 447Ter alleles were 0.02, 0.02, 0.03 and 0.10, respectively. The genotype distributions were consistent with HWE (p>0.05), except for the -93T/G polymorphism. Therefore, this promoter-polymorphism was excluded from further analysis. Comparisons of baseline characteristics among the genotypes are shown in table 1a. The low frequency of carriers of 9Asn and 291Ser has prompted us to pool heterozygotes and homozygotes for these two LPL variants. No statistically significant differences were observed between the groups, with the following two exceptions: heterozygotes and homozygotes for the allele encoding for a 291Ser-genotype had a higher rate of previous CABG (p<0.05).
Table 1a. Baseline Clinical Characteristics of the Patients according to the Genotypes of LPL Polymorphisms

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<td>54.4 53.5</td>
</tr>
<tr>
<td>β-blocker medication</td>
<td>79.0 65.3*</td>
<td>78.7 75.5</td>
</tr>
</tbody>
</table>

*p<0.05 in comparison with the other genotypes; p=not significant for all comparisons. Age is mean ± SD; other variables are percentage of patients. LPL, lipoprotein lipase; Asp, aspartic acid; Asn, asparagine; Ser, serine; Ter, stop; MI, myocardial infarction; CABG, coronary artery bypass grafting
Furthermore, heterozygotes and homozygotes for the allele encoding for the 9Asn LPL-variant used less lipid lowering medication, but had a higher use of β-blocker medication. Lesion-related and procedural parameters of the genotype groups are presented in table 1b. The only significant difference was found for carriers of the allele encoding for 9Asn, who were treated more often for total occlusion compared to homozygotes for the common allele (p<0.05). Interestingly, homozygotes for the 447Ter-genotype presented with a high statistically significant reduction in multivessel-disease compared to the other genotypes (p=0.002). In fact, we observed a gene dosage effect underlining a relation between these two parameters.

At follow-up 304 patients (9.8%) had to undergo TVR. We did observe a significant association between the 447Ter-genotype and the rates of TVR after univariate analysis (RR=0.6, 95%CI: 0.44-0.83, p=0.004). In contrast, the LPL Asp9Asn, Asn291Ser variants did not show a significant association with TVR (p>0.1) (Table 2).

<table>
<thead>
<tr>
<th>Table 1b. Lesion and Procedural Characteristics at the Time of Intervention According to the Genotypes of the LPL Polymorphisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPL G/A (Asp9Asn) LPL A/G (Asn291Ser) LPL C/G (Ser447Ter)</td>
</tr>
<tr>
<td>N</td>
</tr>
<tr>
<td>-----------------------</td>
</tr>
<tr>
<td>Restenotic lesion</td>
</tr>
<tr>
<td>Total occlusion</td>
</tr>
<tr>
<td>Type C lesion</td>
</tr>
<tr>
<td>LAD proximal</td>
</tr>
<tr>
<td>RCX</td>
</tr>
<tr>
<td>Multivessel disease</td>
</tr>
<tr>
<td>Stable angina</td>
</tr>
<tr>
<td>Residual stenosis &gt;20%</td>
</tr>
<tr>
<td>Stenting</td>
</tr>
<tr>
<td>Glycoprotein IIB/IIA</td>
</tr>
<tr>
<td>antagonist</td>
</tr>
</tbody>
</table>

*p<0.05 in comparison with the other genotypes of the same polymorphism; p=not significant for all other comparisons. LPL, lipoprotein lipase; Asp, aspartic acid; Asn, asparagine; Ser, serine; Ter, stop; LAD, left anterior descending coronary artery; RCX, circumflex branch of the left coronary artery; Type C lesion, according to the American College of Cardiology and American Heart Association.
The LPL 447Ter genotype remained associated with a decreased risk of TVR (RR=0.6, 95%CI: 0.44-0.86) upon multivariable analysis, including all three polymorphisms. Finally, in the regression model, we included patient and intervention-related characteristics that were previously described to be related to TVR risk or with genotype (such as age, gender, diabetes, stenting, residual stenosis>20%, current smoking, total occlusion, lipid lowering medication, β-blocker use, multi-vessel-disease and previous CABG). This backward stepwise selection yielded similar results (RR=0.6, 95%CI: 0.44-0.86) (Table 3).

Furthermore, as the severity of the stenosis before angioplasty as well as (especially) the severity of the stenosis immediately post-angioplasty are key determinants of the risk of restenosis, we have examined the effect of pre-and post-procedural lesion diameter for the different genotypes in a subpopulation of 478 patients with additional angiographic data. However, pre-and post percentage stenosis values did not differ significantly between the three genotypes of the 447-polymorphism, (p=0.75 and p=0.83, respectively).

The polymorphisms were also combined into haplotypes for further analysis. Seven out of eight possible haplotypes were indeed observed (data not shown). The 9G/291A/447C haplotype was

<table>
<thead>
<tr>
<th>Polymorphisms</th>
<th>Number of patients</th>
<th>TVR (%)</th>
<th>Model used</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp9Asn</td>
<td>3031</td>
<td>9.7</td>
<td>Dominant</td>
<td>0.77</td>
</tr>
<tr>
<td>GG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GA/AA</td>
<td></td>
<td>10.4</td>
<td></td>
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<tr>
<td>Asn291Ser</td>
<td>3021</td>
<td>9.9</td>
<td>Dominant</td>
<td>0.62</td>
</tr>
<tr>
<td>AA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AG/GG</td>
<td></td>
<td>8.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ser447Ter</td>
<td>3054</td>
<td>10.5</td>
<td>Dominant</td>
<td>0.004</td>
</tr>
<tr>
<td>CC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CG</td>
<td></td>
<td>70</td>
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</tr>
<tr>
<td>GG</td>
<td></td>
<td>0</td>
<td></td>
<td></td>
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</tbody>
</table>

Table 2. Univariate Analysis of LPL Polymorphisms in Association with TVR and the Distributions of the Polymorphisms

TVR, target vessel revascularization; LPL, lipoprotein lipase, Asp, aspartic acid; Asn, asparagine; Ser, serine; Ter, stop
most common in both TVR-cases and controls (89.7% and 85.1%, respectively). Smallest relative risk was seen with respect to the 9G/291A/447G-haplotype (RR=0.62, 95% CI; 0.45-0.85). When evaluating bilocus-haplotypes it became evident that this effect was caused only by the LPL 447-variant.

Lipid profiles were investigated in a subgroup of patients (N=942, data not shown). We were not able to find a significant correlation between carriers and non-carriers of the polymorphisms investigated with regard to HDL-cholesterol, LDL-cholesterol, and triglycerides (p>0.20). Correction for lipid levels in this subgroup-analysis was of no influence on the association of the different polymorphisms and TVR.

Subsequently, we evaluated if LPL-gene expression played a role in the development of restenosis by analysing arterial mRNA in an established model of restenosis in hypercholesterolemic mice. At the time of surgery, total plasma cholesterol level was 13.9 ± 3.6 mM. The mRNA encoding LPL, isolated from the cuffed right femoral artery and untreated left femoral artery, was quantified at various time points after cuff-placement. The mRNA encoding LPL showed peak expression 6 hours after cuff-placement, where it showed a 40-fold increase compared to the normal artery. LPL mRNA levels were back to baseline after 24 hours of the induction of the restenotic process. Sham operated vessels (femoral artery prepared free, but without cuff placement) showed essentially the same results vs. cuff placement as the non-operated vessels.

<table>
<thead>
<tr>
<th>Table 3. Multivariable Cox Regression of Clinical Variables and LPL Ser447Ter Polymorphism Associated with TVR</th>
</tr>
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<tbody>
<tr>
<td></td>
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<tr>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
</tr>
<tr>
<td>Current smoker</td>
</tr>
<tr>
<td>Stenting</td>
</tr>
<tr>
<td>Total occlusion</td>
</tr>
<tr>
<td>Residual stenosis-20%</td>
</tr>
<tr>
<td>LPL 447Ter</td>
</tr>
</tbody>
</table>

TVR, target vessel revascularization; LPL, lipoprotein lipase; Ser, serine; Ter, stop;
Discussion

In a large prospective multicenter follow-up study of consecutive patients we demonstrated that the LPL Ser447Ter variant, present in approximately 20% of the general population, is associated with a decreased risk of TVR after PCI. This polymorphism has been shown in most but not all studies to modulate lipid levels as well as LPL. In fact, it was shown that this polymorphism is associated with decreased triglycerides (TG), increased HDL-cholesterol, and a decreased risk of coronary artery disease. Furthermore, a recent meta-analysis confirmed that the Ser447Ter variant has an effect on the lipoprotein profile, by decreasing plasma triglycerides and increasing HDL-cholesterol. The mechanism responsible for cardiovascular protection and beneficial lipid profile changes observed in this LPL-variant is not entirely clear. The data suggest that this variant may be catalytically normal with normal stability, but may be present at higher concentrations in the circulation associated with a higher level of LPL-activity. We did not find any associations for the other two polymorphisms and TVR.

The Asp9Asn substitution at the N-terminal end is situated near a glycosylation site that may influence overall catalytic activity, whereas the Asn291Ser substitution is located in a heparin-binding cluster and may thus affect the interaction of LPL with the cell wall glycosaminoglycans. Both these two amino acid substitutions are located in the N-domain and likely reduce enzyme activity and consequently increase triglyceride levels. The Ser447Ter substitution is located in the C-domain and thus may cause increased binding affinity of the truncated LPL to receptors or may affect its subunit interaction, either facilitating or otherwise affecting the formation of
dimers, which would explain the opposite effect of this substitution compared with the other two, what could form the basis of the observed association. In our study we did not find a significant association between the LPL447-polymorphism and HDL-, LDL-cholesterol levels and triglycerides (data not shown). This could be due to the use of lipid lowering medication in many of our patients. Haplotype analyses showed that the difference in TVR rate between the various haplotypes was completely explained by the LPL 447 variant.

In addition to the well-known role of LPL in the hydrolysis of the triglycerides packaged in chylomicrons and VLDL, several other functions of the enzyme have recently been identified. In particular, it has been shown that LPL increases monocyte adherence via a mechanism that requires interaction between the C-terminal domain of the LPL, heparin sulfate proteoglycans (HSPGs) and integrins. However, Zhang et al. showed no difference in the affinity of LPL Ser447Ter for HSPGs from wild-type LPL. Since, LPL is also expressed in smooth muscle cells in the arterial media, the LPL Ser447Ter polymorphism may affect the level of interaction between smooth muscle cells and the extracellular matrix; The former could result in less arterial stiffness, leading to more distensible arteries, and thus lesser propensity for restenosis.

On a completely different note, the LPL protein may also influence vascular tone by affecting the synthesis or degradation of endothelium-derived relaxing factors such as nitric oxide (NO). Endothelium-dependent vascular relaxation is abnormal in the setting of atherosclerosis, associated with subnormal NO-synthase (eNOS) activity, the key enzyme in basal endothelial cell NO-production. NO dilates coronary arteries and promotes blood flow by inhibiting smooth muscle contraction, platelet aggregation and platelet adhesion to endothelial cells by a cyclic guanosine monophosphate (cGMP)-mediated mechanism. In fact, LPL has been reported to increase NO-synthase production and consequential increased NO-production in culture macrophages. LPL may well have a similar function in vivo in both macrophages and endothelial cells and may therefore have an effect on vascular tone. Therefore, mutated levels of LPL, as observed with the Ser447Ter polymorphism, may be beneficial for endothelial function, an important contributor involved in restenosis. In addition, Ziouzenkova et al. found a link between LPL and peroxisome proliferators-activated receptor (PPAR) activation, suggesting that impaired LPLs enzymatic activity might decrease endogenous PPARα activation and its subsequent downstream effects, including anti-inflammatory responses. Inflammation has
been previously reported as a very important component of restenosis. Clee et al. found a decrease in blood pressure independent of the lower level of triglycerides in patients with LPL Ser447Ter polymorphism. Another study also showed that LPL Ser447Ter polymorphism was associated with lower systolic blood pressure (SBP) and pulse pressure (PP) levels in women. Since inflammation and elevated blood pressure have been implicated in increased risk for restenosis, any positive effect of this polymorphism on arterial tone, inflammatory status and elevated blood pressure may ultimately translate into lesser risk for restenosis.

Since we hypothesize a potential interaction between LPL activity by genotype and inflammatory activity, we also investigated several inflammatory markers, which could be of influence on the development of restenosis and their effect on TVR. These markers examined are the fibrinogen level, the erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP). These markers were determined in a subgroup of patients from the GENDER-study (in 753 patients for fibrinogen, in 1000 patients ESR and in 888 patients CRP, respectively). All three factors, determined pre-PCI, did not have a statistically significant effect on TVR (p>0.20), nor did they influence the general results for the LPL polymorphisms.

We further studied LPL gene expression during the development of restenosis in an established mouse model for diet-induced atherosclerosis. In the mouse model of restenosis, LPL mRNA levels were increased 40-fold compared to control arteries at 6 hours post cuff-placement. This indicates that LPL may play an important role in the early stages of the restenotic process development.

Limitations of the study
In our study we lack data on LPL concentration and LPL activity in plasma. However, we believe that plasma determinations have no added value, due to a number of reasons. Circulating LPL protein levels, were not assessed here, since basal (pre-PCI) plasma measurements of the gene product are not likely to reflect the genetically determined differences in reaction to a trauma such as PCI. Moreover, local differences in LPL sensitive reactions, such as occurring in the vessel wall at the site of PCI, cannot be measured systemically, as it is not yet possible to measure gene products in the vessel wall locally in the acute phase of treatment and the following days. Furthermore, we made use of an atherosclerotic mouse model to study the effect of LPL on restenosis, in this model we were not able to test the LPL polymorphisms found in humans.
However, we believe that this model contributes to a better understanding of the involvement of LPL in the process of restenosis. Although the mice studies can be used for the analysis, it should be realized that perivascular cuff-placement result initially in adventitial injury whereas in patients PCI results in intimal injury. It is not certain to what extent these apparently different ways of vascular injury differ in their reaction regarding vascular activation and the resulting intimal hyperplasia. In addition, genotyping of some patients was missing due to lack of DNA or inconclusive genotyping and mistakes could have been made in the genotyping. However, patients who could not be genotyped did not differ in any characteristic from those who could be genotyped. Furthermore, the PCR procedure was performed in replicate on 10% and there was a difference observed in less than 1% of the samples. The -93T/G polymorphism was not in HWE, the observed numbers of patients were: T/T; 2914, T/G; 109, G/G; 5 opposed to the expected number of patients of; 2910 T/T, 117 T/G and 1 G/G. This discrepancy could be explained by the fact that it is possible that this polymorphism is associated with one or more in-/exclusion criteria, however, we cannot confirm that with our data since all participants fulfilled those criteria. The most important inclusion criterion was that all participants were scheduled for PCI, and obviously the classical risk factors of cardiovascular disease will be enriched in such a population. The -93T/G polymorphism is sometimes found to be especially associated with HDL2-cholesterol, and ApoA-I levels: with lower levels in -93G/G carriers. Based on this assumption one might expect more heterozygote, or homozygote carriers, and the latter were indeed slightly more present than expected. Drug eluting stents (DES) are now more widely used. In our study we do not have any data on these stents, since no drug eluting stents were used for our study, which can be seen as a limitation of our study. However, genes involved in the process of restenosis after DES are probably different from the process of restenosis after bare-metal stent placement or plain balloon angioplasty. Therefore, new studies have to be set up to investigate genes involved in the process of restenosis after DES. Finally, the polymorphism associated with TVR in our study may be in linkage disequilibrium with other polymorphisms in the gene or with other nearby genes that are actually responsible for the development of this condition.

**Conclusions**

In conclusion, we have demonstrated, that LPL is significantly associated with TVR. The LPL C/G polymorphism, which results in a 447 Serine → Stop (X) mutation, appears to be an important independent protective factor in this. Furthermore, LPL mRNA was highly upregulated in the first six hours after vascular damage in a mouse model of restenosis. Determination of this genotype
could contribute to better risk stratification and more tailored therapy for the individual patient to prevent TVR after PCI.

Acknowledgement
We thank S. Cheng, M. Grow and their colleagues at Roche Molecular Systems (Alameda, USA) for developing and providing their multilocus genotyping assays under a research collaboration. We thank P. Schiffers from the University of Maastricht for assistance with the genotyping assay. The contribution of the members of the clinical event committee, J.J. Schipperheyen MD PhD, J.W. Viersma MD PhD, D. Düren MD PhD, and J. Vainer MD is greatly acknowledged.

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Reference List


Lipoprotein Lipase gene Polymorphisms and the Risk of Target Vessel Revascularization after Percutaneous Coronary Intervention


Lipoprotein Lipase gene Polymorphisms and the Risk of Target Vessel Revascularization after Percutaneous Coronary Intervention
CHAPTER 7

LIPOPROTEIN LIPASE S447X DECREASES MICROALBUMINURIA RELATED CARDIOVASCULAR RISK IN MALES

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² Department of Medical Genetics, University Medical Center Groningen, University of Groningen, the Netherlands
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⁵ Department of Clinical Pharmacology, University Medical Center Groningen, University of Groningen, the Netherlands

Manuscript in preparation for publication
Abstract

Objectives Microalbuminuria (MA) has emerged as an important risk indicator for cardiovascular disease (CVD). Part of this relation has been attributed to progressive metabolic derangements characteristic during the various albuminuric stages, amongst which dyslipidemia (i.e. high triglycerides (TG) and low high-density lipoprotein cholesterol (HDL-C) is a prominent feature. The frequent lipoprotein lipase (LPL) gene variant S447X (LPLS447X) is associated with beneficial lipid changes (low TG and high HDL-C). In the present study, we evaluated whether LPLS447X was able to modify CVD risk increase in different categories of albuminuria.

Methods We used data from the PREVEND (Prevention of Renal and Vascular Endstage Disease) study (n=8592), a prospective general population-based cohort study. Subjects with baseline CVD or macroalbuminuria were excluded. Remaining subjects were categorized according to their albuminuric status (I: 0-15, II: 15-30 and III: 30-300 mg/24hr). Primary endpoint was a major cardiovascular event (MACE). Multivariate Cox regression analysis was used to calculate hazard ratios (HRs) as an estimate of the relative risk of LPLS447X on MACE.

Results Carriers and non-carriers of the LPLS447X variant were categorized according to their albuminuric status (I: n=5427, 1037/4390, II: n=1115, 229/886 and III: n=956, 185/771). LPLS447X was associated with decreased TG (p<0.0001) and elevated HDL-C levels (p<0.0001). After correction for age, smoking, mean arterial pressure, low-density lipoprotein cholesterol, HDL-C and TG, LPLS447X was still significantly associated with reduced CVD risk in males (adjusted HR cat. II vs. I: 0.31 [95% CI: 0.11-0.88], p=0.028; and adjusted HR cat. III vs. I: 0.44 [95% CI: 0.19-0.99], p=0.048). In females, LPLS447X was not significantly associated with cardiovascular protection (adjusted HR cat. II vs. I: 0.83 [95% CI: 0.19-3.55], p=0.80 and adjusted HR cat. III vs. I: 0.63 [95% CI: 0.12-3.30], p=0.59).

Conclusion We confirm that LPLS447X is associated with decreased TG as well as elevated HDL-C levels. In addition, a significant interaction between LPLS447X and albuminuria was found. Thus, a reduction of CVD risk was present predominantly in males with higher albumin excretion rates (≥15 mg/24hr), part of which appeared to be independent of the improved lipid profile in LPLS447X carriers. Presumably, the lifelong LPLS447X mediated beneficial effect on lipid modulates risk at later years, at least in males.
Introduction

Microalbuminuria (MA) is associated with increased cardiovascular disease (CVD) risk in patients with type 2 diabetes\textsuperscript{1,2}, with hypertension\textsuperscript{3} as well as in the general population\textsuperscript{4}. More recently, it has been shown that albumin excretion rates within the 'normal' ranges, i.e. below 30 mg/24hr, are already associated with increased CVD risk.\textsuperscript{5} Interestingly, it has been a matter of intense debate whether albuminuria merely reflects vascular and renal damage or whether it is also causally related to progressive vascular disease.\textsuperscript{6,9} A potential causal role of microalbuminuria has been supported by the metabolic derangements associated with increases in albumin excretion in the physiological range. One of these metabolic sequelae includes a pro-atherogenic lipid profile. In fact, a multivariate analysis in the DCCT/EDIC cohort recently confirmed that increased albumin excretion was associated with alterations in triglyceride/HDL concentrations.\textsuperscript{10} However, the actual contribution of lipid changes to the microalbuminuria associated increase in CVD risk remains to be proven.

Lipoprotein lipase (LPL) is involved in regulating both TG and HDL-C levels via the enzymatic as well as non-enzymatic function of this protein.\textsuperscript{11-13} In line, various common LPL gene mutations resulting in loss of LPL function have been associated with high TG and low HDL-C, as well as increased CVD risk.\textsuperscript{14} In contrast, the frequent LPL gene variant S447X (LPLS447X) has been associated with low TG and high HDL-C. These favourable lipid changes have been associated with a lower CVD risk in LPLS447X carriers,\textsuperscript{15-18} whereas a recent meta-analysis showed that the protective effect was gender-specific, providing benefit only to males with 18% reduced risk of future CVD.\textsuperscript{14}

We hypothesized that in categories of albuminuria, the LPLS447X variant would attenuate the albuminuria-associated dyslipidemia. If MA-associated dyslipidemia is an important factor contributing to CVD risk in MA-subjects, LPLS447X can be expected to partly attenuate the CVD risk increase. In order to test this hypothesis, we investigated the effect of the LPLS447X variant on lipids and CVD risk in subjects in different categories of albuminuria. We tested this hypothesis in the PREVEND (Prevention of Renal and Vascular Endstage Disease) study, a large population cohort derived from the general population of Groningen, the Netherlands.
Materials and Methods

Study population
The population analysed in this study was obtained from the PREVEND (Prevention of REnal and V ascular ENdstage Disease) study, which was designed to investigate the natural course of MA and its relation with renal and cardiovascular disease in the general population. The study cohort consists of 8592 subjects (aged 28-75 years) from the city of Groningen, The Netherlands, which completed an extensive screening program, as described previously in detail. The population is enriched for subjects with a morning urine albumin concentration of more than 10 mg/24hr (N=6000), while the remainder of the population had a baseline urine albumin excretion of <10 mg/24hr (N=2592). All participants gave written informed consent and subsequently completed a questionnaire on demographics and cardiovascular and renal history. The PREVEND study was approved by the local institutional review board and conducted in accordance with the guidelines of the declaration of Helsinki. For the present study, subjects with missing albuminuria data, baseline macroalbuminuria (>300 mg/24hr) or with a reported baseline history of a major adverse cardiovascular event were excluded.

Methods
All subjects visited the outpatient clinic twice during which physiological parameters were measured. Urinary albumin excretion and highly sensitive C-reactive protein (CRP) were determined by nephelometry as described previously. Blood pressure was calculated as the average of the two last out of ten consecutive measurements using an automatic Dinamap XL model 9300 series device (Johnson-Johnson, Medical INC, Tampa, Florida). HDL-C was assessed using a commercially available assay (Abbott Inc, IL, USA). Serum TG was measured enzymatically. LPL genotyping was performed as previously described.

Definitions
The primary end-point of this study, major adverse cardiovascular event (MACE), included death from myocardial infarction and ischemic heart disease, hospitalization for myocardial infarction, ischemic heart disease, and cerebrovascular accident (CVA) or hospitalization for CVA. Causes of death and information on other cardiovascular events were obtained from the Central Bureau of Statistics (CBS, Voorburg, The Netherlands) and national hospital information system (Prismant, Utrecht, The Netherlands). The vital status of the participants from the time...
of inclusion was checked through the municipal register and the first MACE of each participant was used for analysis. Mean arterial pressure (MAP) was calculated using systolic blood pressure (SBP) and diastolic blood pressure (DBP) as follows: \( \text{MAP} = \frac{\text{SBP} + 2 \times \text{DBP}}{3} \). Body mass index was defined as weight/height\(^2\). Average urinary albumin excretion (UAE) was calculated from the determined albumin content in two consecutive 24h urinary sample collections. Hypertension was defined as blood pressure > 140/90 mmHg. Hypercholesterolemia was defined as baseline total cholesterol (TC) > 6.5 mmol/L or in case of secondary prevention or treatment with lipid lowering medication, TC > 5 mmol/L. Smoking was defined as current active smoking or when quitted smoking less than one year and non-smoking was defined as currently not active smoking or when quitted smoking more than one year ago. Information regarding drug use was obtained via questionnaires.

**Statistical Analysis**

Continuous (baseline) variables were compared between LPLS447X carriers and non-carriers using an independent sample t-test. Chi-square test was applied for comparison of distributions of dichotomous data. In order to observe the effect of cardiovascular protection in different categories of microalbuminuria, three categories of albuminuria were created: category I: 0-15 mg/24hr, category II: 15-30 mg/24hr and category III: 30-300 mg/24hr. Hazard ratios (HR) and corresponding 95% confidence intervals (95% CI) as an estimate of the relative risk of LPLS447X on MACE in different categories of albuminuria were calculated using Cox regression analysis. The lowest category of albuminuria was used as the reference category. Models were tested for the interaction between LPLS447X and the different categories of albuminuria. In addition, adjustments were made for potential confounders using multivariate models. Variables with a skewed distribution were log-transformed before analyses (UAE, TG and CRP). P-values < 0.05 were considered statistically significant. All data were analyzed with SPSS (version 12.0.1, SPSS Inc., Chicago, Illinois, USA).

**Results**

**Study population**

From a total of 8592 participants, 735 subjects were excluded for the present study due to missing albuminuria data (n=15) or in case of baseline macroalbuminuria (n=109) or reported
baseline history of MACE (i.e. myocardial infarction (n=335), hospitalization for myocardial infarction (n=149), cerebrovascular accident (n=96) and hospitalization for cerebrovascular accident (n=31); total: n=611). LPLS447X genotyping was performed in the remaining 7857 subjects, but failed in 359 subjects. Of all remaining 7498 subjects, 6047 were non-carriers (80.6%), 1355 (18.1%) were heterozygous and 96 (1.3%) homozygous for LPLS447X variant. Subjects were dichotomised in non-carriers (n=6047, 80.6%) and carriers (n=1451, 19.4%), because of the low CVD incidence rate in and relative low numbers of homozygote LPLS447X carriers.

**Table 1 Baseline Characteristics of LPLS447X Carriers and Controls**

<table>
<thead>
<tr>
<th></th>
<th>Carriers (n=1451)</th>
<th>Non-carriers (n=6047)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>48.6 ± 12.5</td>
<td>48.4 ± 12.4</td>
<td>0.58</td>
</tr>
<tr>
<td>Males</td>
<td>713 (49.1%)</td>
<td>2898 (47.9%)</td>
<td>0.41</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.9 ± 4.1</td>
<td>26.0 ± 4.2</td>
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</tr>
<tr>
<td>W/H ratio</td>
<td>0.88 ± 0.10</td>
<td>0.88 ± 0.09</td>
<td>0.87</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>128.2 ± 19.8</td>
<td>128.2 ± 19.9</td>
<td>0.93</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>73.5 ± 9.7</td>
<td>73.7 ± 9.7</td>
<td>0.43</td>
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<tr>
<td>MAP (mmHg)</td>
<td>91.7 ± 12.3</td>
<td>91.9 ± 12.3</td>
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<tr>
<td>TC (mmol/L)</td>
<td>5.67 ± 1.15</td>
<td>5.63 ± 1.11</td>
<td>0.22</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>1.37 ± 0.40</td>
<td>1.33 ± 0.40</td>
<td>0.0001</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>3.72 ± 1.08</td>
<td>3.66 ± 1.05</td>
<td>0.06</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.08 (0.80 - 1.55)</td>
<td>1.16 (0.84 - 1.69)</td>
<td>0.0001</td>
</tr>
<tr>
<td>CRP (mmol/L)</td>
<td>1.24 (0.52 - 2.90)</td>
<td>1.20 (0.54 - 2.80)</td>
<td>0.93</td>
</tr>
<tr>
<td>UAE (mg/24 hours)</td>
<td>9.25 (6.27 - 17.08)</td>
<td>91.9 (6.26 - 13.60)</td>
<td>0.44</td>
</tr>
</tbody>
</table>

BMI: body mass index; W/H ratio: waist/hip ratio; SBP: systolic blood pressure; DBP: diastolic blood pressure; MAP: mean arterial pressure: (SBP + 2xDBP)/3; TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; TG: triglycerides; CRP: C-reactive protein; UAE: mean urinary albumin excretion

**Baseline characteristics**

Overall, there were no differences in age, gender, body mass index (BMI), waist/hip ratio, SBP, DBP, MAP or CRP between carriers and non-carriers as shown in table 1. Whereas TC levels were
comparable (5.67 ± 1.15 vs. 5.63 ± 1.11 mmol/l, P=0.22), HDL-C was 3.0% higher (1.37 ± 0.40 vs. 1.33 ± 0.40 mmol/l, P=0.0001) and median TG were 6.9% lower (1.08 [0.80 - 1.55] vs. 1.16 [0.84 - 1.69] mmol/l, P=0.0001) in carriers compared to controls. In contrast, carriers presented with similar median urinary albumin excretion (UAEx) levels (P=0.44). In addition, both groups presented with similar numbers of patients with hypertension (29.2 vs. 28.2 %, P=0.63) or hypercholesterolemia (12.9 vs. 12.9%, P=0.94). No significant differences could be found in smoking status (P=0.52) or racial distribution (P=0.67).

Baseline characteristics of carriers and non-carriers in different categories of albuminuria.
Since our current study set out to evaluate whether LPLS447X modified CVD risk in different albuminuria categories, carriers and non-carriers were categorized according to their albuminuric status, referred to as 'low normal' (category I: 0-15 mg/24hr) and 'high normal' (category II: 15-30 mg/24hr) levels of albuminuria and micro-albuminuria (category III: 30-300 mg/24hr). Baseline characteristics of carriers and non-carriers in these different categories of albuminuria are listed in table 2. There were no differences in age, gender, BMI, waist/hip ratio, SBP, DBP, MAP, hs-CRP or UAEx levels or use of medication between carriers and controls in any category of albuminuria. In the lowest category of albuminuria, carriers presented with 2.9% higher HDL-C (P=0.006) and 5.4% lower TG level (P<0.0001). In the 'high normal' category, HDL-C was 8% (P=0.002) and TG 16.1% lower (P=0.001). Interestingly, in microalbuminuric subjects, HDL-C and TG levels were unaffected by the LPL variant (table 2).

Albuminuria categories and CVD risk.
Median follow-up time during the study was comparable for carriers, 2147 (2037 - 2232) and non-carriers, 2142 (2031 - 2226) person days. Distribution of MACE in the three categories of albuminuria in carriers and non-carriers are listed in table 3. As expected, multivariate Cox regression analysis showed that high normal levels of albuminuria were associated with increased CVD risk as compared to low albuminuria levels in males (HR cat. II vs. I: 2.94 [95 % CI 2.01 - 4.28], P<0.0001) as well as in females (HR cat. II vs. I: 2.37 [95% CI 1.33 - 4.22], P=0.003). In addition, microalbuminuria was also associated with increased CVD risk in males (HR cat. III vs. I: 3.85, [95 % CI 2.69 - 5.50], P<0.0001) as well as in females (HR cat. III vs. I: 3.35 [95 % CI 1.90 - 5.88], P<0.0001). Most markedly, our analysis revealed a significant interaction between LPLS447X and different categories of albuminuria with respect to CVD incidence in males. Specifically, LPLS447X was associated with reduced CVD risk in 'high normal' albuminuria
<table>
<thead>
<tr>
<th></th>
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<th>30 - 300 mg/24hr</th>
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<tr>
<td></td>
<td>Carriers</td>
<td>Non-carriers</td>
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<tr>
<td>n</td>
<td>1037 (19.1%)</td>
<td>4390 (80.9%)</td>
<td>P</td>
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<td>Age</td>
<td>47.1 ± 11.9</td>
<td>46.7 ± 11.9</td>
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<td>Males (%)</td>
<td>45.4</td>
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<td>BMI (kg/m²)</td>
<td>25.5 ± 3.8</td>
<td>25.5 ± 4.0</td>
<td>0.66</td>
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<td>W/H ratio</td>
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<td>0.86 ± 0.09</td>
<td>0.83</td>
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<td>SBP (mmHg)</td>
<td>1241 ± 171</td>
<td>1244 ± 175</td>
<td>0.62</td>
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<td>DBP (mmHg)</td>
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<td>72.0 ± 8.7</td>
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<td>MAP (mgHg)</td>
<td>89.2 ± 10.8</td>
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<td>0.44</td>
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<td>TC (mg/dL)</td>
<td>5.61 ± 1.14</td>
<td>5.56 ± 1.12</td>
<td>0.18</td>
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<tr>
<td>HDL-C (mg/dL)</td>
<td>1.40 ± 0.40</td>
<td>1.36 ± 0.40</td>
<td>0.006</td>
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<tr>
<td>LDL-C (mg/dL)</td>
<td>3.66 ± 10.8</td>
<td>3.60 ± 10.5</td>
<td>0.98</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>1.05 (0.78-1.43)</td>
<td>1.11 (0.81-1.58)</td>
<td>&lt;0.0001</td>
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<tr>
<td>CRP (mg/dL)</td>
<td>1.05 (0.48-2.44)</td>
<td>1.05 (0.48-2.55)</td>
<td>0.88</td>
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<tr>
<td>UAE (mg/24hr)</td>
<td>73.2 (5.67-99.3)</td>
<td>73.1 (5.66-98.8)</td>
<td>0.72</td>
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</table>

BMI: body mass index; W/H ratio: waist/hip ratio; SBP: systolic blood pressure; DBP: diastolic blood pressure; MAP: mean arterial pressure; TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; TG: triglycerides; CRP: C-reactive protein; UAE: mean urinary albumin excretion.
category (HR cat. II vs. I: 0.28 [95% CI 0.01 - 0.77], P=0.01) as well as in the microalbuminuria category in males (HR cat. III vs. I: 0.43 [95% CI 0.19 - 0.98], P=0.04). In contrast, LPLS447X was not significantly associated with cardiovascular protection in females (HR cat. II vs. I: 0.83 [95% CI: 0.20 - 3.53], p=0.80; HR cat. III vs. I: 0.67 [95% CI: 0.13 - 3.49], P=0.64). After correction for age, smoking, MAP and LDL-C, HDL-C and TG, LPLS447X was still significantly associated with reduced CVD risk in males (adjusted HR cat. II vs. I: 0.31 [95% CI: 0.11 - 0.88], P=0.028); and adjusted HR cat. III vs. I: 0.63 [95% CI: 0.12 - 3.30], P=0.59). Of note, LPLS447X also interacted with albumin excretion as continues variable with respect to CVD incidence in males (HR: 0.31 [95% CI: 0.11 - 0.87], P=0.026) but not in females (HR: 0.61 [95% CI: 0.14 - 2.88], P=0.61). After correction for age, smoking, MAP and LDL-C, HDL-C and TG, this interaction in males remained significant (HR: 0.30 [95% CI: 0.17 - 0.91], P=0.027) and not in females (HR: 0.54 [95% CI: 0.09 - 3.25], P=0.51).

Discussion

In the present study, we show that increased albumin excretion, even in normal range, is associated with atherogenic lipid profile changes, comprising increased levels of TC, LDL-C and decreased HDL-C levels. The LPLS447X variant was associated with beneficial changes in lipids, i.e. lower TG and higher HDL-C levels, predominantly in carriers with 'low normal' albuminuria (moderate effect) and 'high normal' albuminuria (strong effect). Most markedly, LPLS447X protected against future CVD in men with higher albumin excretion rates (>15mg/24hr). These effects imply that LPLS447X may convey cardiovascular protection in part through its beneficial lipid profile changes. Presumably, the lifelong LPLS447X mediated beneficial effect on lipid modulates risk at later years, at least in males.
LPLS447X and cardiovascular risk factors

Recently, LPLS447X has been characterized as a ‘gain-of-function’ mutation, resulting in both increased enzymatic as well as non-enzymatic LPL function. These effects are thought to lead to enhanced conversion of triglyceride-rich lipoproteins (TRL) as well as increased LDL clearance from the circulation. The latter may be related to increased concentrations of (monomeric) LPL in the circulation of carriers of this LPL variant. Indeed, large observational studies have substantiated that LPLS447X is associated with beneficial effects on fasting (increased HDL-C, lower TG),14,16,18,22,24-32 as well as on postprandial lipid profile,23,26,33,34 most markedly present in obese males.28 In line, we also found lower TG and higher HDL-C levels in LPLS447X carriers compared to non-carriers. Beside beneficial effects on lipids, LPLS447X has also been associated with blood pressure changes in women as well as in patients with familial hypercholesterolemia. However, in the present study we were unable to confirm such an interaction between blood pressure and LPLS447X.

Microalbuminuria and cardiovascular risk

Microalbuminuria has been generally acknowledged as a marker for increased CVD risk in subjects with type 2 diabetes,2,3 hypertensive subjects as well as the general population.1 Even albumin excretion rates within the ‘normal’ range (10-20 mg/24 hrs) have been associated with cardiovascular risk factors and cardiovascular morbidity. Klausen et al.7 showed that in the Third Copenhagen City Heart Study low urinary albumin excretion (UAE) levels (>6.9 mg/24 hrs) were already associated with a two-fold increase in CVD risk. Arnlov reported a significantly increased CVD risk (HR: 2.92; 95% CI, 1.57 - 5.44; P<0.001) at even lower levels of albuminuria (>5.6 mg/24 hrs) in the Framingham Offspring Study. Accordingly, data from our current study confirm that ‘high normal’ levels of albumin excretion (15-30 mg/24 hrs) are associated with increased CVD risk compared to ‘low normal’ albumin excretion levels (0-15 mg/24 hrs). At the same time, increasing albumin excretion rates were also associated with adverse lipid profile changes, comprising increased TG levels and decreased HDL-C levels.

Interaction between LPLS447X and microalbuminuria

LPLS447X was consistently associated with reduced CVD risk in different albuminuria categories in males, even after adjustment for traditional risk factors. Interestingly, LPLS447X was also associated with a beneficial lipid profile, particularly in males characterized by ‘low normal’ as well as ‘high normal’ albumin excretion rates. These beneficial lipid changes coincided with a
reduction of albuminuria-associated CVD risk in male subjects. Remarkably, LPLS447X was also associated with a reduction in cardiovascular risk in males with microalbuminuria, whereas in this category beneficial effects on lipid levels could no longer be demonstrated in carriers versus non-carriers. The latter finding suggests that, at least in the microalbuminuric subjects, LPLS447X-associated lipid changes cannot fully account for the cardiovascular benefit of this mutation. In this respect, multivariate regression analysis in the high normal category revealed that the reduction in risk remained unaffected after adjustment for lipid changes in males. Collectively, these data indicate that, whereas LPLS447X has a favourable impact on CVD risk through beneficial lipid changes, additional factors are likely involved in cardiovascular protection in LPLS447X carriers.

**Potential mechanisms contributing to cardiovascular prevention**

Many studies have shown that serum LPL concentration (i.e. preheparin LPL concentration) is inversely related to cardiovascular disease. Serum LPL concentration has been shown to reflect insulin sensitivity and low levels of serum LPL concentration can be found in subjects with increased CVD risk like subjects with type 2 diabetes. The impact of serum LPL concentration on CVD progression has been highlighted by a strong correlation between serum LPL and severity of CAD and more importantly, low serum LPL concentration was shown to be an independent risk factor for CAD in multiple linear regression analysis (p=0.007). Recently, prospective data confirmed these findings and finally proved that increased LPL concentration indeed is associated with reduction in CVD risk. Interestingly, LPLS447X has been associated with markedly increased levels of serum LPL concentration. This may be associated with increased LPL activity as well as non-enzymatic functions of LPL. Possibly, serum LPL has beneficial effects leading to reduced CVD risk or increased levels of serum LPL represent a vascular system with lower CVD risk as in reduced endothelial dysfunction e.g. direct effects on endothelial function.

Even though lipid level correction in our current Cox regression model did not affect outcome (future CVD risk), the lifelong beneficial effects on lipid levels are likely to have contributed to differences in CVD risk in later years. Most likely, LPLS447X-associated CV protection is due to various mechanisms, like increased lipolytic activity and/or concentration in the circulation, increased stability of LPL dimers and better binding to heparan sulphate containing proteoglycans and lipoproteins, promotion of hepatic uptake of lipoproteins resulting in a better clearance of atherogenic remnant lipoproteins, reduced expression of LPLS447X by macrophages,
or even reduced LPL-mediated uptake of modified lipoproteins by subendothelial macrophages in carriers of the mutation.\textsuperscript{43}

**Study limitations**

Two points in our current study require closer attention. First, we find lowering of albuminuria-associated CVD risk only in males, not in females. These results are in line with other studies showing LPLS447X mediated cardiovascular protection only in males.\textsuperscript{14,16,18} The lack of a statistically significant risk reduction in females may in part be due to lower baseline CVD risk in females. Women suffer from CVD later in life as compared to men. Hence, follow-up time in the present study may have been too short to observe the effect of LPLS447X on albuminuria associated risk in women. The alternative, i.e. a potential pathway for a true gender-specific modification of risk, needs further elaboration. Second, the fact that TG levels between microalbuminuric carriers and controls were no longer significantly different is unexpected. Most likely, this pertains to the large variations in TG in this albuminuria category, combined with the limited number of LPLS447X carriers in the microalbuminuric cohort.

**Conclusions**

In the present study, we confirm that albuminuria is associated with dyslipidemia as well as an increased CVD risk. In subjects with 'low normal' and 'high normal' albuminuria, LPLS447X is associated with decreased TG as well as increased HDL-C levels. These beneficial effects on lipid levels no longer reached statistical significance in subjects with microalbuminuria. Markedly, LPLS447X was associated with a reduction of CVD risk in males with higher albumin excretion rates (≥ 15mg/24hr), even after adjustment for lipid changes. These findings imply that LPLS447X is associated with cardioprotective effects, only in part attributable to beneficial lipid changes.
Reference List


Lipoprotein Lipase S447X Decreases Microalbuminuria Related Cardiovascular Risk in Males
SERUM LIPOPROTEIN LIPASE CONCENTRATION AND RISK FOR FUTURE CORONARY ARTERY DISEASE; THE EPIC-NORFOLK PROSPECTIVE POPULATION STUDY

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Department of Vascular Medicine (JR, MCN, JNIM, JJPK, JAK), Cardiology (SMB) and Clinical Epidemiology and Biostatistics (BAH), Academic Medical Center, Amsterdam, The Netherlands; Medical Research Council Epidemiology Unit, Cambridge, United Kingdom (NJW); Medical Research Council Dunn Nutrition Unit, Cambridge, United Kingdom (SAB); Department of Public Health and Primary Care, Institute of Public Health, University of Cambridge, Cambridge, United Kingdom (RL, NED, KTK)

Abstract

Background. Lipoprotein lipase (LPL) is associated with coronary artery disease (CAD) risk, but prospective population data are lacking. This is mainly due to the need for cumbersome heparin injections which are necessary for LPL measurements. Recent retrospective studies, however, indicate that LPL concentration can be reliably measured in serum which enabled evaluation of the prospective association between LPL and future CAD.

Methods and results. LPL concentration was determined in serum samples of men and women in the EPIC-Norfolk population cohort who developed fatal or non-fatal CAD during 7 yrs follow-up. For each case (n=1006) two controls, matched for age, sex and enrolment time were identified. Serum LPL concentration was lower in cases compared to controls (median(interquartile range): 61 (43–85) vs. 66 (46–92) ng/mL; p<0.0001). Those in the highest LPL concentration quartile had a 34% lower risk for future CAD compared to those in the lowest quartile (odds ratio (OR) 0.66, CI:0.53–0.83; p<0.0001). This effect remained significant after adjustment for blood pressure, diabetes, smoking, body mass index and low-density lipoprotein cholesterol (OR 0.77, CI:0.60–0.99; p=0.02). As expected from LPL biology, additional adjustments for either high-density lipoprotein cholesterol (HDL-c) or triglyceride (TG) levels rendered loss of statistical significance. Of interest, serum LPL concentration was positively linear correlated with HDL and LDL size.

Conclusions. Reduced levels of serum LPL are associated with an increased risk for future CAD. The data suggest that high LPL concentrations may be atheroprotective through decreasing TG levels and increasing HDL-C levels.
Introduction

Lipoprotein lipase (LPL) hydrolyses plasma triglycerides (TG) that are packaged in chylomicrons and very-low density lipoproteins (VLDL). This catalytic activity results in the formation of cholesterol-rich lipoprotein remnants and generates constituents for the anti-atherogenic high-density lipoprotein (HDL) pool. LPL also enhances the hepatic clearance of atherogenic lipoprotein remnants via the low-density lipoprotein (LDL) receptor. The crucial role of LPL in lipid metabolism is illustrated by genetic LPL deficiency, a rare disorder characterized by severe hypertriglyceridemia and low HDL-c levels.

Over the last 25 years, the relation between LPL and coronary artery disease (CAD) has been addressed using various approaches. Patients with genetic LPL deficiency have been studied in detail and although there are indications that these patients suffer from premature atherosclerosis, there are also reports indicating that a complete lack of LPL, and thus a lack of formation of atherogenic lipoprotein remnants, does not underlie increased CAD risk. In the families in which LPL deficient probands were identified, the heterozygotes were shown to be at increased risk for atherosclerosis. Nevertheless, reliable data on CAD risk are unavailable as only small groups of affected individuals were studied. The use of (multiple) variants at the LPL gene locus has provided more insight. In fact, numerous genetic association studies, and studies on frequent functional variants such as LPL D9N and N291S have shown that loss of LPL function is associated with CAD. In addition, the vast majority of animal studies clearly indicate that LPL protects against (diet-induced) atherosclerosis. Biochemical assessment of LPL function, has also frequently been used to assess the role of LPL in atherogenesis. However, these studies are hampered by the need to administer heparin intravenously to release sufficient LPL from the endothelium to measure reliably LPL activity and LPL concentration. Since heparinisation is time-consuming, not-standardized and induces bleeding risk, most investigators have only studied limited numbers of diseased and/or non-diseased individuals. The bulk of these studies have indicated that (post-heparin) LPL activity is decreased in hypertriglyceridemic subjects and other patients at increased risk for CAD. Recently, the availability of a highly sensitive enzyme linked immunosorbent assay which can measure accurately freely circulating LPL concentration in non-heparinized serum has provided a tool to more easily assess the relationship between LPL and CAD. Olivecrona and colleagues were the first who studied how preheparin LPL (from now on referred to as serum LPL) relates to plasma lipoproteins and post-heparin LPL.
It was recognized that the majority of serum LPL is catalytically inactive\textsuperscript{21} and likely represents a mere catabolic product of catalytically active LPL that is bound to the endothelium. Also, it was demonstrated that serum LPL concentration is not associated with post-heparin LPL concentration or LPL activity. This was not unexpected since LPL levels are controlled by many factors, including differential transcriptional regulation in adipose and skeletal muscle tissue, post-translational modification and translocation over the endothelium, retro-endocytosis, binding to heparan sulfate-containing proteoglycans, lipoproteins and receptors, and hepatic clearance.\textsuperscript{22-25} Despite this, serum LPL concentration was strongly positively related with HDL-C and negatively with VLDL-TG although the latter relation was weak. Japanese investigators, using a commercially available LPL ELISA, have recently confirmed that serum LPL is not associated with post-heparin LPL concentration and LPL activity\textsuperscript{26,27} but at the same time their data suggest that serum LPL concentration reflects whole body LPL production or the systemic potential to hydrolyze plasma TG. In agreement with the studies of Tornval and Vilella, they showed that serum LPL is strongly correlated with HDL-C, and inversely related to plasma levels of TG while no correlations with total cholesterol and low-density lipoprotein cholesterol (LDL-C) were found.\textsuperscript{22,23} Two cross-sectional analyses have shown that men with acute myocardial infarction have lower serum LPL concentration compared to healthy controls.\textsuperscript{29} In addition, serum LPL is reported to be inversely related with the extent of coronary atherosclerosis.\textsuperscript{30} However, prospective data in humans showing that LPL is atheroprotective, are lacking. Based on the above data, we hypothesized that in apparently healthy individuals, low concentrations of serum LPL are associated with an increased risk for future CAD. We tested this hypothesis in a large prospective nested case-control study.

**Methods**

We performed a nested case-control study among participants in the EPIC-Norfolk cohort study (EPIC, European Prospective Investigation into Cancer and Nutrition), a population of 25,663 men and women between age 45 and 79. EPIC-Norfolk is part of the ten-country collaborative EPIC study designed to investigate determinants of cancer.\textsuperscript{31} From the outset, additional data were obtained in EPIC-Norfolk to enable the assessment of determinants of other diseases. Recruitment of participants was done by mail from age-sex registers of general practices. The recruitment rate was relatively low as addressed by Day et al. in one of the first study reports.\textsuperscript{32} At
the baseline survey between 1993 and 1997, participants completed a detailed health and lifestyle questionnaire which included questions about cigarette smoking habit and past medical history, and attended a clinic visit where additional data collection was undertaken by trained nurses using standardized protocols as previously described. This included anthropometry, blood pressure measurements and a non-fasting blood sample. Body mass index (BMI) was estimated as weight in kg divided by height in meters squared. All individuals have been flagged for mortality at the UK Office of National Statistics, with vital status ascertained for the entire cohort. Death certificates for all decedents were coded by trained nosologists according to the International Classification of Diseases (ICD) 9th revision. Death was considered due to CAD if the underlying cause was coded as ICD 410-414. In addition, participants admitted to hospital were identified using their unique National Health Service number by data linkage with ENCORE (East Norfolk Health Authority database), which identifies all hospital contacts throughout England and Wales for Norfolk residents. Participants were identified having CAD during follow-up if they had a hospital admission and/or died with CAD as underlying cause. We report results with follow-up to January 2003, an average of about 6 years. The study was approved by the Norwich District Health Authority Ethics Committee and all participants gave signed informed consent.

Participants
We have previously described similar designed nested case-control studies of the EPIC-Norfolk cohort. All individuals who reported a history of heart attack or stroke at the baseline clinic visit were excluded. Cases were individuals who developed a fatal or non-fatal CAD during follow-up until November 2003. Controls were study participants who remained free of any cardiovascular disease during follow-up. We matched two controls to each case by sex, age (within 5 years), and time of enrolment (within 3 months).

Biochemical analysis
Levels of total cholesterol, HDL-c, and TG in non-fasted serum samples were measured with the RA 100 (Bayer Diagnostics, Basingstoke, UK), and LDL-c levels were calculated using the Friedewald formula. LDL size and HDL size were assessed by proton nuclear magnetic resonance spectroscopy as described previously. Serum LPL concentrations were measured using a commercially available sandwich enzyme-linked immunosorbent assay (Dainippon Pharmaceutical Co, Ltd, Japan). Pooled plasma from healthy volunteers (n=200) was used as a control in each individual LPL assay and the interassay variance was found 8.2%. Samples were analyzed in...
random order to avoid systemic bias. Researchers and laboratory personnel were blinded to identifiable information, and could identify samples by number only.

Statistical analysis
Baseline characteristics were compared between cases and controls using a mixed effect model for continuous variables or conditional logistic regression for categorical variables which takes into account the matching for sex and age and enrolment time. Because TG and serum LPL levels had a skewed distribution, values were log-transformed before being used in the statistical analyses as continuous variables. In the tables, we show untransformed medians and corresponding interquartile ranges. Serum LPL levels were categorized in quartiles based on the distribution in the controls. Mean levels of traditional cardiovascular risk factors were calculated per LPL quartile. Linear associations between LPL quartiles and traditional risk factors were calculated using linear regression for continuous variables and chi-square tests for categorical variables. In addition Pearson’s correlation coefficients and corresponding p-values were calculated to assess the relationship between log-transformed LPL and other continuous risk factors. Odds ratios (OR) and corresponding 95% confidence intervals (95%CI) as an estimate of the relative risk of incident CAD were calculated using conditional logistic regression analysis, which takes into account the matching for sex and age and enrolment time. The lowest LPL quartile was used as the reference category. Odds ratios were adjusted for the following traditional CAD risk factors: systolic blood pressure, smoking (never, previous, current), BMI, LDL-C and diabetes. We also performed analyses that additionally adjusted for HDL-C and TG. Statistical analyses were performed using SPSS software (version 12.0.1, Chicago, Illinois). A p-value <0.05 was considered significant.

Results
Baseline characteristics
We identified 1006 individuals who developed CAD during an on average 6 years follow-up. A total of 974 cases could each be matched to two controls. For the remaining 32 cases, we could identify only one control per case. Thus, the control group consisted of 1980 people. At baseline, cases were more likely to have diabetes and be smokers compared to controls (table 1). Furthermore, BMI, systolic blood pressure, diastolic blood pressure, and plasma levels of total
cholesterol (TC), LDL-C and TG were significantly higher in cases compared to controls. In contrast, HDL-c levels were significantly lower in cases compared to controls. Serum LPL concentration in serum was significantly lower in cases compared to controls: 61 (43-85) ng/mL versus 66 (46-92) ng/mL (p<0.0001).

<table>
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<td>Triglycerides, mmol/l</td>
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<td>Serum LPL concentration, ng/ml</td>
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</table>

Data are presented as mean ± SD, percentage (n), or median (interquartile range). BMI, body mass index; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; LPL, lipoprotein lipase.

Serum LPL and other CAD risk factors
Linear negative associations with serum LPL concentration quartiles were observed for BMI, the number of subjects with diabetes and TG (p for linearity for all was <0.0001, table 2). For HDL-C, HDL size and LDL size, we identified a linear positive association with serum LPL quartiles (p for linearity <0.0001 for all three parameters). Similar significant linear associations between serum LPL quartiles and LDL-C or TC were not observed.
Serum LPL relation to CAD

The risk of future CAD decreased with increasing LPL quartiles such that people in the highest quartile had an OR of 0.66 (95%CI: 0.53-0.83), compared to those in the lowest quartile (p for linearity <0.0001; model 1 - table 3). Looking for pathways through which LPL might offer the observed protection, we used multivariate analyses. After adjustment for systolic blood pressure, diabetes, BMI, LDL-C and smoking a significant association between LPL quartiles and risk for CAD remained present (OR: 0.77, 95%CI: 0.60-0.99; model 2) for the comparison of extreme quartiles (p for linearity = 0.02).

Table 2 Distribution of CAD risk factors by serum LPL quartiles

<table>
<thead>
<tr>
<th>LPL quartile</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>P*</th>
<th>R</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPL range (ng/ml)</td>
<td>&lt;46</td>
<td>47 - 65</td>
<td>66 - 91</td>
<td>&gt;92</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case/control</td>
<td>315/495</td>
<td>259/495</td>
<td>215/495</td>
<td>217/495</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>o Never</td>
<td>13 (98)</td>
<td>11 (88)</td>
<td>9 (70)</td>
<td>11 (84)</td>
<td>0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>o Previous</td>
<td>56 (439)</td>
<td>49 (388)</td>
<td>52 (403)</td>
<td>49 (387)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>o Current</td>
<td>32 (249)</td>
<td>39 (309)</td>
<td>40 (310)</td>
<td>40 (316)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>274 ± 3.5</td>
<td>267 ± 3.5</td>
<td>263 ± 3.6</td>
<td>261 ± 3.9</td>
<td>-0.0001</td>
<td>-0.141</td>
<td>-0.0001</td>
</tr>
<tr>
<td>Diabetes</td>
<td>6.7 (54)</td>
<td>3.0 (24)</td>
<td>2.6 (21)</td>
<td>1.6 (13)</td>
<td>-0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>6.3 ± 1.1</td>
<td>6.3 ± 1.2</td>
<td>6.4 ± 1.3</td>
<td>6.4 ± 1.2</td>
<td>0.945</td>
<td>0.058</td>
<td>0.002</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>4.1 ± 1.0</td>
<td>4.1 ± 1.0</td>
<td>4.2 ± 1.1</td>
<td>4.2 ± 1.1</td>
<td>0.441</td>
<td>0.034</td>
<td>0.072</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>1.17 ± 0.32</td>
<td>1.27 ± 0.35</td>
<td>1.40 ± 0.37</td>
<td>1.53 ± 0.43</td>
<td>-0.0001</td>
<td>0.349</td>
<td>-0.0001</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>2.4 ± 1.3</td>
<td>2.1 ± 1.1</td>
<td>1.8 ± 1.3</td>
<td>1.6 ± 0.8</td>
<td>-0.0001</td>
<td>-0.246</td>
<td>-0.0001</td>
</tr>
<tr>
<td>LDL size (nm)</td>
<td>20.7 ± 0.6</td>
<td>20.9 ± 0.6</td>
<td>21.1 ± 0.6</td>
<td>21.2 ± 0.5</td>
<td>-0.0001</td>
<td>0.301</td>
<td>-0.0001</td>
</tr>
<tr>
<td>HDL size (nm)</td>
<td>8.7 ± 0.4</td>
<td>8.8 ± 0.4</td>
<td>9.0 ± 0.5</td>
<td>9.1 ± 0.5</td>
<td>-0.0001</td>
<td>0.331</td>
<td>-0.0001</td>
</tr>
</tbody>
</table>

P* indicates linearity between serum LPL quartiles and risk factor levels. R indicates Pearson’s correlation between log-transformed serum LPL levels and risk factor levels and P† indicates the corresponding p-value. (Total cholesterol, LDL cholesterol, HDL cholesterol and Triglycerides in mmol/l)

Additional adjustment for either TG (model 3) or HDL-C (model 4) levels, two parameters that are intrinsically correlated with LPL, rendered loss of statistical significance (p for linearity 0.17 and 0.16; respectively). This suggest that LPL mediates is protective effects through these parameters. This is in accordance with LPL biology in that LPL is the sole enzyme responsible for the clearance of plasma triglycerides and also provides constituents that contribute the pool of HDL.
Lipoprotein lipase and coronary artery disease

This prospective study shows that levels of serum LPL are inversely related to future CAD in apparently healthy men and women. In agreement with previous studies, we observed that this parameter is strongly associated with diabetes, HDL-C and TG, but not with TC and LDL-C levels. To obtain insight in the pathways through which LPL concentration can offer atheroprotection, we performed multivariate analyses. Corrections for systolic blood pressure, diabetes, BMI and LDL-C levels, did not strongly affect the relationship between serum LPL concentration and CAD. Further correction of HDL-C and TG levels, however, rendered loss of statistical significance indicating that the relationship of serum LPL concentration with CAD is largely explained by these factors. This result agrees with the fact that LPL is at the start of a cascade that culminates in the breakdown of plasma triglycerides thereby releasing apolipoproteins, phospholipids and other constituents from chylomicrons and VLDL to the HDL pool. Since others recently provided evidence for a positive association between serum LPL concentration and LDL size, an important player in atherogenesis, we examined whether LDL

<table>
<thead>
<tr>
<th>LPL quartile</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range, (ng/ml)</td>
<td>&lt;46</td>
<td>47-65</td>
<td>66-91</td>
<td>&gt;92</td>
<td></td>
</tr>
<tr>
<td>Cases / controls</td>
<td>315/495</td>
<td>259/495</td>
<td>215/495</td>
<td>217/495</td>
<td></td>
</tr>
<tr>
<td>Model 1</td>
<td>1</td>
<td>0.81</td>
<td>0.66</td>
<td>0.66</td>
<td>0.66</td>
</tr>
<tr>
<td>Model 2</td>
<td>1</td>
<td>0.93</td>
<td>0.73</td>
<td>0.77</td>
<td>0.77</td>
</tr>
<tr>
<td>Model 3</td>
<td>1</td>
<td>0.97</td>
<td>0.80</td>
<td>0.88</td>
<td>0.87</td>
</tr>
<tr>
<td>Model 4</td>
<td>1</td>
<td>0.95</td>
<td>0.79</td>
<td>0.79</td>
<td>0.87</td>
</tr>
</tbody>
</table>

OR and corresponding 95% confidence intervals calculated by conditional logistic regression, taking into account matching for age, sex, and enrolment time. LPL, levels and TG levels were log-transformed before analysis. P* indicates p-value for linearity between LPL quartiles and CAD risk.

Model 1 unadjusted;
Model 2 adjusted for systolic blood pressure, diabetes, BMI, LDL-C and smoking;
Model 3 adjusted for the same variables as in model 2 and also TG levels;
Model 4 adjusted for the same variables as in model 2 and also HDL-C levels.

Discussion

Lipoprotein lipase and coronary artery disease

This prospective study shows that levels of serum LPL are inversely related to future CAD in apparently healthy men and women. In agreement with previous studies, we observed that this parameter is strongly associated with diabetes, HDL-C and TG, but not with TC and LDL-C levels. To obtain insight in the pathways through which LPL concentration can offer atheroprotection, we performed multivariate analyses. Corrections for systolic blood pressure, diabetes, smoking, BMI and LDL-C levels, did not strongly affect the relationship between serum LPL concentration and CAD. Further correction of HDL-C and TG levels, however, rendered loss of statistical significance indicating that the relationship of serum LPL concentration with CAD is largely explained by these factors. This result agrees with the fact that LPL is at the start of a (catalytic) cascade that culminates in the breakdown of plasma triglycerides thereby releasing apolipoproteins, phospholipids and other constituents from chylomicrons and VLDL to the HDL pool. Since others recently provided evidence for a positive association between serum LPL concentration and LDL size, an important player in atherogenesis, we examined whether LDL
size as measured by nuclear magnetic resonance differed between individuals in the four concentration quartiles. The data confirmed a strong positive correlation between serum LPL concentration and LDL size. In addition, we also found a positive correlation with HDL size, a parameter which is associated with decreased CAD risk.\textsuperscript{39} The latter observations support the association of a high serum LPL concentration with a more beneficial lipid profile.

\textbf{Lipoprotein lipase biology}

In trying to understand how low levels of LPL in the circulation are associated with increased cardiovascular risk, we would like to refer to the idea of Tornval et al. that this parameter may represent a catabolic product of biologically active LPL.\textsuperscript{20} There are several lines of evidence in support of the hypothesis that this parameter somehow reflects total LPL body production.\textsuperscript{27} First, peroxisome proliferated activated receptor alpha and gamma agonists that are known to increase LPL gene expression increase serum LPL concentration.\textsuperscript{26,38} Second, insulin concentrations which control LPL gene expression levels also affect serum LPL concentration.\textsuperscript{39,40} A recent study furthermore shows that variation at the LPL gene locus also affects serum LPL concentration.\textsuperscript{21} Specifically, it was reported that carriers of a common LPL gene variant (LPLS447X) have increased levels of serum LPL while others have shown that this mutation protects against CAD. Thus, serum LPL concentration may be a marker for the amount of systemically available (catalytically) active LPL, when taken into notice that LPL is the sole lipolytic enzyme that is responsible for the breakdown of plasma triglycerides. Serum LPL mass may, however, also have a direct atheroprotective role in mediating the clearance of atherogenic lipoproteins remnants.\textsuperscript{5} These assumptions need confirmation in mechanistic studies into triglyceride catabolism.

\textbf{Considerations}

Several aspects of the current study warrant attention. First, CAD events were ascertained through death certification and hospital admission data, which is likely to lead both to underascertainment and to misclassification of cases. However, previous validation studies in our cohort indicate high specificity of such case ascertainment.\textsuperscript{33} Second, the recruitment rate for the EPIC Norfolk study was relatively low, but the study population is representative of the general British population for all classical risk factors except for a low smoking rate. Third, serum levels of LPL and other lipid-related variables were determined in a single non-fasting sample that was obtained at a non-uniform time of the day. Diurnal variation, variation over time and differences in the time span since the last meal could have affected these variables. The latter is especially
true for TG levels. We wish to underline, however, that in the Western World, people live under constant postprandial conditions. Therefore, studies into the associations between lipids, lipoproteins and CAD risk, are in our opinion best performed under non-fasting conditions. Random measurement error in both case ascertainment and time variations would lead to an underestimation of any relationships between risk factors and CAD risk. The extent of measurement error, however, is unlikely to differ from those for other risk factors or from other prospective studies.

Conclusions
We here show that apparently healthy men and women with reduced levels of serum LPL have an increased risk for future CAD. The data suggest that high LPL concentrations may be athero-protective through associations with decreased TG levels and increased HDL-C levels.

Acknowledgements
We thank the participants, general practitioners and staff in EPIC-Norfolk. EPIC-Norfolk is supported by program grants from the Medical Research Council UK and Cancer Research UK and with additional support from the European Union, Stroke Association, British Heart Foundation, Department of Health, Food Standards Agency and the Wellcome Trust. Part of the lipid measurements described in this article was funded by an educational grant from the Future Forum. The Hague, The Netherlands.

Conflict of Interest Disclosures
No conflicts to disclose
Reference List


Chapter 1


PART III

LIPOPROTEIN LIPASE DEFICIENCY; FROM DIAGNOSIS TO MANAGEMENT
LIPOPROTEIN LIPASE GENE ANALYSES IN ONE TURKISH AND THREE DIFFERENT CHINESE FAMILIES WITH SEVERE HYPERTRIGLYCERIDEMIA; ONE NOVEL AND SEVERAL ESTABLISHED MUTATIONS

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Abstract

Lipoprotein lipase (LPL, triacylglycerol acylhydrolase; EC 3.1.1.3) deficiency (McKusick 238600) is an autosomal recessive inherited condition caused by mutations in the LPL gene, either in a homozygous or a compound heterozygous state, leading to loss of lipolytic activity and resulting in severe hypertriglyceridemia and subsequent risk for developing pancreatitis. Numerous LPL gene mutations leading to loss of catalytic function have been described. In this present study, we describe full clinical, biochemical and molecular analyses of severe hypertriglyceridemic individuals in one Turkish and three Chinese families. We established one novel mutation (delCT\textsuperscript{1312-1313}), a new combination of mutations (S\textsuperscript{193}R and I\textsuperscript{194}T) and four previously reported mutations (L\textsuperscript{252}R, L\textsuperscript{252}V, S\textsuperscript{193}R and I\textsuperscript{194}T) of the LPL gene and report phenotypes for these and four previously described mutations. Finally, we show that two patients homozygous for the LPL delCT\textsuperscript{1312-1313} gene mutations are characterized by absence of LPL activity that coincided with absence of LPL protein.
Introduction

Lipoprotein lipase (LPL, triacylglycerol acylhydrolase; EC 3.1.1.3) deficiency (McKusick 238600) is an autosomal recessive inherited condition caused by mutations in the LPL gene, either in a homozygous or a compound heterozygous state, leading to loss of lipolytic activity and resulting in severe hypertriglyceridemia and subsequent risk for developing pancreatitis. At present, over 221 LPL gene mutations leading to loss of catalytic function have been described not only in western populations and western hypertriglyceridemic subjects, but also in the Chinese subjects with coronary artery disease (CAD), in Chinese hypertriglyceridemic subjects, in Chinese diabetics, in Chinese women with pregnancy-induced chylomicronemia as well as in Chinese newborns.

LPL deficient patients have genuinely lactescent plasma due to extreme high concentrations of circulating triglyceride-rich lipoproteins (TRL) for which currently no effective treatment exists. This extremely rare genetic disorder typically manifests itself in early childhood with the hyperchylomicronemia syndrome: a presentation of symptoms including severe abdominal pain, repetitive colicky pain, ‘failure-to-thrive’ and even acute pancreatitis. On physical examination eruptive xanthomas, lipaemia retinalis and hepato- and/or splenomegaly can be found. The increased concentration of triglycerides (TG) is thought to be mainly responsible for the increased risk of pancreatitis, which can occur at TG concentrations >10 mmol/L.

In this present study, we describe the clinical, biochemical and molecular analyses of one Turkish and three Chinese families with LPL related hypertriglyceridemia.

Materials and methods

Patient selection
Members of four families of Chinese and Turkish descent were screened for LPL mutations because of the presence of the clinical hyperchylomiconeremia syndrome in their family. All Chinese subjects were diagnosed at a Lipid Clinic in Kuala Lumpur, Malaysia, whereas the Turkish family was living in The Netherlands and was referred to our hospital for further diagnosis and treatment. Not only was DNA analysis performed in all probands, but we also screened for possible LPL gene mutations in their first degree family members. The study conforms to the principles outlined in the Declaration of Helsinki. All participants gave written informed consent.
Biochemical measurements
All blood samples were taken in fasting conditions (>10hrs). Blood for lipid analysis was drawn in EDTA-coated tubes. Plasma was isolated by centrifugation and aliquoted for storage at -80°C. Total cholesterol (TC) was measured by standard enzymatic methods (CHOD-PAP, Roche Diagnostics, GmbH, Mannheim, Germany). HDL-C was measured in the supernatant fraction after precipitation of apoB-containing lipoproteins with dextran sulphate and magnesium chloride. TG was measured using a commercially available kit (Triglyceride GPO-Trinder, Sigma Diagnostics Inc., St. Louis, MO, USA). LDL-C was calculated using the Friedewald formula.19

Measurement of LPL concentration and activity
Blood for post-heparin LPL concentration and activity measurements was collected in heparin-containing tubes 15 minutes after an intravenous injection of heparin (50 IU/kg body weight). LPL activity was analyzed as previously published.19 LPL concentrations were measured using a commercially available kit (Markit-M LPL, Dainippon Pharmaceutical Co, Osaka, Japan) as previously published.20-24

LPL gene sequencing
Genomic DNA was prepared from 10 mL whole blood on an AutopureLS apparatus according to a protocol provided by the manufacturer. (Gentra Systems, Minneapolis, USA). For PCR and sequence reactions, 10 primer pairs were designed to cover all coding regions and intronic boundaries (table 1). For all PCR amplifications 50 ng of DNA was added to a mixture containing 2.5 µl of 10x Qiagen Taq buffer (Qiagen GmbH, Hilden, Germany), 1 µl of each dNTP (1.25 mM), 1 µl of each primer (10 µM) and 1.0 U Qiagen Taq polymerase (Qiagen GmbH, Hilden, Germany), in a total volume of 25 µl. PCR reactions were performed in a PCR apparatus (Biocycler, T3 Biometra, Germany) using a initial denaturation (5 minutes, 95°C) followed by 30 cycles of 95°C (1 minute), 64°C (exon 1) or 58°C (exon 2-10) (1 minute) and 72°C (1 minute), completed with an additional extension at 72°C for 10 minutes. Sequence reactions were performed using fluorescently labeled dideoxy chain terminations with the Big Dye Terminator ABI Prism Kit (Applied Biosystems, Foster City, Ca., USA) and run on a Applied Biosystems Model 3730 automated DNA sequencer Applied Biosystems, Foster City, Ca., USA). Sequences were analyzed with the Sequencer package (GeneCodes Co, Ann Arbor, MI, USA). ApoE genotyping was performed as described by Reymer et al.25
Table 1 PCR and Sequence Primers

<table>
<thead>
<tr>
<th>Exon</th>
<th>Forward (5' - 3')</th>
<th>Reverse (5' - 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ACGCCCCGGTCTGCAAGTGGAAGGG</td>
<td>GTAGAGTGGAAACCCCTTAAGCTAAGCG</td>
</tr>
<tr>
<td>2</td>
<td>AACCCTCCAGTTACCTCATATCC</td>
<td>CACCACCCCAATCCACCTTTCCC</td>
</tr>
<tr>
<td>3</td>
<td>GAACAGCCGGTTTTCTGGCTCAGTC</td>
<td>GCTAGGTTGGTAATTAAAGAAGCTTGTG</td>
</tr>
<tr>
<td>4</td>
<td>GAATTAGTTTTACATATTCTATATTTTGG</td>
<td>CTCTGAGAATGAAGTCTTTCACCC</td>
</tr>
<tr>
<td>5</td>
<td>GCAGTGAGCATGCGAATGTCATACGC</td>
<td>GGACATTGGTCATAAGGGTTAAGG</td>
</tr>
<tr>
<td>6</td>
<td>CCACATCTCACTATTTTAGACTGCC</td>
<td>GCAGTGAGCATGATGAAATAGGACTCC</td>
</tr>
<tr>
<td>7</td>
<td>GAGTTCCATGTGTGCACTTTCGAG</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>AAAGTGAGGGCGAGAGAGCTGATC</td>
<td>CATCAGGTTGGGGCTAAAGAAGCTGAG</td>
</tr>
<tr>
<td>9</td>
<td>GATTCGTGATGTCGGCTAGTGAAGCAG</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>CACATCTACCCCTGGTTATTTTCAC</td>
<td>GCTAAAGTTGAGAAGGCTCAGTCC</td>
</tr>
</tbody>
</table>

Patients and results

General characteristics of all participants (i.e. eight genetic LPL deficient patients and available first-degree family members) are shown in table 2. All probands were screened for LPL gene mutations and each available family member was examined for the presence of LPL mutations observed in their family proband. Only in the Turkish family, post-heparin LPL concentration and activity were measured.

Family 1

The proband of this Chinese family developed jaundice for which she was hospitalized two weeks after birth. Her blood was found to be chalky and her plasma was severely lipemic, exhibiting floating chylomicrons. After one week of extensive fasting, her TG level was still severely elevated (TG: 13 mmol/L) and on physical examination, no stigmata for the hyperchylomicronemia syndrome were found. She was given a medium chain triglyceride (MCT) based diet (Enfalac Pregestimil, Mead Johnson Nutritional, Ottawa, Ontario) and her jaundice gradually improved. Growth and development were considered normal, but her plasma continually revealed increased TG levels. When her sister was born, she was screened for hypertriglyceridemia too and subsequently exhibited a moderate increased TG level (TG: 2.0 mmol/L) and thus also received
an MCT substituted low-fat diet. Her growth and development were considered normal and her plasma revealed clearly lower TG levels as compared to her older sister but still evidently elevated. Notably, the TG levels of the sister of the proband progressively worsened over time even on a similar low-fat diet as the proband (TG max: 4.8 mmol/L). Both parents were free of any clinical symptoms and information concerning consanguinity is currently missing. DNA analysis revealed that both sisters were compound heterozygous for two mutations in the LPL gene (L252V and L252R in exon 6). Both parents were found to be heterozygous carriers. Unfortunately, information on post-heparin LPL concentration and activity were not available in this family.

Family 2
Two month after birth, the proband of this Chinese family was accidentally found to have severely elevated TG levels. Secondary causes for hypertriglyceridemia were excluded and additional testing of apolipoprotein E revealed an ApoE2E3 genotype. Her plasma showed floating CM and subsequently she was thought to have a type I hyperlipidemia. When plasma apoCII was found to be present, LPL deficiency was diagnosed and a low-fat diet was described, but strongly elevated TG levels persevered. Her brother also exhibited a moderately increased triglyceride level without any clinical symptoms (TC: 4.2 and TG: 2.9 mmol/L). Her father’s first lipid profile, tested several years before the current investigation, showed a clear dyslipidemia with only a moderate hypertriglyceridemia (TC: 8.0 and TG: 2.1 mmol/L). After recent retesting, a substantial change in lipid profile had occurred and severe hypertriglyceridemia was recognized (TC: 8.8 and TG: 22.3 mmol/L) for which antilipemic treatment was immediately started. Even though clear hypertriglyceridemia was established in the father, he has always been free of symptoms. Her mother also showed a moderately increased TG level (TC: 3.6 and TG: 3.8 mmol/L). Both parents were free of any clinical symptoms and information on consanguinity is not available. DNA analysis revealed that proband was compound heterozygous carrier of two LPL gene mutations (L252V and L252R in exon 6). Both parents were found to be heterozygous carriers of single mutations and no LPL gene mutations could be found in the brother. Unfortunately, additional information about the post-heparin LPL concentration and activity were not available in this family.
Table 2 General Characteristics of LPL-Deficient Patients and First Degree Family Members at Time of Testing.

<table>
<thead>
<tr>
<th>Family</th>
<th>Age (yrs)</th>
<th>BMI</th>
<th>TC (mmol/L)</th>
<th>HDL-C (mmol/L)</th>
<th>TG (mmol/L)</th>
<th>Clinical symptoms and signs</th>
<th>Allele 1</th>
<th>Allele 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>proband</td>
<td>15</td>
<td>12.1</td>
<td>4.9</td>
<td>0.4</td>
<td>11.3</td>
<td>Neonatal jaundice</td>
<td>L252V</td>
<td>L252R</td>
</tr>
<tr>
<td>sister</td>
<td>12</td>
<td>12.8</td>
<td>4.3</td>
<td>0.7</td>
<td>48</td>
<td>None</td>
<td>L252V</td>
<td>L252R</td>
</tr>
<tr>
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<td>48</td>
<td>n.a.</td>
<td>n.a</td>
<td>n.a</td>
<td>2.9</td>
<td>None</td>
<td>L252V</td>
<td>WT</td>
</tr>
<tr>
<td>mother</td>
<td>43</td>
<td>n.a.</td>
<td>n.a</td>
<td>n.a</td>
<td>n.a</td>
<td>None</td>
<td>L252R</td>
<td>WT</td>
</tr>
<tr>
<td>proband</td>
<td>10</td>
<td>n.a.</td>
<td>6.2</td>
<td>0.2</td>
<td>19.5</td>
<td>None</td>
<td>L252V</td>
<td>L252R</td>
</tr>
<tr>
<td>brother</td>
<td>12</td>
<td>n.a.</td>
<td>4.2</td>
<td>0.2</td>
<td>2.9</td>
<td>None</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>father</td>
<td>67</td>
<td>n.a.</td>
<td>8.8</td>
<td>0.88</td>
<td>22.3</td>
<td>None</td>
<td>L252R</td>
<td>WT</td>
</tr>
<tr>
<td>mother</td>
<td>49</td>
<td>n.a.</td>
<td>3.8</td>
<td>0.36</td>
<td>3.6</td>
<td>None</td>
<td>L252V</td>
<td>WT</td>
</tr>
<tr>
<td>proband</td>
<td>18</td>
<td>n.a.</td>
<td>4</td>
<td>0.37</td>
<td>11.8</td>
<td>recurrent episodes of abdominal pain and vomiting, acute pancreatitis at age of 5, splenomegaly</td>
<td>S193R</td>
<td>I194T</td>
</tr>
<tr>
<td>sister</td>
<td>20</td>
<td>n.a.</td>
<td>4.7</td>
<td>2.27</td>
<td>0.6</td>
<td>overweight</td>
<td>I194T</td>
<td>WT</td>
</tr>
<tr>
<td>sister</td>
<td>24</td>
<td>n.a.</td>
<td>4.3</td>
<td>0.39</td>
<td>14.5</td>
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<td>S193R</td>
<td>I194T</td>
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<tr>
<td>sister</td>
<td>26</td>
<td>n.a.</td>
<td>n.a</td>
<td>n.a</td>
<td>n.a</td>
<td>recurrent episodes of abdominal pain, splenomegaly</td>
<td>S193R</td>
<td>I194T</td>
</tr>
<tr>
<td>father</td>
<td>52</td>
<td>n.a.</td>
<td>9.3</td>
<td>2.03</td>
<td>2.6</td>
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<td>S193R</td>
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<td>22</td>
<td>5.3</td>
<td>n.a</td>
<td>20.14</td>
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Age (yrs), BMI: Body Mass Index (kg/m^2), TC: Total Cholesterol (in mmol/L), HDL-C: High Density Lipoprotein Cholesterol (in mmol/L), TG: Triglycerides (in mmol/L), Allele: LPL gene mutation, n.a.: not available.
Family 3
The proband in this Chinese family had been admitted to the hospital with symptoms of acute pancreatitis at the age of 14. Hypertriglyceridemia and splenomegaly were found. Her medical history revealed that she suffered from recurrent episodes of abdominal pain and vomiting starting one year after birth. At the age of five, she was admitted to the hospital with severe abdominal pain and vomiting and was readmitted one year later with identical symptoms. Since then, she was treated with a low fat diet, but still her TG levels remain seriously elevated. The medical history of her eight years older sister also revealed episodes of recurrent abdominal pain starting from 2.5 years after birth. At that time, she also revealed hypertriglyceridemia and splenomegaly. Since then, she was prescribed a low-fat diet. Unfortunately, her initial and current TG values are unknown. Her six years older sister was also screened for hypertriglyceridemia, even though she never showed any clinical signs of abdominal pain. She exhibited identically elevated TG levels as the proband and she was then also treated with a low-fat diet, which unfortunately was unable to successfully lower her TG levels. Her youngest sister showed initial increased lipid levels (values unknown) on screening. This elevated TG level coincided with clear overweight (BMI unknown) that interestingly normalized upon weight reduction. Her father has been symptom-free throughout his entire life. On presentation, he exhibited a clearly abnormal lipid profile with increased LDL-C levels (6.1 mmol/L) as well as increased TG levels (2.6 mmol/L) possibly as a result of enhanced VLDL production. He was successfully treated with antilipemic drugs since. Her mother exhibited a normal lipid profile. Her three hypertriglyceridemic sisters were all compound heterozygous carriers of two LPL gene mutations: S193R and I194T in exon 5. Her youngest sister with overweight related TG levels was heterozygous carrier of the I194T mutation. Both parents were heterozygous carriers of a single mutation in the LPL gene; the father presented as heterozygous S193R carrier and the mother presented as heterozygous I194T carriers of the LPL gene. The combination of LPL gene mutation and dyslipidemia from the father has never been described before. However, the S193R variant of the LPL gene has previously been associated with very low but consistently detectable LPL activity. Unfortunately, information about the post-heparin LPL concentration and activity were not available in this family.

Family 4
Two months after birth, the proband of this Turkish family was screened for congenital hypothyroidism (CHT) which was repeatedly tested positively. When he was hospitalized for further analysis, no abnormalities on physical examination were observed, and hepatomegaly was not
reported. His medical history revealed several episodes of abdominal pain and unexplained diarrhea. His lipemic plasma showed extremely elevated lipid levels (TC: 28 and TG: 280 mmol/L). Immediately, a low-fat diet was started consisting of 20-energy percentage (en%) from fat with MCT as a source of alternative energy. Subsequently, his lipid profile improved dramatically but remained abnormal (TC: 9.7 and TG: 8.3 mmol/L). After this initial TG improvement, the CHT test was repeated for the third time and strikingly found to be normal. At the age of five, he was admitted to the hospital with acute pancreatitis, which was later ascribed to hypertriglyceridemia due to poor low-fat diet adherence. Currently, his growth is considered normal and he experiences severe abdominal pain occasionally after periods of poor diet compliance. When his sister was born, she was also screened for hypertriglyceridemia and was shown to suffer from the same condition as her brother. She was treated with the same MCT-based low-fat diet since. Up until now, she never experienced any clinical symptoms or complaints associated with her increased TG levels. Her lipid profile currently shows an impressive hypertriglyceridemia (TC: 6.90 and TG: 31.20 mmol/L) comparable to her brother's current profile (TC: 5.27 and TG: 20.11 mmol/L). Both parents revealed moderate hypertriglyceridemia. Notably, the mother revealed lipemic plasma when was she was pregnant (values unknown). Both parents were free of any clinical symptoms and information on consanguinity was not available. Direct sequencing of the LPL gene of the proband revealed a dinucleotide deletion on both alleles of the LPL gene as illustrated in figure 1.

Figure 1: Electropherograms of part of exons 7 of the LPL gene

The upper panel shows the control LPL gene. The lower panel shows the homozygous mutation deletion CT\(^{1312-1313}\) in the proband in the sense direction. The mutation covers the last two bases of exon 7, interrupting codon Leu 353 so that a premature translation termination codon will be formed at codon 354 (TGA 354). The mutation sites are indicated by arrows.
This deletion was confirmed in his sisters LPL gene, together with heterozygous deletions in both parents. This del CT\(^{1312-1313}\) covers the last two bases of exon 7, interrupting codon Leu353 so that a premature translation termination codon will be formed at codon 354 (TGA 354). In line with these findings, we found a complete absence of LPL protein in post-heparin plasma together with a complete absence of post-heparin LPL activity in both affected children. (Controls: post-heparin LPL activity 192 ng/mL and post-heparin LPL concentration 477 mU/mL, del CT\(^{1312-1313}\) homozygotes: post-heparin LPL activity 0 ng/mL and post-heparin LPL concentration: 0 mU/mL). Both parents were found to be heterozygote carriers of the LPL gene mutation. Unfortunately, data on LPL concentration and activity in both parents was not available.

Discussion and conclusion

Our present study describes a classical clinical phenotype of LPL deficiency in one Turkish and in three Chinese families. Clinical LPL deficiency was diagnosed in each family at relatively young age. Also, severe hypertriglyceridemia was shown in one parent carrying a heterozygous LPL mutation. Our study illustrates the inability to effectively lower plasma TG levels of the genetic LPL deficient patients in all but one of the LPL deficient patients identified. Of note, without such effective treatment, all LPL-deficient patients remain at risk for potential life-threatening pancreatitis. Next to four well-known LPL deficiency causing LPL mutations, we characterized two hypertriglyceridemic patients suffering from the hyperchylomicronemia syndrome due to homozygosity of a novel LPL mutation, delCT\(^{1312-1313}\). Homozygosity for this LPL mutation caused complete absence of LPL activity, due to complete absence of LPL protein. Moreover, we identified a novel combination of LPL gene mutations (S193R and I194T in exon 5) in three hypertriglyceridemic patients. The current treatment of LPL deficiency is a harsh dietary regimen since lipid lowering medication has been shown to be unsuccessful\(^{12,27}\). At the present time, additional triglyceride lowering therapy is not available.

At present, over 221 LPL gene mutations leading to loss of catalytic function have been described\(^1\) not only in western populations and western hypertriglyceridemic subjects\(^2\), but also in Chinese subjects with coronary artery disease (CAD)\(^{13,14}\), in Chinese hypertriglyceridemic subjects\(^{15}\), in Chinese diabetics\(^{16}\), in Chinese women with pregnancy-induced chylomicronemia\(^10\) as well as in Chinese newborns\(^{16,17}\).
The LPL Leu252Val (L252V) mutation in exon 6, a single nucleotide C1009→G substitution in codon 252 in exon 6 of the LPL gene, results in a substitution of a leucine by a valine residue in the mature protein. This LPL mutation has previously been shown to cause hypertriglyceridemia and recurrent pancreatitis. In that study, homozygous L252V carriers exhibited hypertriglyceridemia with nearly undetectable LPL activity and heterozygous carriers also exhibited low post-heparin LPL activity. In vitro experiments confirmed the L252V mutation resulted in loss of LPL activity. The LPL Leu252Arg (L252R) mutation in exon 6, a single nucleotide T1010→G substitution has also previously been described and is associated with reduced LPL mass (32% of the normal) and absent LPL activity. In addition, the occurrence of LPL deficiency due to compound heterozygosity for the L252V and L252R mutations in exon 6 of the LPL gene has previously been described causing severe hypertriglyceridemia and recurrent pancreatitis, and was associated with low post-heparin LPL activity and concentration; this study showed that in vitro L252R expression abolished both catalytic function and secretion of LPL, while L252V also completely abolished the catalytic function but only fairly halved the LPL secretion. Clearly, compound heterozygous carriers exhibit detectable levels of LPL protein, but completely lack post-heparin LPL activity. Interestingly, thus far, mutations in codon 252 of the LPL gene have only been described in Chinese families. Families 1 and 2 share the same combination of LPL gene mutations, but a familial relation could not be established. Interestingly, the father of one of the probands presented with severe hypertriglyceridemia whereas LPL gene analysis proved he was only a heterozygous carrier of the variant. This combination of severe hypertriglyceridemia and the heterozygous L252R presence has been reported previously; heterozygous carrier of the L252R variant presented with a severely reduced post-heparin LPL activity that coincided with a similar hypertriglyceridemic profile (TC 9.8 and TG 24.6 mmol/L) as the father of one of the probands in our current study. That study elegantly showed that the L252R variant is associated with a clear reduction in post-heparin LPL activity in heterozygous carriers. Since our LPL gene analysis method basically sequences the entire functional LPL gene (exons, part of the introns, splice sites and promoter) chances are small that an additional LPL gene variant would be responsible for the current hypertriglyceridemia. However, we cannot exclude that an additional intron mutations of the LPL gene could result in this current phenotype.

The LPL Ser193Arg (S193R) mutation in exon 5 has previously been described to be associated with hyperchylomicronemia due to a greatly reduced LPL activity (< 10% of normal), which was confirmed in vitro experimenting (2% of normal). Also, the LPL Ile194Thr (I194T) mutation in
exon 5 has been reported previously\textsuperscript{29,30}. In vitro, I194T exhibited absent LPL activity with normal LPL concentration\textsuperscript{29}. Comparison of the I194T variant to control LPL in vitro showed no differences in dimer conformation, heparin binding, esterase activity, surface activation, or lipid binding, which was confirmed by the observation of absent LPL activity and normal LPL concentration in post-heparin plasma\textsuperscript{30}. Notably, compound heterozygosity for both LPL mutations (S193R and I194T), however, has never been published before, but clearly carriers exhibit detectable levels of post-heparin LPL protein with extreme low to absent post-heparin LPL activity.

For the first time, homozygosity for a novel small deletion in the LPL gene, delCT\textsuperscript{1312-1313} is shown to create a premature translation termination codon. This resulted in absence of post-heparin LPL protein and a concomitantly complete absence of post-heparin LPL activity thus causing severe hypertriglyceridemia. Possibly, this mutation causes a truncated LPL protein, but we cannot exclude the very real possibility that this mutation causes nonsense mediated decay of mRNA resulting in absence of LPL protein. Therefore, further investigation is warranted. Of note, the fact that no mass could be established in post-heparin plasma does not rule out that some immunoreactive LPL mass is actually circulating. With our current analysis techniques however, we were not able to detect any LPL protein. Interestingly, both parents were heterozygous carriers and both revealed moderate hypertriglyceridemia whereas pregnancy enhanced the condition, a phenomenon previously shown in heterozygous carriers of LPL mutations\textsuperscript{2,10}.

Finally, LPL deficient patients are at clear risk of developing potential life-threatening pancreatitis due to severely elevated TG levels. Therefore, the primary target for treatment of LPL deficiency is reducing this risk by lowering TG levels below 10 mmol/L\textsuperscript{2}. The sole effective triglyceride-lowering option currently is harsh dietary modifications since medical interventions have shown to be unsuccessful\textsuperscript{13,27}. However, treatment of LPL-deficient patients is difficult and maintaining these strict dietary regulations can sometimes be unfeasible, especially at the long term. This leads to increased TG concentrations and sometimes recurrent pancreatitis and other manifestations of LPL deficiency. Consequently, a novel triglyceride-lowering strategy will be investigated in the near future that might help lowering TG levels in LPL deficient patients and thereby reduce the risk for (recurrent) pancreatitis\textsuperscript{21,31}. 


Reference List


CHAPTER 10

GENE THERAPY FOR GENETIC LIPOPROTEIN LIPASE DEFICIENCY: FROM PROMISE TO PRACTICE

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Chapter 10
Abstract

Lipoprotein lipase (LPL) deficiency is a rare, hereditary disorder of lipoprotein metabolism characterized by severely increased triglyceride levels, and associated with an increased risk for pancreatitis. Since no adequate treatment modality is available for this disorder, we set out to develop an LPL gene therapy protocol. This paper focuses on the clinical presentation of LPL deficiency, summarizes the preclinical investigations in animal models and describes the rationale to evaluate gene therapy for this monogenetic disorder of lipid metabolism in humans.
Lipoprotein Lipase

Lipoprotein lipase (LPL) is one of the key enzymes in the metabolism of triglyceride-rich lipoproteins (TRLs) and is produced in fat tissue, skeletal muscle, and heart muscle. Activated by its cofactor apolipoprotein (apo) CII, LPL mediates the hydrolysis of triglycerides (TG) in chylomicrons (CM) and very-low density lipoproteins (VLDL) at the luminal side of the endothelium. The generated free fatty acids (FFA) are subsequently used for energy production in muscle tissue or stored as fat in adipose tissue. LPL also contributes to the high-density lipoprotein (HDL) pool by shedding of phospholipids and apolipoproteins during the hydrolysis of these lipoproteins. Besides the enzymatic activity, LPL also enhances hepatic clearance of triglyceride-rich lipoproteins (TRL) by mediating receptor-mediated uptake of these atherogenic lipoprotein particles (ligand or bridging function). Through these actions LPL exerts anti-atherogenic effects. Of note, subendothelially located LPL has been described to have pro-atherogenic effects by increasing oxidative susceptibility of LDL facilitating the uptake of TRLs by macrophages. The latter promotes foam cell formation, i.e. the hallmark of atherogenesis. In view of these heterogeneous effects, the exact role of LPL in atherogenesis is still a matter of debate. The delicate balance between pro- en anti-atherogenic properties of LPL has been shown to depend in part on the exact location of this enzyme.

LPL mutations

More than 100 mutations in the LPL gene have been described to date. While some mutations result in total loss of function, others only exert a moderate effect on LPL activity such as the D9N, N291S, and S447X mutations. The latter are frequently found in the general population and have provided valuable insight into the relationship between LPL and the progression of atherosclerosis. Carriers of LPLN291S and PLPD9N, with a combined frequency of 5% in the general population, are characterised by low HDL cholesterol and increased TG with a concomitant increased risk of cardiovascular disease. Conversely, carriers of LPLS447X with a frequency of 18-22% in the general population are characterised by increased levels of HDL cholesterol and lower TG levels. In line, this mutation has been reported to have a protective effect against cardiovascular disease (CVD).
Genetic LPL deficiency

Clinical presentation and diagnosis of genetic LPL deficiency
LPL deficiency is an autosomal recessive hereditary disease caused by mutations in the LPL gene. Homozygosity or compound heterozygosity for mutations in the LPL gene, resulting in loss of catalytically active LPL, is the basis of genetic LPL deficiency. The resulting clinical chylomicro-nemia syndrome (see further in text), was first described by Bürger and Grütz in 1932 and 56 years later, in 1989, the first LPL mutation responsible for this phenotype was revealed.16

LPL deficiency typically manifests itself in early childhood with a variety of symptoms including severe abdominal pain, repetitive colicky pain, hepatosplenomegaly, failure-to-thrive and acute pancreatitis.17 Increased irritability, diarrhoea and intestinal bleedings can occur even shortly after birth. Although the clinical presentation is non-specific, especially at a younger age19, the plasma of the patients is always milky white or lipemic (figure 1a), even under fasting conditions. On physical examination, eruptive xanthomas (figure 1b and 1c) are frequently present. These xanthomas consist of small erythematous-based yellow papules ranging in size from one to several millimetres in diameter. Eruptive xanthomas frequently exhibit the Koebner phenomenon, also called the isomorphic response, which refers to the appearance of lesions at a site of injury or pressure. Therefore, eruptive xanthomas are usually formed on the buttocks, elbows, back, and knees, but they can also occur on any cutaneous surface including the oral mucosa. These lesions generally recede with reduction of the TG levels. In addition to these skin lesions, lipaemia retinalis and hepatosplenomegaly can be observed.

Figure 1a: Lipemic Plasma

Figure 1 b + c: Eruptive xanthomas

Lipemic plasma and eruptive xanthomas found on the upper legs and knees of a patient with severe hypertriglyceridemia (fasting TG=46 mmol/L)
In clinical practice, this combination of symptoms is often not recognized to be directly related to the hyperchylomicronemia syndrome\(^2\) and the diagnosis often becomes clear only after the first occurrence of pancreatitis. Lipid analysis reveals 10-100 times increased plasma TG while HDL cholesterol levels are markedly decreased. In addition, LPL deficiency is characterised by reduced LDL cholesterol levels. In line, levels of apoB100, the main structural apolipoprotein of LDL and VLDL are reduced. The increased TG concentration increases the risk of pancreatitis\(^21\), which can occur from TG concentrations of 10 mmol/L onwards\(^22\). This clinical complication, often recurrent in LPL deficient patients, can be lethal. It is noteworthy that in these patients, pancreatitis cannot be excluded on the basis of normal plasma amylase concentrations, since high TG concentrations can interfere with the analytical method resulting in false negative results\(^2\)\(^2\)\(^3\). Assessment of urine amylase excretion is more reliable as a diagnostic test for pancreatitis in a hypertriglyceridemic patient\(^2\)\(^6\)\(^7\). Other laboratory investigations can also be disturbed as a result of increased TG levels, such as sodium (artificially low)\(^2\)\(^8\), haemoglobin (artificially increased)\(^2\)\(^9\), HbA1c (artificially low)\(^10\) en bilirubin (artificially increased)\(^3\)\(^1\)\(^\)\(^1\)\(^2\).

Prevalence of genetic LPL deficiency

Exact data on the prevalence of LPL deficiency are not available. The reported prevalences for genetic LPL deficiency vary between 1:1,000,000\(^3\)\(^3\) and 1:5,000 in French Quebec (caused by a so-called “founder effect”)\(^2\)\(^1\)\(^3\)\(^4\). Based on extensive efforts to track down all LPL deficient patients in the Netherlands, we estimate a prevalence of approximately 1:500,000.

LPL deficiency and clinical complications

The main clinical risk of LPL deficient patients is the development of pancreatitis\(^2\)\(^2\). The exact aetiology of pancreatitis in hypertriglyceridemia is unclear but it is believed that the high concentrations of CM in the pancreatic microcirculation result in increased ‘free radical’ activity, which can result in episodes of pancreatic ischemia. Inflammation of the pancreas is supposed to be the result of local fatty acids generation due to small amounts of free lipases in the microcirculation of the pancreas. A disrupted microcirculation, caused by hyperchylomicronemia, is suggested to damage pancreatic cells with ensuing increased release of lipolytic enzymes. The latter causes hydrolysis of abundantly present CM contributing to a strong increase in local FFA, followed by local pancreatic inflammation. This cascade of events is thought to eventually cause pancreatitis\(^2\)\(^1\).
Elevated TG levels are a strong independent risk factor for CVD. It is unclear, however, whether LPL deficiency is associated with an increased CVD risk. Whereas two publications have reported premature atherosclerosis in LPL deficient patients, LPL deficiency has also been described to be not associated with dramatic increase in CVD. The reported lack of atherosclerosis has even been described to relate to the low concentrations of LDL cholesterol in these patients (a direct consequence of a disturbed catabolism of the precursor of LDL, i.e. VLDL). The latter phenomenon was nicely illustrated by a homozygous LPL deficient patient suffering from familial hypercholesterolemia (FH) with clearly lower LDL cholesterol levels compared to FH siblings and the absence of signs of CVD during follow up. Another potential mechanism antagonizing atherogenesis is the inability of CM to penetrate in the vascular wall. In line, the accumulation of TRLs in macrophages in the vascular wall has been shown to be reduced in patients with LPL deficiency.

**Therapeutic options in LPL deficiency**
The primary objective of treating LPL deficient patients is reducing the risk for pancreatitis. To reduce this risk, TG lowering below 10 mmol/L is desired.

**Diet** The intake of dietary fats has to be lowered 20-25% of the total daily caloric intake, i.e. 40-50 grams dietary fat per day. If this has insufficient effect on TG, part of the fat can be replaced by Medium Chain Triglycerides. These TG are transported to the organs for hydrolysis without the need for CM packaging, thus excluding the need for LPL. In our western society, characterized by dietary fat intakes of approximately 120 grams per day, maintaining these strict dietary regimes has been proven to be most difficult, resulting in poor adherence. Consequently, the prevention of pancreatitis is often unsuccessful in LPL deficient patients and additional therapeutic modalities are mandatory. Of note, it should be emphasized, that consequent and strict adherence to a stringent low-fat diet is likely to be associated with effective lowering of the hypertriglyceridemia with ensuing decrease in risk for pancreatitis.

**Fibrates** Fibric acids normally affect TG metabolism by reducing the hepatic production of VLDL and enhancing VLDL clearance from the circulation. Fibric acids are agonists of a family of transcription factors, i.e. peroxisome proliferators activated receptors (PPARs). These factors have been shown to reduce the production of hepatic apoCIII (an inhibitor of LPL activity) and thereby increase LPL mediated lipolysis. Also, via direct stimulation of the LPL gene promoter
LPL synthesis is upregulated. However, in LPL deficiency, upregulation of defective LPL will not render the desired effects. In addition, a decreased VLDL synthesis may help managing TG levels; the primary problem of these patients is the lack of lipolytic activity. As a consequence, plasma TG levels in LPL deficient patients are generally unaffected upon fibrate therapy.\(^{33,44}\)

**Statins** Statins inhibit HMG-CoA reductase, leading to a reduced hepatic cholesterol production and upregulation of LDL receptors. This results in enhanced hepatic uptake of LDL and TRL (VLDL en IDL)\(^{45,46}\), reducing the concentrations of plasma LDL cholesterol and of plasma TG. LPL deficient patients, however, are characterized by reduced concentrations LDL cholesterol via decreased turnover of VLDL to LDL\(^{47}\) as well as increased LDL catabolism\(^{48}\). As a consequence, neither TG nor LDL levels are lowered by statin therapy\(^{33,44}\).

**Nicotinic acid derivates** Nicotinic acid derivates (vitamin B3) normally inhibit the hepatic synthesis and esterification of FFA, resulting in a reduced hepatic VLDL production\(^{49,50}\). Nicotinic acid derivates also induce accelerated intracellular degradation of apoB\(^{51}\) whereas a reduced hepatic clearance of apoAI results in an increase of HDL cholesterol\(^{52}\). In LPL deficient patients, the response to nicotinic acid derivates has been shown to be marginal\(^{33,44}\).

**Omega-3 Fatty Acids** Daily use of high dose of omega-3 fatty acids (4 grams/day) leads to enhanced clearance of plasma CM\(^{53}\) in combination with an reduced production of hepatic VLDL\(^{54}\) without affecting LPL activity\(^{55}\). In primary hypertriglyceridemia by causes other than LPL deficiency, the effect of this treatment varies from a TG reduction of 29% to 50%\(^{56-60}\). Treatment of with omega-3 fatty acids in genetic LPL deficient patients has never been published and therefore may warrant further investigation.

Due to the lack of effective pharmacological interventions, modern treatment options are currently restricted to intensive dietary modifications. These strict dietary regimes has been proven to be most difficult resulting in poor adherence. Consequently, the prevention of pancreatitis is unsuccessful in LPL deficient patients and additional, effective therapeutic modalities are needed.
LPL gene therapy

Rationale
Several facts have contributed to the development of gene therapy for LPL deficient patients. First, as described above, LPL deficiency currently lacks an effective and successful therapy. Second, the diagnosis of genetic LPL deficiency can be accurately made. Third, the LPL gene is rather small, which allows the incorporation of the gene into a wide range of viral vectors. Fourth, appropriate animal models for the extensive testing of this gene therapy are available (LPL `knock-out` mice and LPL deficient kittens). Fifth, LPL is naturally produced in skeletal muscle. Not only can this tissue be easily reached via intramuscular injections, but it can also be targeted with vectors with a natural tropism for this tissue. Sixth, most patients present with detectable levels of inactive LPL protein in the circulation. This strongly diminishes the risk of a significant immune response against the transgenic LPL upon effective gene therapy. Finally, increases of LPL activity in the human circulation are only associated with beneficial effects. Not only does increased LPL activity result in significant lowering of both fasting and postprandial TG levels, it will likely increase anti-atherogenic HDL cholesterol levels.

LPL gene therapy, choice of virus and pre-clinical experiments
Effectiveness of LPL gene therapy using adenovirus has long been established in animal models. Since the duration of transgene expression upon adenoviral infection is limited, the non-pathological adeno-associated virus (AAV) has been put forward, a virus that has been used in several gene therapy studies in men. As transgene, we have chosen for a naturally occurring LPL variant (LPLS447X) that has been shown to exhibit beneficial effect on lipids profiles and a concomitant decreased CVD risk.

In murine LPL deficient models, a single intramuscular injection of AAV1-LPLS447X (dosage: 8x10^12 AAV genome copies/kg body weight) resulted in a highly significant TG reduction of 97% for more than 12 months. We have recently been able to confirm these promising results in LPL deficient cats (dosage: 1x10^11 AAV genome copies/kg body weight; unpublished). The result of bio distribution and toxicity studies with the recombinant virus are excellent and have paved the way for further development.
LPL deficient patients
Awaiting the initiation of the AAV₃-LPL^{S447X} gene therapy trial, the first six LPL deficient patients have been thoroughly investigated. All patients were characterized by TG levels > 10 mmol/L, despite compliance to dietary restrictions. In addition, all patients had suffered from (recurrent) pancreatitis. The patients showed complete loss of enzymatic LPL activity, whereas circulating inactive LPL protein could be demonstrated in all (protein concentration: 19%-103% of normal). We furthermore cultured myoblasts from needle biopsies of the right upper leg of all six patients (Pro-mag 2.2 automatic biopsy system, N14GA/10cm needle; MDTECH, USA). These myoblasts were infected with AAV₃-LPL^{S447X}, after which all myocytes were shown to secrete catalytically active LPL (unpublished data).

Conclusion

LPL deficiency is a rare hereditary condition characterized by high TG levels that correlate with an increased risk for potentially lethal (recurrent) pancreatitis. In view of the lack of effective pharmacological agents, TG levels remain seriously elevated. We here report the successful implementation of an LPL gene therapy protocol using an AAV₃-LPL^{S447X} vector in both murine and feline models of LPL deficiency. In addition, we demonstrated that the myocytes of our LPL deficient patients are able to produce and secrete catalytically active LPL into culture media upon infection with AAV₃-LPL^{S447X}. Based on these promising results, the initiation of the first human LPL gene therapy trial in the Netherlands is expected soon. Other patient populations that may benefit from LPL gene therapy include heterozygote LPL deficient patients with a clinical phenotype of the chylomicronemia syndrome, patients with therapy resistant hypertriglyceridemia and maybe patients with hypertriglyceridemia formerly characterized as (Frederickson) type V hyperlipidemia. For now, we will first evaluate the effectiveness in LPL deficient patients.
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GENE THERAPY FOR LIPOPROTEIN LIPASE DEFICIENCY: WORKING TOWARDS CLINICAL APPLICATION

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Abstract

Lipoprotein lipase (LPL) deficiency causes hypertriglyceridemia and recurrent, potentially life-threatening pancreatitis. There currently is no adequate treatment for this disease. Previously, we showed that intramuscular (IM) administration of an AAV1 vector encoding the human LPLS447X variant cDNA (AAV1-LPLS447X) normalized the dyslipidemia of LPL-/- mice for over a year. In preparation for a clinical trial, we evaluated the safety and biodistribution of AAV1-LPLS447X in wild-type mice and fully characterized six LPL deficient patients. Toxicological analysis in mice showed that IM administration was well-tolerated. Acute inflammatory response markers were transiently increased, and anti-AAV1 antibodies were generated. Histological analyses indicated a dose-dependent reversible spleen hyperplasia, and myositis at the injection sites. Biodistribution data showed short-term vector leakage from injection sites into the circulation, followed by liver-mediated clearance. Persistence of vector DNA was limited to the injected muscle and draining lymph nodes, while spread to reproductive organs was limited. Characterization of LPL deficient patients showed that all patients presented with hypertriglyceridemia and recurrent pancreatitis. LPL catalytic activity was absent, but LPL protein levels were 20-100% of normal. Myoblasts derived from skeletal muscle biopsies of these patients were efficiently transduced by AAV1-LPLS447X and secreted active LPL. These data support the initiation of a clinical trial in LPL deficient patients, for which regulatory approval has now been granted.
Introduction

Over the past decade, our laboratories have contributed to the development of human gene therapy for lipoprotein lipase (LPL) deficiency. LPL is expressed in adipose tissue, skeletal muscle and heart muscle, where it catalyzes the hydrolysis of triglycerides (TG) which are packaged in chylomicrons and very-low density lipoproteins. This action results in the generation of free fatty acids (FFA) which serve as a source of energy in muscle cells, or can be stored in adipose tissue. In addition to its catalytic activity, LPL also mediates the hepatic clearance of lipoproteins, further illustrating its key role in lipid homeostasis.

Complete LPL deficiency is a rare disorder of lipid metabolism with an estimated prevalence of 1 in 1,000,000 in the general population. LPL gene mutations, underlying complete loss of function of the enzyme, result in the accumulation of chylomicrons and excessive TG levels in the circulation. This is associated with abdominal pain, eruptive xanthomata, hepatosplenomegaly, and recurrent and potentially life-threatening pancreatitis. Since chylomicronemia is directly related to fat intake, LPL deficient patients are to keep to a strict low-fat diet (<10% of total caloric intake). However, adherence to such a diet is difficult, as illustrated by recurrent hospitalization of these patients due to pancreatitis. In addition, currently available lipid-lowering medication is not effective as a treatment for LPL deficiency. Gene therapy is, in our opinion, a feasible approach to treat LPL deficiency for several reasons. Firstly, LPL is synthesized in skeletal muscle, an organ that can be easily targeted with appropriate viral vectors. Secondly, limited LPL expression (~10% of normal) in mice has been shown to be sufficient to markedly reduce plasma TG levels. Thirdly, determination of biological response is straightforward since plasma TG correlate well with the clinical severity of this disease. Finally, approximately 60% of LPL deficient patients exhibit mutations that underlie complete loss of catalytic function but detectable levels of protein in the circulation. This is important since absence of LPL may provoke an immune response against transgenic LPL upon successful treatment. From an ethical point of view, gene therapy for this disease is warranted since no other effective treatment exists and TG levels in patients remain above the critical level associated with pancreatitis (>10 mmol/l).

As a vector for DNA delivery, we have chosen adeno-associated virus (AAV). In our studies with LPL deficient mice and cats, we have used an AAV2 vector pseudotyped with AAV1 capsids (AAV1), since the latter mediates efficient transduction of skeletal muscle. Intramuscular (IM) administration of AAV1 was used to express a common variant of human LPL, lacking the two
C-terminal amino acids. This LPLS447X is associated with a favorable lipid profile and a reduced risk for cardiovascular disease. In LPL deficient mice, IM injection of AAV1-LPLS447X was previously shown to induce life-long complete normalization of TG levels. In the feline model, IM injection of AAV1-LPLS447X also induced a dramatic TG reduction.

Following these proof of principle studies, we now present the results of in vivo safety and biodistribution studies in mice, following IM administration of AAV1-LPLS447X. We furthermore assessed feasibility of clinical implementation through characterization of LPL deficient patients, and tested the biological activity of our vector in primary myoblasts isolated from these patients.

Methods

Production of AAV1-LPLS447X
AAV1-LPLS447X was produced essentially as previously described. The ampicillin resistance gene of the AAV1 helper plasmid pDP1 and vector plasmid pVD5 was exchanged for the kanamycin resistance gene, resulting in pVD20 and pVD23 respectively. The latter vector plasmid contained the transgene expression cassette, including the cytomegalovirus (CMV) immediate early promoter, the cDNA sequence of lipoprotein lipaseS447X, a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE), and the bovine growth hormone polyadenosine transcription termination signal (bGH polyA). The expression cassette was flanked by two inverted terminal repeats (ITRs) derived from AAV serotype 2. Following production in HEK293 cells, the vector was purified by a process including anion chromatography and hydrophobic interaction chromatography followed by diafiltration and concentration (Hermens et al., unpublished). Viral titer was determined by quantitative PCR. Total protein content was measured with the NanoOrange Protein Quantification Kit (Molecular Probes, N-6666). Viral purity was visualized by SDS-PAGE. Infectivity and transgene expression was tested in vitro in HEK293 cells.

Safety and biodistribution studies in mice
For the safety studies (conducted at Covance labs., Harrogate, UK), both male and female C57Bl/6 mice were housed singly in cages conform the 'Code of practice for the housing and care of animals used in scientific procedures' (Home Office, London, 1989). Groups of six mice
(per timepoint) were injected with AAV1-LPLS447X at a dose of $1 \times 10^{11}$, $1 \times 10^{12}$, $1 \times 10^{13}$ gc/kg, or with PBS supplemented with 5% sucrose as a control (formulation buffer). Animals were dosed by slow manual injection using a 27G needle, in the gastrocnemius and adductor muscles of both hind legs (2 sites per leg; 25 µl injection volume per site). Local viral doses in muscle thus ranged from $6 \times 10^{8}$-6$ \times 10^{10}$ gc/site. Following IM administration, the animals were observed daily for signs of illness or overt toxicity. Body weight and food consumption was recorded. Animals were sacrificed at 7, 28 and 90 days following dosing and tissues from all treatment groups were examined macroscopically. Microscopic analysis was performed on all organ samples from the high dose group and controls. Organs examined were: adrenals, aorta, brain, coecum, colon, duodenum, eyes, femur, gall bladder, gross lesions, heart, ileum, injection sites, jejunum, kidney, larynx, liver, lungs, mammary, mandibular lymph nodes, mesenteric lymph nodes, muscle (quadriceps), esophagus, optic nerves, ovaries, pancreas, pituitary, prostate, salivary glands, sciatic nerves, seminal vesicles, skin, spinal cord, spleen, sternum, stomach, testes, epididymis, thymus, thyroids, tongue, trachea bifurcation, trachea, urinary bladder, uterus, and vagina. Terminal blood samples were taken from each animal for blood hematology and clinical chemistry parameters. Hematology parameters examined were: hemoglobin concentration, packed cell volume, mean cell volume, mean cell hemoglobin concentration, red cell distribution width, platelet crit, platelet distribution width, red blood cell count, reticulocytes, mean cell hemoglobin, hemoglobin distribution width, platelet count, mean platelet volume, total and differential white cell count. Clinical parameters examined were: aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, sodium, potassium, calcium, inorganic phosphorus, chloride, total protein, albumin, globulin, albumin/globulin ratio, total cholesterol, glucose, urea, total bilirubin, creatinine, creatine phosphokinase, lactate dehydrogenase. In the event of pathology, relevant organs were also examined in the lower dose groups. Grading was defined as follows: grade 1 and 2 represent minimal to slight changes within tissue, with limited significance. At grade 3, visible changes are moderate and may indicate limited tissue or organ dysfunction/alteration correlating with changes in organ weights, gross changes, and/or changes in hematological and clinical chemistry parameters. At grades 4 and 5, tissue changes are marked to severe and tissue or organ dysfunction probable to expected, respectively. At grade 5, visible changes within affected tissue correlate with clinical signs, mortality, changes in organ weights, and clinical chemistry, and lesions may be life-limiting if in a critical organ.

For the biodistribution studies, groups (n=5/group/time point) of male and female C57Bl/6 mice were injected with either $1 \times 10^{11}$ or $1 \times 10^{12}$ gc/kg AAV1-LPLS447X, while the control group
was injected with formulation buffer. Animals were sacrificed at 7, 28 and 90 days following dosing. Tissue samples were collected and incubated with proteinase K before DNA isolation using the DNeasy tissue extraction kit (Qiagen). DNA was extracted from blood samples using the QIAamp® DNA Blood Mini Kit (Qiagen). 1 µg of DNA per reaction was analyzed by quantitative PCR (Q-PCR) to detect the presence of AAV1-LPLS447X vector DNA. In the event of low recovery of DNA from tissue, or if DNA samples were deemed inhibitory, less DNA was used. The limit of detection of the Q-PCR was <10 copies/reaction, the limit of quantification 10 copies/reaction. DNA samples were spiked with 100 copies of pVD23 positive control DNA to monitor sample-specific inhibition. Tissues examined were; testes, epididymis, ovaries, liver, adrenals, brain, contralateral mandibular lymph nodes, draining inguinal lymph nodes, heart, biceps (uninjected) muscle, spleen, kidneys, lungs, marrow from femur, and injected muscles. Separate groups (n=6/group) of male and female mice were treated as indicated to determine LPL expression in post-heparin plasma. Following termination, LPL expression was also measured in muscle homogenates, as described below. Blood samples were also analyzed for anti-AAV antibodies using a sandwich ELISA specific for AAV1, and for anti-LPL using a similar ELISA specific for human LPL. Acute responses were measured in short-term samples (taken 4 and 24 hours after vector administration) using an ELISA specific for murine serum amyloid A (SAA) (Biosource).

Selection of LPL deficient patients
Six patients were selected from the database of the Lipid Clinic of the Academic Medical Center. These patients have previously been diagnosed with LPL deficiency, on the basis of low LPL activity, LPL gene defects, and classical LPL deficiency phenotype. All patients gave informed consent and the protocol used to obtain skeletal muscle biopsies was approved by the Medical Ethical Committee of the Academic Medical Center.

Lipids, lipoproteins and apolipoproteins in plasma of LPL deficient patients
EDTA plasma samples were collected from fasted patients and used for plasma lipid and lipoprotein measurements. TC and TG concentrations were measured using standard enzymatic methods. The cholesterol content of the lipoprotein fractions was determined with high-performance gel permeation chromatography (HPLC).
Lipase activities and LPL concentration in post-heparin plasma of LPL deficient patients

Fasting blood samples were collected before and after an intravenous bolus of heparin (50 U/kg body weight). LPL concentrations were measured in post-heparin plasma using a Markit-F LPL Kit (Dainippon Pharmaceutical Co, Ltd, Japan). Total post-heparin plasma lipolytic activity (PHLA) was measured using tri-1-[14C]-oleate as a substrate and human serum as source of apoCII in a phosphatidylcholine emulsion. HL activity was determined as the lipase activity remaining after addition of 1M NaCl (final concentration). LPL activity was calculated after subtraction of HL activity from PHLA. Both LPL concentration and activity were expressed as percentage of a pool plasma of 11 healthy volunteers.

Isolation of myoblasts from LPL deficient patients

Muscle biopsies were taken from the vastus lateralis of the quadriceps muscle with a Pro-mag 2.2 automatic biopsy system (MDTECH, USA) and a N14GA/10cm needle through a small incision in the anesthesized skin (lidocainehydrochloride 10mg/ml, Fresenius Kabi, Netherlands). All muscle specimens were taken up in 10% DMSO, 40% serum and 50% skeletal muscle cell growth medium (Promocell). Muscle biopsies were minced and subjected to 4 cycles of digestion at 37°C with a trypsin/collagenase mixture (1mg/ml collagenase (Sigma), 1 mg/mL BSA (Sigma) dissolved in trypsin-EDTA solution; Gibco-BRL). Released cells were grown in collagen-coated wells using SKMC growth medium. To avoid myotube formation, cells were grown to a maximum of 70% confluency before propagation. After two passages, myoblasts were isolated using dynabeads (Dynal, Biotech) coated with anti-myosin. The efficacy of this procedure was investigated by immunohistochemistry. Cells were grown until confluency, washed and fixed in methanol. After blocking with 5% horse serum / 0.05% Tween / PBS for 30min, the cells were incubated with anti-myosin antibody for 1 hr at room temperature. Labeling was carried out with fluorescent labeled anti-mouse IgG (Alexa-Fluor594, Molecular Probes) for 1 hr in the dark. The number of myogenic positive cells was estimated using a fluorescence microscope.

Infection of primary human myoblasts with AAV1-LPLS447X

Myoblasts were grown well until 70% confluency and the cells were infected with AAV1-LPLS447X (approximately 1.6x10⁴ gc/cell) in absence or presence of wild-type adenovirus. The media were collected after 48-96 hours, for the determination of LPL activity and LPL (protein) concentration. All values were corrected for the background values measured in non-infected cells. Cellular production of LPL was verified by immunohistochemistry. Cells were fixed with 4%
paraformaldehyde and incubated with mouse anti-bovine LPL for 1 hr at 37°C. After incubation with horseradish peroxidase-labeled goat anti-mouse IgG (Sigma), the cells were washed and stained with 2 mg 3-amino-9-ethyl-carbazole dissolved in 0.5 mL di-methyl-formamid, 9.5 mL sodium acetate buffer (0.05M pH 5.0) and 5 µl 30% H₂O₂.

**Results**

Transgene expression following IM administration of AAV1-LPLS447X in wild type C57Bl/6 mice

We previously showed efficacy of AAV1-LPLS447X in LPL-/- mice, at doses ranging between 8x10¹¹ and 8x10¹² gc/kg. Intermediate efficacy has also been shown in LPL-/- cats, at a dose of 1x10¹¹ gc/kg. To obtain approval for the use of this vector in the clinic, we conducted a GLP study to assess the safety and vector biodistribution associated with IM delivery of AAV1-LPLS447X in C57Bl/6 mice. Doses of vector used were 1x10¹¹ (low dose), 1x10¹² (mid dose), and 1x10¹³ gc/kg (high dose), thus exceeding a minimal efficacious dose in LPL-/- cats 100-fold.

To confirm transgene expression, human LPL expression was measured in post-heparin plasma (at day 7 and day 28 following administration) and in homogenates of injected muscle (at day 90 following administration). At 28 days post-administration of the vector (IM administered in 4 sites), LPL was dose-dependently expressed in post-heparin plasma, but only in the intermediate- and high dosed animals (table 1). A similar dose-dependent LPL expression was found when analyzing homogenates of the injected muscle. This may reflect a critical threshold level or multiplicity of infection (MOI) needed to induce transgene expression in muscle. Based on the average number of cells contained within murine muscle and the local dose administered, we estimate the in vivo MOI to range from 4x10⁻¹⁷-4x10⁻¹⁶. Expression in the gastrocnemius muscle was consistently higher (~2-fold) than in the adductor. Levels of LPL expression were similar in male and female mice. It should be noted that LPL expression and efficient reduction of plasma TG levels was noted in LPL-/- cats, following IM administration of the low dose (1x10¹ⁱ gc/kg) of AAV1-LPLS447X. The larger muscle mass available for each injection in cats may positively affect transduction efficiency (less leakage), and in addition there may be differences between species with respect to the ability of AAV1 to transduce muscle.
Toxicity of AAV1-LPLS447X following IM administration in wild type C57Bl/6 mice

The data of this toxicological analysis are shown in table 1. There were no deaths or significant changes in overall health or food consumption between groups that had received 1x10^11, 1x10^12 or 1x10^13 gc/kg of AAV1-LPLS447X. A significant decrease in body weight gain (~30%) was noted primarily in high dose females in the day 90 subset. Analysis of hematology and serum clinical chemistry parameters did not reveal any consistent changes.

Macroscopic evaluation of organs showed no effects of AAV1-LPLS447X at any time point. Microscopically, a minor and reversible lymphoid hyperplasia was noted in the spleen of high dose females.

### Table 1  Efficacy and toxicity assessment after intramuscular administration of AAV1-LPLS447X in C57BL/6 mice

<table>
<thead>
<tr>
<th>Dose (gc/kg)</th>
<th>1x10^11 gc/kg</th>
<th>1x10^12 gc/kg</th>
<th>1x10^13 gc/kg</th>
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</thead>
<tbody>
<tr>
<td>LPL expression¹</td>
<td>males/females</td>
<td>males/females</td>
<td>266±75 / 174±80 ng/mL</td>
</tr>
<tr>
<td>Plasma</td>
<td>-</td>
<td>76±49 / 42±26 ng/mL</td>
<td>346±134 / 294±58 ng/mL</td>
</tr>
<tr>
<td>Muscle Homogenate (A⁺)</td>
<td>-</td>
<td>76±49 / 91±66 ng/mg</td>
<td>888±358 / 631±106 ng/mg</td>
</tr>
<tr>
<td>Muscle Homogenate (G⁺)</td>
<td>-</td>
<td>147±50 / 133±26 ng/mg</td>
<td>888±358 / 631±106 ng/mg</td>
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<tr>
<td>Clinical Signs³</td>
<td>none</td>
<td>none</td>
<td>Reduced body weight gain (~30%) by day 90, females only</td>
</tr>
<tr>
<td>Changes in haematology and clinical chemistry parameters³</td>
<td>Minor and sporadic change</td>
<td>Minor and sporadic change</td>
<td></td>
</tr>
<tr>
<td>Histopathology⁴</td>
<td>Assessment limited to day 90, injection site: minimal (grade 1) myositis around injection area (variable between muscle groups, between 2 of 12 and 10 of 12)</td>
<td>Minimal reversible lymphoid hyperplasia in spleen: on day 7 (7 of 12) and day 28 (9 of 12), but not on day 90 (0 of 12); progressive myositis at injection site: day 90, minimal (grade 1d) to mostly slight (grade 2d) myositis around injection area (12 of 12)</td>
<td></td>
</tr>
</tbody>
</table>

gc, genome copies; LPL, lipoprotein lipase. ¹LPL expression in postheparin plasma 28 days after administration of vector. ²LPL expression in homogenates from adductor (A) and gastrocnemius (G) muscle samples taken 90 days postadministration. ³See Materials and Methods.

**Toxicity of AAV1-LPLS447X following IM administration in wild type C57Bl/6 mice**

The data of this toxicological analysis are shown in table 1. There were no deaths or significant changes in overall health or food consumption between groups that had received 1x10^11, 1x10^12 or 1x10^13 gc/kg of AAV1-LPLS447X. A significant decrease in body weight gain (~30%) was noted primarily in high dose females in the day 90 subset. Analysis of hematology and serum clinical chemistry parameters did not reveal any consistent changes.

Macroscopic evaluation of organs showed no effects of AAV1-LPLS447X at any time point. Microscopically, a minor and reversible lymphoid hyperplasia was noted in the spleen of high dose females.
dose male and female animals at day 7 and 28, which dissipated by day 90. Such transient lymphoid hyperplasia was characterized by an increase in the number of germinal centers in the white pulp of the spleen. Minimal (grade 1) myositis around the injection area was observed in both control and high dose animals at day 7, consistent with needle track lesions. Histology of the injection area was normal at day 28. A slight (grade 2) myositis was observed at the injection sites of all high dose animals at day 90 (not observed in the control group). This myositis was characterized by multifocal to diffuse muscle fiber degeneration (muscle cell wasting, loss of intracellular structure, variation in muscle fiber diameter) associated with regeneration of muscle fibers (increase in number of centralized nuclei), and perivascular and perifascicular infiltration of inflammatory cells (mainly lymphocytes). Lesions were generally chronic, as determined by the fact that the inflammatory component consisted of cells displaying exclusively non-segmented nuclei. The myositis was observed throughout the injected muscle, mostly confined to these injected muscles, but was sometimes also observed in other (non-injected) muscles close to the injection site and in adjacent fat tissue. Analysis of the medium dose animals at day 90 indicated minimal (grade 1) and very local myositis around the injection area in some animals (varying between 2 out of 12, to 10 out of 12). Histology of the injection area was normal in the low dose and in control groups.

Analysis of plasma samples at 4 and 24 hours, 3 and 7 days after transfer showed high levels of murine serum amyloid A (mSAA) in plasma at 4 hours, as a measure of general inflammatory response (data not shown). The mSAA levels dissipated rapidly beyond this time point. Further analysis of blood samples showed a high titer of antibodies against AAV1, regardless of the viral dose used, but with a trend towards a lower response at a dose of $1 \times 10^{11} \text{ gc/kg}$ particularly in females. These antibodies were inhibitory in an in vitro assay testing infection of AAV1-LPLS447X (data not shown). We did not observe antibodies directed against the transgenic product LPLS447X.

**Biodistribution of AAV1-LPLS447X following IM administration in wild type C57Bl/6 mice**

During the course of this experiment, a reduction in body weight gain (-30%) was again observed in both males and females in the high dose group ($1 \times 10^{11} \text{ gc/kg}$), which was not accompanied by reduced food intake (table 2). Tissues from the control group that had received formulation buffer were negative for the presence of AAV1-LPLS447X DNA at day 7. Control group animals were therefore not analyzed at the later time points.
Whole blood samples from the high dose group tested positive for viral DNA until day 28, but viral DNA in plasma was rapidly cleared within 3-4 days (data not shown). At day 7, vector DNA (expressed as copies per \(\mu g\) of tissue DNA) was mainly detected in the injected muscles (\(7.1 \times 10^6\) copies/\(\mu g\) for both gastrocnemius and adductor muscles), spleen (\(150,000\) copies/\(\mu g\)), liver (\(2.9 \times 10^6\) copies/\(\mu g\)), inguinal lymph nodes (8 out of 10 samples; \(2.7 \times 10^6\) copies/\(\mu g\)), and marrow (\(120,000\) copies/\(\mu g\)) at day 7. Levels of vector DNA in the various organs and tissues declined with time. At day 28, vector DNA was mainly detected in the injection sites (\(4.2 \times 10^6\) and \(3.2 \times 10^6\) copies/\(\mu g\) for gastrocnemius and adductor muscles, respectively), liver (\(41,000\) copies/\(\mu g\)), and inguinal lymph nodes (7 out of 10 samples; \(460,000\) copies/\(\mu g\)). At day 90, high levels of vector DNA were still detected in the injection sites (\(610,000\) and \(790,000\) copies/\(\mu g\) for gastrocnemius and adductor muscle, respectively), liver (\(13,000\) copies/\(\mu g\)), inguinal lymph nodes (8 out of 10 samples; \(140,000\) copies/\(\mu g\)). Analysis of AAV1-LPLS447X-derived DNA sequences in the low dose group demonstrated a similar biodistribution. Absolute levels of vector sequence in the various organs and tissues analyzed were lower, proportional to the lower dose administered.

**Dissemination of AAV1-LPLS447X to reproductive organs following IM administration in wild type C57Bl/6 mice**

To assess transfer of AAV1-LPLS447X sequences to the germ line, male and female gonads were analyzed (table 2). In the high dose group at day 7, levels of vector DNA in the gonads amounted to \(19,000\) copies/\(\mu g\). At day 90, these levels had diminished to \(10\) to \(550\) copies/\(\mu g\). In the low dose group, vector sequences were detectable at low levels at day 7 but undetectable beyond this time point (most gonads). In the few gonads that did show vector DNA presence at this time point, the amount was just above background (\(10\) copies/\(\mu g\)). On average, the levels per \(\mu g\) DNA were \(-1,000\) to \(1,500\)-fold lower than those detected for injected muscle.

**Characteristics of LPL deficient patients**

Six patients, previously diagnosed to suffer from genetic LPL deficiency 10-13 were investigated to assess whether they would be eligible for a future clinical trial. Important criteria included severe hypertriglyceridemia, low post-heparin LPL activity yet residual LPL (protein) concentration, and confirmation of LPL gene mutations. Prior to diagnosis of LPL deficiency, all patients had a history of pancreatitis (table 3). Upon diagnosis, each patient was advised to adhere to a strict low-fat diet (\(10\%\) of caloric intake). Four patients were using TG-lowering medication (patient I, III and IV used modalim, and patient II used bezofibrate).
<table>
<thead>
<tr>
<th>Dosage</th>
<th>Control</th>
<th>1x10^6 gc/kg</th>
<th>1x10^7 gc/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical signs</td>
<td>None</td>
<td>None</td>
<td>Reduced body weight gain (-30%) by day 90, males and females</td>
</tr>
<tr>
<td>Vector DNA distribution</td>
<td>No sequences detectable</td>
<td>Day 7, low-dose samples: Injected muscles (&lt;400 to &lt;150,000 c/µ), Liver (&lt;3,800 to &lt;13,000 c/µ), Spleen (&lt;100 to &lt;10,000 c/µ), Inguinal LN (&lt;10 to &lt;11,000 c/µ)</td>
<td>Day 7, high-dose samples: Injected muscles (&lt;130,000 to &lt;71<em>10^6 c/µ), Liver (&lt;840,000 to &lt;2.9</em>10^6 c/µ), Spleen (&lt;11,000 to &lt;150,000 c/µ), Inguinal LN (&lt;1300 to &lt;2.7*10^6 c/µ), Marrow (&lt;16,000 to &lt;120,000 c/µ)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Day 28, low-dose samples: Injected muscles (&lt;440 to &lt;23,000 c/µ), Inguinal LN (&lt;10 to &lt;4800 c/µ)</td>
<td>Day 28, low-dose samples: Injected muscles (&lt;71,000 to &lt;4.2*10^6 c/µ), Liver (&lt;2000 to &lt;41,000 c/µ), Inguinal LN (&lt;10,000 to &lt;460,000 c/µ)</td>
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<tr>
<td></td>
<td></td>
<td>Day 90, low-dose samples: Injected muscles (&lt;100 to &lt;12,000 c/µ), Inguinal LN (negative to &lt;3600 c/µ)</td>
<td>Day 90, high-dose samples: Injected muscles (&lt;40,000 to &lt;790,000 c/µ), Liver (&lt;1500 to &lt;13,000 c/µ), Inguinal LN (&lt;870 to &lt;140,000 c/µ)</td>
</tr>
<tr>
<td>Vector DNA in gonads</td>
<td>No sequences detectable</td>
<td><strong>Tests</strong> Day 7: &lt;10 to &lt;60 c/µ Days 28 and 90: &lt;10 c/µ</td>
<td><strong>Tests</strong> Day 7: &lt;2800 to &lt;5700 c/µ Day 28: &lt;130 to &lt;1200 c/µ Day 90: &lt;80 to &lt;330 c/µ</td>
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<td><strong>Epididymis</strong> Day 7: &lt;10 to &lt;30 c/µ Days 28 and 90: &lt;10 c/µ</td>
<td><strong>Epididymis</strong> Day 7: &lt;3000 to &lt;19,000 c/µ Day 28: &lt;100 to &lt;5500 c/µ Day 90: &lt;30 to &lt;550 c/µ</td>
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<tr>
<td></td>
<td></td>
<td><strong>Ovaries</strong> Day 7: &lt;10 to &lt;30 c/µ Days 28 and 90: &lt;10 c/µ</td>
<td><strong>Ovaries</strong> Day 7: &lt;10 to &lt;7100 c/µ Day 28: &lt;70 to &lt;840 c/µ Day 90: &lt;10 to &lt;200 c/µ</td>
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</tbody>
</table>

*c/µ, copies of AAV1-LPLS447X sequence per microgram of DNA; gc, genome copies; LN, lymph node. Male and female C57BL/6 mice were injected intramuscularly with the indicated doses of AAV1-LPLS447X. The numbers of AAV1-LPLS447X vector copies in the various organs, on days 7, 28, and 90 post-administration, were determined by quantitative PCR. The range of genome copies found per microgram of genomic DNA is indicated.*
Characteristics of LPL deficient patients

Six patients, previously diagnosed to suffer from genetic LPL deficiency\(^{10-13}\) were investigated to assess whether they would be eligible for a future clinical trial. Important criteria included severe hypertriglyceridemia, low post-heparin LPL activity yet residual LPL (protein) concentration, and confirmation of LPL gene mutations. Prior to diagnosis of LPL deficiency, all patients had a history of pancreatitis (table 3). Upon diagnosis, each patient was advised to adhere to a strict low-fat diet (\(<10\%\) of caloric intake). Four patients were using TG-lowering medication (patient I, III and IV used modalim, and patient II used bezofibrate).

On their initial visit to the clinic, plasma TG levels of these patients ranged from 22.5 to 78.8 mmol/l, indicative of a severely disturbed TG metabolism. Following a TG-lowering diet (and, in some cases, lipid-lowering medication), plasma TG levels decreased by an average of 50%. On recent multiple visits, most patients still presented with plasma TG levels of 15-30 mmol/l (data not shown). Pancreatitis persisted even when on diet (table 3), plasma TG levels remaining above the critical threshold level (\(>10\) mmol/l).\(^4\) On average, high-density lipoprotein (HDL) cholesterol levels and low-density lipoprotein (LDL) cholesterol levels were markedly reduced. All patients had normal total cholesterol levels.

Sequence analysis in each case confirmed the presence of missense mutations within the LPL gene (see table 3) predicting amino acid substitutions and complete loss of activity in the mature LPL protein. The effect of these mutations on LPL function was further analyzed: testing of the capacity for TG hydrolysis in post-heparin plasma, using \(^{3}H\) triolein substrate, revealed a complete loss of LPL activity (0\% as compared to a pool of post-heparin plasma of 11 healthy volunteers set at 100\%). Each patient showed LPL protein in post-heparin plasma, varying from 20\% to 100\% of normal LPL protein concentration levels.

Infection of human myoblasts with AAV1-LPLS447X

Although earlier experiments clearly established the potency of AAV1-LPLS447X in LPL-/- mice\(^1\) and cats\(^5\), there are no data to support efficacy of AAV1 in human muscle tissue. We therefore set out to test the vector in skeletal muscle cells obtained from LPL deficient patients. For this purpose, we cultured satellite cells from skeletal muscle biopsies from five out of the six patients described. Immunohistochemistry using antibodies against myosin indicated that the purification procedure resulted in nearly 100\% myosin-positive cells.
<table>
<thead>
<tr>
<th>Patient</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>VI</th>
<th>V</th>
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<tbody>
<tr>
<td>Sex</td>
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<td>Male</td>
<td>Male</td>
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<td>Current age</td>
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<td>BMI</td>
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BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides. 1Before diagnosis of LPL deficiency; 2After diagnosis of LPL deficiency. 3Reference values are for the general population. 4Concentrations are given as percentage of a pool of postheparin plasma drawn from 11 healthy volunteers.
The resulting primary skeletal muscle cell culture was exposed to AAV1-LPLS447X (1.6x10^4 gc/cell). 48 hours following infection, the culture media contained hLPLS447X, ranging from 60 to 200% of an infected control cell culture derived from a healthy control (table 4). Addition of wild-type adenovirus boosted transgene expression in most cultures. The transgenic product displayed detectable lipolytic activity compared to infected myoblasts isolated from a healthy control subject, LPL activity levels in the culture media from the patient’s cells ranged between 22-90%. The varying transduction efficiency observed may be due to varying propagation rates of the primary cell cultures. Immunohistochemistry revealed that, upon infection with AAV1-LPLS447X, approximately 40% of the cells expressed LPL (figure 1).

| Table 4 AAV1-LPLS447X Infection of Primary Skeletal Muscle Cells |
|------------------|------------------|------------------|
|                  | LPL concentration | LPL activity     |
|                  | (ng/ml)           | (mU/ml)          |
| Patient          | -Adenovirus       | +Adenovirus      | +Adenovirus      |
| I                | 150               | 215              | 16               |
| II               | 78                | 304              | 8                |
| III              | 650               | 657              | 18               |
| IV               | 26                | 300              | 14               |
| VI               | 619               | 716              | 30               |
| Control¹         | ND                | 351              | 34               |

LPL, lipoprotein lipase; ND, not determined. Infections were carried out with 1.5x10^10 AAV1-LPLS447X gc/3x10^5 cells (an MOI of 1.6x10^4) in the absence or presence of wild-type adenovirus. LPL measurements were carried out in heparin-containing medium 48 hr (presence of adenovirus) and 96 hr (absence of adenovirus) after infection. All values were corrected for background values measured in culture medium from noninfected cells. ¹Myoblasts from healthy volunteer.
Discussion

Currently, there is no effective treatment for LPL deficiency, an orphan disease characterized by excessive hypertriglyceridemia and pancreatitis. Restriction of dietary fat intake is the only option to lower plasma TG below a critical level of 10 mmol/l. However, as also demonstrated in this study, restriction of dietary fat does not eliminate painful and potentially lethal episodes of pancreatitis and alternative strategies are warranted. Enzyme-replacement is not an option because of the short half-life of exogenous LPL (<10 minutes in the circulation). Conventional medications primarily directed at lipid metabolism downstream of LPL cannot correct the inactive protein in these patients. We have studied the option of gene therapy for LPL deficiency in LPL deficient mice, and have shown complete and long-term resolution of hypertriglyceridemia following IM administration of an AAV1 vector expressing LPLS447X. A complete normalization of hypertriglyceridemia was also observed in a feline model of complete LPL deficiency treated with AAV1-LPLS447X. In preparation for clinical application, we now evaluated the toxicology and vector biodistribution associated with IM delivery of AAV1-LPLS447X to wild type C57BI/6 mice, and fully characterized 6 LPL deficient patients who could be eligible for enrolment in a clinical trial.

Biodistribution and germ line transmission

Safety and biodistribution of intramuscular application of AAV serotype 1 has not been described.
in detail to date. Shortly after IM administration of AAV1-LPLS447X, vector DNA was detected in muscle, but there was also considerable leakage into the circulation. Plasma clearance was rapid (within days), resulting in accumulation of vector in filtering organs, particularly the liver. AAV DNA sequence also accumulated in lymph nodes close to the injection site, indicating drainage via the lymphatic system. While initial liver uptake accounted for at least 50% of the viral DNA recovered from various tissues (muscle accounting for most of the remainder), persistence of vector sequence over time was limited to the injected muscles, and to the proximal lymph nodes. Short-term clearance of the vector by the liver is likely followed by efficient degradation, e.g. by liver Kupffer cells, since levels of vector DNA sequence in liver decline rapidly. In agreement with this assumption, spread of AAV1-LPLS447X to distant organs such as liver did not result in transgene expression. The slow clearance of AAV2 sequence from whole blood (over weeks) as opposed to rapid clearance from plasma (within days), as also observed for AAV1 in the current study, may be attributed to persistence of vector in circulating PBMC’s. Accumulation in liver and lymph nodes was also observed, and lymph nodes may act as a preferential reservoir for AAV1-transduced mononuclear cells, with occasional release into the circulation. Such a scenario may well have contributed to the immunological responses against AAV1 capsid proteins observed in this study.

Vector DNA was detected in both male and female gonads but the levels declined over time to undetectable (low dose) or low (high dose) levels after 3 months. In a separate study in which LPL deficient cats were injected IM with AAV1-LPLS447X (data not shown), vector DNA was also detected in epidymides and testis (at a medium dose of $1 \times 10^{12}$ gc/kg of vector, at 8-10 weeks post-administration: up to ~170 copies/µg DNA). However, the corresponding motile sperm fraction contained little (~10 copies) to no vector DNA sequence. Similar findings have been reported for AAV2 vectors in mice. Also, exposure of mouse spermatozoa to high concentrations of AAV2 did not result in germ cell transduction, and IM administration of the same vector in a clinical trial did not result in the presence of AAV2 vector DNA in sperm cells.

**General toxicity and inflammation**

Others have shown that intramuscular AAV-mediated gene delivery is safe in cats, dogs, monkeys, and man. In the current analysis, AAV1 was shown to be well-tolerated in mice. The only dose-related clinical observation in wild-type mice was a reduced body weight gain (~30% at the high dose). Marked over-expression of LPL in skeletal muscle has also been shown
to result in a reduced body weight gain in transgenic mice and rabbits. As in the current study, this reduction was not accompanied by reduced food intake, perhaps indicating increased energy expenditure.

There were no changes in blood clinical chemistry or hematology parameters. At high dosage, exposure of muscle (and liver) to the vector did not result in elevations in markers for muscle- or liver-specific markers in serum (CPK for muscle; ALT, AST, and LDH for liver), which would otherwise indicate tissue damage and possible organ dysfunction. In agreement, there were no macroscopic findings of note in any of the tissues analyzed.

General microscopic analysis revealed a dose-dependent and transient hyperplasia of the spleen, and activation of draining lymph nodes proximal to the treated muscle. Consistent with an acute inflammatory response after exposure to a viral capsid protein load, serum mSAA levels were transiently elevated (<24 hours). We furthermore detected antibodies directed against AAV1 irrespective of the dose used, although there was a trend towards a lower response at $1 \times 10^{11}$ gc/kg AAV1-LPLS447X. In a separate study in wild-type mice, we have found such titers of anti-AAV1 antibodies to persist for >1 year. Being inhibitory in an in vitro infection assay, these neutralizing antibodies prevent transgene expression following a second administration of AAV1 encoding for a different transgene (unpublished data).

Microscopic analysis of the injection site indicated myositis in the mid- and high dose groups which became most apparent at later time points (day 90) during the study. A transient myositis (at 7 days post-administration), irrespective of treatment regimen, indicated physical trauma to the muscle in response to the injections per se. In contrast, the myositis observed 90 days after administration correlated with the viral dose administered, and with the level of transgene expression (table 1). The current study does not allow us to discriminate between the effects induced by the virus or by LPL expression. At this point, we can only refer to studies by other investigators. Louboutin et al. and Poirier et al. also reported the induction of focal mild myositis after IM application of AAV1 in both wild-type mice and New Zealand White rabbits, expressing either alpha-1 anti-trypsin or LacZ. The results of these studies point towards a response against AAV1 capsid proteins. In support of this, anti-AAV1 antibodies were detected in the current study. On the other hand, local over-expression of LPL’s biological activity has been reported deleterious, as further discussed below.
Overall, the side effects of IM administration of AAV1-LPLS447X are limited to local inflammation within the injected area. Importantly, such effects were observed at a dose greatly exceeding the minimal efficacious dose of $1 \times 10^{11}$ gc/kg as determined in LPL-/- cats. Experiments are underway to further investigate these local responses.

**Over-expression of LPL.**

In the current study, we observed limited effects on body weight after IM application of AAV1-LPLS447X. Marked muscle-specific over-expression of human LPL has been shown to result in weight loss, and even premature death in transgenic mice. This discrepancy may be explained by the differences and extent of LPL expression. We induced a local 15-fold increase of lipase activity levels in only four injected muscles while in the respective transgenic models, skeletal muscle tissue in general showed a 5.5, 8.1 or 25.5-fold increase in lipase activity.

In the current study, local effects of treatment on muscle morphology were minor and mainly of inflammatory nature. In mice over-expressing human LPL in skeletal muscle, myopathy is attributed to severe dysregulation of FFA uptake following LPL mediated TG hydrolysis. We wish to underline that in contrast to the transgenic mice, the increased muscle tissue lipid levels of LPL-/- mice are normalized upon IM administration of AAV1-LPLS447X, and myopathy was not observed. Our previous and current data therefore indicate that local IM treatment with AAV1-LPLS447X does not cause marked deleterious effects. Interestingly, myopathy was also not observed in rabbits over-expressing human LPL in many tissues, a.o. in muscle, but also in adipose and liver. Perhaps pathological over-expression of LPL in muscle is observed only when the capacity of muscle for TG hydrolysis and lipoprotein uptake greatly outweighs that of other tissues normally involved in the metabolism of TG-rich lipoproteins.

**Feasibility of treatment of LPL deficient patients**

As a preparation for the first clinical trial for which official approval has already been obtained, six LPL deficient patients were characterized in detail. Four patients were treated with fibrates which are known to increase LPL gene transcription. Since the LPL alleles in these patients are defective, this did not yield substantial effects. Nevertheless, moderate TG-lowering effects may have been achieved through fibrate-induced reduction in hepatic VLDL output. In the 6 patients studied, it was impossible to differentiate between the TG-lowering effect of fibrates and that of (uncontrolled) reduced dietary fat intake. All individuals reported that adherence to a
low-fat diet was almost impossible to comply with, as described by others.³² Importantly, the measures taken to treat these patients were ineffective. This may be concluded from the fact that plasma TG levels remained above a threshold level associated with an increased risk of pancreatitis (>10 mmol/l).³³ In accordance, recurrent pancreatitis was not prevented (table 3).

To answer the question whether human skeletal muscle would be receptive for AAV1-mediated transduction, a question unanswered to date, muscle biopsies were obtained from the vastus lateralis muscle of the LPL deficient patients. Myoblasts were isolated and exposed to AAV1-LPLS447X, resulting in substantial levels of LPLS447X and LPL activity in the culture media, indicating that human muscle cells are amenable to transduction by AAV1 vectors. In vivo, however, transduction may be hampered by the presence of pre-existing (neutralizing) antibodies against AAV1 capsid protein. We have studied this by checking for AAV1 antibody (Ab) titers using a novel ELISA: while neutralizing anti-AAV1 Ab were detected in sera from 3 out of 160 (<2%) apparently healthy volunteers, anti-AAV1 Ab titers in sera from the LPL deficient patients were below the 80% confidence interval of this control population and neutralizing antibodies were not detected (data not shown). While promising, only in vivo data may tell us whether the presently observed Ab titers will ultimately affect efficacy of AAV1-LPLS447X gene therapy.

From a technical point of view, LPLS447X gene therapy for the treatment of human LPL deficiency seems feasible. Experiments in LPL-/- mice have indicated that little LPL activity (<10% of normal) is needed to have a large impact on plasma TG levels. In addition, transduction of a limited muscle mass was shown to provide complete resolution of lipemia.¹ Toxicology and biodistribution studies have now demonstrated safety (at least for 90 days post-treatment; additional studies are ongoing). Furthermore, evidence is provided that the vector is able to induce production and secretion of catalytically active LPL in the skeletal muscle cells that were derived from the patients, the exact cell type that will be targeted when this therapy will be tested in the clinic.

A remaining issue of concern is the possibility of an immune response against the transgenic protein, preventing efficacy of gene therapy in LPL deficient patients. In fact, in LPL-/- and LPL+/+ cats, AAV1-mediated LPL gene transfer to muscle results in the generation of antibodies against human LPLS447X, preventing long-term transgene expression (unpublished data). The presence of significant amounts of endogenous but catalytically inactive LPL in the circulation of our patients hopefully provides a better starting point in this regard.
Conclusions

LPL deficiency in man is associated with recurrent pancreatitis despite attempts to manage excessive hypertriglyceridemia. The need of additional treatment is illustrated by the fact that 2 of the patients described in the current study suffered from pancreatitis during the preparation of this manuscript. Whereas one patient recovered after receiving intensive care treatment, the second 25 yr old patient died as a direct result from this complication. Intramuscular expression of a natural variant of LPL, LPLS447X, via AAV1-mediated gene transfer is effective in isolated human skeletal muscle cells and results in dramatic TG lowering in LPL deficient animal models. Toxicity and biodistribution studies in mice, spanning 90 days, indicate that this treatment is safe and does not cause serious side effects. During the preparation of this manuscript, we obtained official approval of the European (EMEA) and Dutch regulatory authorities to conduct a first clinical trial with AAV1-LPLS447X gene therapy in patients with genetic LPL deficiency. In short, it was decided to start an open label dose escalation study (doses ranging from $1 \times 10^{10}$, $3 \times 10^{10}$ and $1 \times 10^{11}$ gc/kg AAV1-LPLS447X) in which the patients will receive 40, 60, and 80 intramuscular injections in the upper and/or lower limbs, respectively. The dosages and number of injection sites were chosen on the basis of our studies in mice showing that doses of $1 \times 10^{12}$ up to $1 \times 10^{13}$ are therapeutic (TG reductions of 70% and 95%, respectively). In addition, we found in cats that a dose of $1 \times 10^{10}$ gc/kg body weight is still efficacious (unpublished data). In this same model, we also observed a linear increase in LPL expression following IM injections in a range of $4 \times 10^{9}$- $1 \times 10^{12}$ gc per injection site suggesting that the limits of LPL production by the transduced muscle were not reached. At the lowest dose of $1 \times 10^{10}$ gc/kg body weight in an average patient weighing 70 kilogram, this would result in a local dose of $1.75 \times 10^{7}$ gc/site, which is within the linear range as defined by the cat model.

With this protocol we hope to soon provide a definite answer to the question whether AAV1-LPLS447X will be safe and efficacious in patients suffering from LPL deficiency.

Acknowledgements

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Conflict-of-Interest Disclosure Statement
ACB, PD, WTH, JT and JMM are employees of AMT. JJPK is an advisor and shareholder of AMT. MRH is a consultant to AMT.

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SUMMARY & CONCLUSIONS
Summary and Conclusions

In the first part of this thesis, the beneficial effects of LPLS447X on lipid levels are studied. Potential pathways explaining the beneficial effects of this LPL variant are discussed with special emphasis on its effects on triglyceride metabolism. The second part focuses on beneficial consequences of the LPLS447X variant on various phenotypes with increased CVD risk. The third and final part of this thesis discusses the impact of complete absence of LPL, i.e. LPL deficiency. Tentatively, the advent of novel therapeutic modalities, such as gene therapy, is introduced.

Part I: How Lipoprotein Lipase S447X exerts its beneficial effects

In chapter two, an updated perspective of LPLS447X is provided, focusing on the beneficial effects on lipoproteins as well as other traditional risk factors. Potential mechanisms by which LPL can contribute to these beneficial effects are summarized.

Since a substantial part of the beneficial effects of the LPLS447X variant is associated with its impact on lipid clearance, chapter three describes the effects of LPLS447X on apoB48 clearance after an oral fat load in 15 male heterozygous LPLS447X carriers compared to 15 matched control subjects. A 25% lower postprandial peak apoB48 value in the LPLS447X carriers indicates enhanced apoB48 clearance. Most interestingly, a two-fold increase in circulating plasma LPL concentration is established in the carriers, without any changes in LPL activity. Based on these results, part of the additional effect of LPLS447X on lipid clearance is hypothesized to pertain to increased LPL-function (i.e. enzymatic function, non-enzymatic ligand function or both).

To substantiate this hypothesis, chapter four and five evaluate whether LPLS447X has increased LPL-function. In chapter four, the effects of LPLS447X on TRL apoB100 metabolism are studied. TRL apoB100 metabolism is determined in five male homozygous LPLS447X carriers and five matched controls during feeding via infusion of stable isotope L-[1-13C]-valine. LPLS447X is associated with enhanced TRL apoB100 conversion as well as enhanced LDL removal. Combined with increased pre-heparin LPL concentration that is bound predominantly to the apoB100 fraction, these data reveal increased enzymatic as well as increased non-enzymatic consequences of LPLS447X in these carriers. In chapter five, the effects of LPLS447X on TRL
apoB48 metabolism are studied. TRL apoB48 metabolism is determined again in the same five male homozygous LPLS447X carriers and five matched controls during feeding via infusion of stable isotope L-[1-13C]-valine. In line with expectation, LPLS447X is associated with enhanced apoB48 clearance. This proves that gain-of-function of this LPL variant not only has an effect on apoB100, but also on apoB48 metabolism. Most importantly, this provides clear evidence that the beneficial impact of LPLS447X mutation is associated to both enzymatic as well as non-enzymatic effects.

Conclusions part I: LPLS447X exerts its beneficial effects via enhanced LPL enzymatic function (enhanced apoB100 TRL turnover), as well as via enhanced non-enzymatic ligand function (enhanced apoB48 and LDL clearance).

Part II: LPLS447X relates to various beneficial phenotypes

To substantiate the in vivo relevance of the LPLS447X mutation, the impact of LPL gene polymorphisms on restenosis as defined by target vessel revascularization (TVR) in a large patient-population undergoing percutaneous coronary intervention (PCI) is examined in chapter six. For this purpose, data from the GENDER (GENetic DEterminants of Restenosis) study, a multicenter prospective trial that enrolled patients after successful PCI (n=3104), is used. LPLS447X is associated with a lower risk of TVR compared to non-carriers. Using a mouse model for restenosis, LPL-mRNA levels in the targeted arterial wall are shown to be 40-fold increased after an intervention, compared to control arteries. Based on the results of these investigations, LPLS447X is concluded to be an important protective factor for TVR in humans. Via the strong expression of target arterial wall LPL, the crucial role of LPL in the process of restenosis is also demonstrated.

To further substantiate the impact of the LPLS447X variant on cardiovascular risk in both controls as well as subjects at increased CV risk, the effect of LPLS447X on future cardiovascular risk in subjects with different levels of albuminuria without prior cardiovascular disease is investigated in chapter seven. Since increased levels of albuminuria are associated with increased CVD risk and LPLS447X is associated with reduced cardiovascular risk, LPLS447X is hypothesized to be able to modify the albuminuria associated risk presumably via beneficial effects on lipids. To test
this hypothesis, data from the PREVEND (Prevention of RÉnal and Vascular ENdstage Disease) study, a prospective general population based cohort study (n=8592), is used. LPLS447X is able to modify microalbuminuria associated cardiovascular risk, even after correction for traditional cardiovascular risk factors including triglycerides and HDL-C. Interestingly, LPLS447X mediated cardiovascular protection was only observed in males and not in females. This is presumably the result of low baseline female risk in combination with relative short follow-up time. Presumably, the lifelong LPLS447X mediated beneficial effect on lipid modulates risk at later years, at least in males.

Chapter eight examines the relation between circulating LPL protein and future cardiovascular disease in a large prospective cohort using data from the EPIC-Norfolk Prospective Population Study. The LPL protein is shown to play an important role in the protection against development of atherosclerosis. Also, reduced levels of serum LPL are associated with an increased risk for future cardiovascular risk. In this chapter, high LPL concentrations are suggested to be beneficial for cardiovascular outcome, potentially via the effects on lipids.

Conclusions part II: LPL plays a crucial role in the process of vascular restenosis and CVD risk. On the short-term, the LPLS447X effects protect against restenosis after a coronary intervention. On the long-term, the lipid lowering LPLS447X effects protect against development of lipid associated cardiovascular risk in males with albuminuria. The pre-heparin LPL concentration is an important predictor of future CVD risk. These findings culminate into a novel paradigm:

_The higher the LPL concentration, the better_

Part III: Lipoprotein Lipase Deficiency; From Diagnosis to Management

Chapter nine describes the full clinical, biochemical and molecular analyses of severe hypertriglyceridemic individuals in one Turkish and three Chinese families. One novel mutation (delCT_{1312-1313}) is established, as well as a new combination of mutations (S193R and I194T) and four previously reported mutations (L252R, L252V, S193R and I194T). The delCT_{1312-1313} LPL gene mutation is shown to be associated with absence of LPL activity via lacking of LPL
protein. Currently, effective therapy for this disease is lacking and LPL deficient patients regularly suffer from recurrent pancreatitis and in some cases even die during such an episode. The only potential risk modulator currently available is a very strict low-fat diet to which absolute adherence is virtually impossible, resulting in frequent costly hospitalisations, and occasionally even in death. Clearly, novel therapeutical modalities are urgently needed.

Chapter ten describes the clinical presentation of LPL deficiency. This condition is associated with extreme hypertriglyceridemia and recurrent pancreatitis for which currently no effective treatment exists. Also, the preclinical investigations in animal models are summarized and the rationale to evaluate gene therapy for this monogenetic disorder of lipid metabolism in humans is described.

Finally, chapter eleven describes the proof of principle or preclinical investigations of LPL gene therapy in animal models in more detail and includes results from toxicology and biodistribution studies. In addition, the first ex vivo LPL gene therapy results are presented as well as detailed characterization of the patients that are aimed to be included in this human LPL gene therapy study, which was actually started in august 2005. The study will expectedly finish in the first quarter of 2007.

Conclusions part III: LPL deficiency needs novel therapeutical interventions in order to properly reduce the risk for recurrent pancreatitis. Data are presented to show that, in fact, LPL gene therapy is a promising and feasible option. So, the novel paradigm may be fulfilled:

More LPL can be established via gene therapy, preferably the LPLS447X variant
Samenvatting

In het eerste deel van dit proefschrift worden de gunstige effecten van de LPLS447X-variant op lipiden bestudeerd. Potentiële mechanismen waardoor deze gunstige effecten worden veroorzaakt, worden besproken. De aandacht gaat specifiek uit naar het effect van deze mutatie op het triglyceridenmetabolisme. De effecten van deze LPLS447X op het toekomstig cardiovasculaire risico bij verschillende groepen mensen met een verhoogd cardiovasculair risico passeren in het tweede deel de revue. Het derde en laatste deel van dit proefschrift beschrijft de effecten van het volledig ontbreken van LPL-functie, LPL-deficiëntie, van diagnose van deze aandoening tot een bescheiden introductie van nieuwe therapeutische interventie: LPL-gentherapie.

Deel I: De wijze waarop Lipoproteïne Lipase S447X gunstig werkt

De huidige inzichten in LPLS447X betreffende de gunstige effecten op lipoproteïnen en op traditionele risicofactoren worden in hoofdstuk twee beschreven. Verder komen de potentiële mechanismen achter de gunstige effecten van deze frequent voorkomende LPL-variant aan bod.


In de volgende twee hoofdstukken wordt bestudeerd of er bij LPLS447X inderdaad sprake is van toegenomen enzymatische dan wel niet-enzymatische effecten op het apoB100- en apoB48-triglyceridenmetabolisme. In hoofdstuk vier wordt het apoB100-metabolisme bestudeerd in vijf homozygote LPLS447X-dragers tijdens gevoede toestand via infusie van de stabiele isotoop L-[1-13C]-valine. De homozygote dragers blijken een verhoogde omzetting van apoB100-gelabelde triglyceridenrijke lipoproteïnen te hebben. Daarnaast is er tevens sprake van een
verhoogde LDL-klaring evenals een verhoogde plasma LPL-concentratie welke voornamelijk gebonden blijkt aan de apoB100-fractie. Deze resultaten tezamen wijzen uit dat er bij dragers van deze LPL-variant inderdaad sprake is van toegenomen enzymatische en niet-enzymatische functie van het LPLS447X-eiwit. In hoofdstuk vijf worden de effecten van LPLS447X op het apoB48-metabolisme onderzocht. Hiervoor worden dezelfde vijf homozygote LPLS447X-dragers bestudeerd tijdens gevoede toestand via infusie van de stabiele isotoop L-\([1-^{13}C]\)-valine zoals dat ook beschreven is in hoofdstuk vier. LPLS447X blijkt, zoals verwacht, geassocieerd te zijn met een toegenomen apoB48-klaring. Hiermee is direct aangetoond dat de toegenomen LPL-functie van LPLS447X niet alleen een gunstig effect heeft op het apoB100-metabolisme, maar ook op het apoB48-metabolisme. Belangrijker nog, hiermee is het bewijs geleverd dat de gunstige effecten van deze LPL-variant geassocieerd zijn met toegenomen enzymatische en ook een toegenomen niet-enzymatische effecten van LPLS447X.

**Conclusies deel I:** de gunstige effecten van LPLS447X worden veroorzaakt door zowel een toegenomen enzymatische LPL-functie (toegenomen omzetting apoB100-gelabelde triglyceridenvervoerende deeltjes), als ook door een toegenomen niet-enzymatische LPL-ligandfunctie (toegenomen apoB48- en toegenomen LDL-klaring).

**Deel II: LPLS447X is gerelateerd aan diverse gunstige fenotypes**

Om het klinische belang van de LPLS447X-variant aan te tonen, beschrijft hoofdstuk zes de impact van polymorfismen in het LPL-gen op restenose, gedefinieerd als target vessel revascularisatie (TVR) na een percutane coronaire interventie (PCI). Hiervoor wordt gebruikt gemaakt van data van de GENDER (GENetic DEterminants of Restenosis) studie, een multicenter prospectieve studie waarin patiënten na succesvolle PCI werden geïncludeerd (n=3104). Dragerschap van LPLS447X is geassocieerd met een lager risico op TVR vergeleken met niet-dragers. In een muismodel voor restenose blijkt tevens dat na een dergelijke interventie de hoeveelheid LPL-mRNA in de wand van dat vat 40-maal verhoogd is in vergelijking met een vat waarin geen enkele interventie is gedaan. Uiteindelijk is de conclusie dat LPLS447X een belangrijke bescherming biedt tegen het ontstaan van restenose na een percutane coronaire interventie bij mensen. Daarnaast is de sterke opregulatie van LPL in de arteriële vaatwand na percutane coronaire interventie bewijs voor de belangrijke rol van LPL bij het proces van restenose.
Om de waarde van LPLS447X op het cardiovasculaire risico bij personen met een primair verhoogd cardiovasculair risico vast te stellen, worden in hoofdstuk zeven de effecten van LPLS447X op het toekomstige cardiovasculair risico in gezonde personen met toenemende albuminurie zonder hart- en vaatziekten bestudeerd. Daar toenemend albuminurie is geassocieerd met een verhoogd cardiovasculair risico en LPLS447X is geassocieerd met een verlaagd cardiovasculair risico, zal LPLS447X theoretisch het cardiovasculaire risico bij albuminurie kunnen beïnvloeden. Om deze theorie te testen, is gebruik gemaakt van data van de PREVEND (Prevention of Renal and Vascular Endstage Disease) studie, een prospectieve op algemene populatie gebaseerde cohort studie (n=8592). De LPLS447X-dragers blijken een lager microalbuminurie-geassocieerd cardiovasculair risico te hebben dan de niet-dragers, zelfs na correctie van dit risico met traditionele cardiovasculaire risicofactoren. Opvallend is dat deze LPLS447X-gemediateerde cardiovasculaire bescherming alleen bij mannen aangetoond kan worden. Dit is waarschijnlijk het gevolg van het lage cardiovasculaire risico van vrouwen in deze studie, evenals de relatief korte volgtijd van deze studie. Duidelijk is wel, dat de levenslange gunstige effecten van deze LPL-variant op de lipidenprofiel uiteindelijk een gunstig effect hebben op het risico op hart- en vaatziekten op latere leeftijd.

Hoofdstuk acht bestudeert de relatie tussen de concentratie van het LPL-eiwit in plasma en het toekomstige risico op hart- en vaatziekten in een groot prospectief cohort (de EPIC-Norfolk Prospective Population Study). Het LPL-eiwit blijkt een belangrijke rol bij de bescherming tegen atherosclerose te spelen. Verder blijkt dat een verlaagd plasma LPL-concentratie geassocieerd is met een toegenomen risico op toekomstig cardiovasculair lijden. In dit hoofdstuk wordt ook gesteld dat een hoge concentratie van LPL gunstig is voor het cardiovasculaire risico en dat dit waarschijnlijk wordt veroorzaakt door de gunstige effecten van het LPL-eiwit op het lipidenprofiel.

Conclusies deel II: LPL speelt een essentiële rol in de ontwikkeling van vasculaire restenose en het toekomstig cardiovasculaire risico. Op de korte termijn verlaagt LPLS447X het risico op de ontwikkeling van een vasculaire restenose na een percutane coronaire interventie. Op langere termijn beschermt LPLS447X via gunstige effecten op het lipidenprofiel tegen de lipiden-gemedieerde ontwikkeling van hart- en vaatziekten bij mannen met albuminurie. De pre-heparine LPL-concentratie heeft een belangrijke voorspellende waarde op toekomstig cardiovasculair risico. Deze bevindingen leiden tot de vorming van een nieuw concept:

**Hoe hoger de LPL-concentratie, hoe beter**
Deel III: Lipoproteïne Lipase Deficiëntie, van diagnose tot behandeling

Hoofdstuk negen beschrijft de volledige klinische, biochemische en moleculaire analyses van personen met een ernstige hypertriglyceridemie in een Turkse en in drie Chinese families. Een nieuwe LPL-mutatie (delCT<sup>1312-1313</sup>) wordt beschreven, evenals nieuwe combinatie van LPL-mutaties (S193R and I194T) en vier eerder beschreven LPL-mutaties (L252R, L252V, S193R and I194T). De delCT<sup>1312-1313</sup> LPL gen-mutatie blijkt geassocieerd te zijn met totale afwezigheid van LPL-activiteit door het ontbreken van LPL-eiwit. Op dit moment bestaat er geen effectieve behandeling voor deze potentieel dodelijke aandoening. De enige optie is een strikt vetarm dieet, welke in de praktijk niet vol te houden blijkt. Dit resulteert in herhaaldelijk dure ziekenhuisopname en leidt soms zelfs tot de dood. Het is duidelijk dat er snel effectieve therapeutische behandelopties moeten komen.


Conclusies deel III: LPL-deficiëntie heeft dringend nieuwe therapeutische interventies nodig om het risico op recidiverende pancreatitis adequaat te kunnen bestrijden. LPL-gentherapie lijkt voor de aandoening tot dusver een geschikte kandidaat te zijn. De toekomst zal uitwijzen of er voldaan kan worden aan het allernieuwste concept:

_Meer LPL door gentherapie met de LPLS447X-variant_
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