# Genetic, clinical and experimental aspects of restenosis : a biomedical perspective

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# Lipoprotein lipase gene polymorphisms and the risk of target vessel revascular ization after percutaneous coronary intervention

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# Abstract

## Background

Variations in the lipoprotein lipase (LPL)-gene have been implicated in a num ber of pathophysiological conditions associated with coronary heart disease. The present study examines the impact of polymorphisms in the LPL-gene on reste nosis (defined by target vessel revascularization, TVR) in a large patient-popula tion 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 success ful PCI. These patients were genotyped for four different LPL gene polymor phisms. In apolipoprotein E(ApoE)*3-Leiden transgenic mice, arterial mes senger ribonucleic acid (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 with 447Ser homozygotes (p=0.005). In the mouse model, LPL mRNA levels were increased 40-fold compared with control arteries at 6 h 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 humans. The role of LPL in this process was fur ther established in a mouse model, where LPL expression was very strongly up-regulated in the target arterial wall, suggesting a contribution of this lipolytic en zyme 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.[1] The hydrolytic function of LPL is essential for the processing of triglyceride-rich chylomicrons and very-low density lipoproteins (VLDLs) to remnant particles and also for the transfer of phospholipids and apolipoproteins to high-density lipoprotein (HDL). Furthermore, LPL plays a key role in the receptor-mediated removal of lipoproteins from the circulation.[2] The gene cod ing for LPL, located on chromosome 8p22, encompasses 10 exons and is rather polymorphic.[3] Abnormal LPL function has been reported to be associated with a number of pathophysiological conditions that underlie coronary heart dis ease.[4;5] 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 choles terol levels, which in turn are implicated in atherosclerotic risk.[6;7]

Percutaneous coronary intervention (PCI), an important treatment for patients with atherosclerosis, is limited by the development restenosis, despite the ad vent of drug-eluting stents. There is increasing evidence that inherited factors may partly explain the excessive risk of restenosis in certain patients. Identify ing such patients may improve stratification of patients to a more individually tailored treatment.[8;9]

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 consecu tive study population whether four different well-known variants in the LPL-gene, denoted as -93T/G, Asp9Asn, Asn291Ser and 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-messenger ribonucleic acid (mRNA) expression in the restenotic vessel in an established mouse model for restenosis.[10-12]

# *Methods*

## Study design

The present study population has been described previously.[13] In brief, the GE Netic DEterminants of Restenosis project (GENDER) 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-segment elevation acute coronary syndromes or silent ischemia by PCI. Patients treated for acute ST-segment elevation myocardial infarction were ex cluded. All patients were treated in four of the 13-referral centers for interven tional 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 in formed consent was obtained from each participant before the PCI procedure.

## PCI procedure

Standard angioplasty and stent placement were performed by experienced oper ators using a radial or femoral approach. Before the procedure, patients received 300 mg aspirin and 7,500 IU heparin. The use of intracoronary stents and addi tional medication, such as glycoprotein IIb/IIIa inhibitors, was at the discretion of the operator. In case of stent implantation, patients received either ticlopi dine 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. Target vessel revascularization (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, as these events were attributable mainly to subacute 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 follow ing standard procedures. The LPL G/A, A/G and the C/G polymorphisms in exons 2, 6 and 9, respectively, resulting in the following amino acid substitutions; Asp9Asn, Asn291Ser and Ser447Ter respectively, were determined by validated multilocus genotyping assay (Roche Molecular Systems, Alameda, California, USA).[14] A similar method was used to detect the LPL -93T/G promoter poly morphism. All four polymorphisms were selected on the basis of their previously described relation to coronary artery disease and/or their influence on LPL ac tivity.[14;15] 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 detec tion method based on incubation with streptavidin-horseradish peroxidase con jugate, 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 rep licate 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). The HDL choles terol level was determined by a turbidimetric assay on a Hitachi 911. Low-density lipoprotein cholesterol was calculated according to the equation of Friedewald et al.[16] Blood was drawn before the PCI procedure. Two of the four participating centers (Leiden University Medical Center and Academic Hospital Maastricht) systematically collected 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 apolipoprotein E (ApoE)*3-Leiden transgenic mice after cuff placement.[10-12] Before cuff placement, the mice were fed a Western-type diet containing 1% cholesterol and 0.05% cholate

(Hope Farms, Woerden, the Netherlands) three weeks before surgery and con tinued after surgery in order to obtain stable plasma cholesterol levels. This diet results in a human-like lipoprotein profile.[10] 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 pro tocol (Invitrogen, Breda, the Netherlands). Subsequently, cDNA synthesis of all RNA samples was achieved using Ready-To-Go real-time (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'TCCACCTCCGTGTAAATCAAGA 3') and probe (5'TTCCCTTCACCCT GCCCGAGGTT 3') for mouse LPL gene were designed using Primer Express™ 1.5 software (Perkin-Elmer Applied Biosystems, Foster City, California, USA). The housekeeping genes, hypoxanthine-guanine phosphoribosyl transferase, cy clophilin and GAPDH were used as controls. RT-PCR was performed on an ABI Prism™ 7700-sequence detection system (Perkin Elmer Biosystems, Bos ton, 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. The RT-PCR analysis was performed using RT-PCR mastermix (Eurogen tec, 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 sam ple 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 co-variates was performed at this stage to al low 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 multivari able regression analysis.[17] The LPL polymorphisms were combined into haplo

types, and the effect of haplotypes on restenosis risk was estimated according to the methods developed by Tanck et al.[18]
Multivariable regression analysis of the TVR risk was performed on all polymor phisms 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, low-density lipoprotein (LDL) cholesterol, and triglyceride levels.
Animal data are presented as mean ± SEM. Data were analysed using the Mann-Whitney U-test.
A p-value < 0.05 was considered statistically significant. Statistical analysis was carried out using SPSS version 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 experi enced 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 Ser447Ter (C/G). Successful DNA genotyping was possible in 3,028, 3,031, 3,021 and 3,054 patients, respectively. The results of the remain ing patients were missing due to lack of DNA or inconclusive genotyping. The frequencies of the rare -93G, 9Asn, 291Ser and 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 1. The low frequency of carriers of 9Asn and 291Ser has prompted us to pool heterozygotes and homozygotes for these two LPL variants. No statistically sig nificant 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). Furthermore, hetero zygotes and homozygotes for the allele encoding for the 9Asn LPL variant used

less lipid-lowering medication but had a higher use of beta-blocker medication.

**Table 1. Baseline clinical characteristics of the patients according to the genotypes of LPL polymorphisms**

| | LPL G/A (Asp9Asn) | | LPL A/G (Asn291Ser) | | LPL C/G (Ser447Ter) | | |
|---|---|---|---|---|---|---|---|
| (N) | GG (2,933) | GA/AA (98) | AA (2,866) | AG/GG (155) | CC (2,452) | CG (571) | GG (31) |
| Age(years) | 62±10.7 | 62±10.8 | 62±10.7 | 62±11.0 | 62±10.7 | 62±10.6 | 62±11.2 |
| Male | 71.3 | 72.4 | 71.3 | 72.9 | 71.4 | 71.6 | 74.2 |
| Hypertension | 40.7 | 33.7 | 40.3 | 43.2 | 41.1 | 38.2 | 32.3 |
| Hypercholesterolemia | 60.7 | 67.3 | 60.7 | 65.8 | 61.8 | 56.9 | 61.3 |
| Diabetes | 14.5 | 15.3 | 14.5 | 14.8 | 14.8 | 14.0 | 3.2 |
| Current smoker | 24.5 | 21.4 | 24.6 | 20.6 | 25.2 | 21.4 | 25.8 |
| Family history | 35.3 | 34.7 | 35.4 | 33.5 | 34.8 | 37.3 | 35.5 |
| Previous MI | 39.9 | 34.7 | 39.7 | 41.3 | 39.8 | 39.9 | 38.7 |
| Previous CABG | 12.3 | 8.2 | 12.1 | 14.2* | 11.9 | 13.5 | 9.7 |
| Lipid lowering medication | 54.0 | 65.3* | 54.4 | 53.5 | 55.1 | 51.5 | 45.2 |
| β-blocker medication | 79.0 | 65.3* | 78.7 | 75.5 | 78.8 | 77.8 | 71.0 |

*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*

Lesion-related and procedural parameters of the genotype groups are presented in table 2. The only significant difference was found for carriers of the allele encoding for 9Asn, who were treated more often for total occlusion compared with 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 with the other genotypes ($p=0.002$). In fact, we observed a gene dosage effect emphasizing a relationship between these two parameters.

**Table 2. Lesion and Procedural Characteristics at the Time of Intervention According to the Genotypes of the LPL Polymorphisms**

| | LPL G/A (Asp9Asn) | | LPL A/G (Asn291Ser) | | LPL C/G (Ser447Ter) | | |
|---|---|---|---|---|---|---|---|
| (N) | GG (2,933) | GA/AA (98) | AA (2,866) | AG/GG (155) | CC (2,452) | CG (571) | GG (31) |
| Restenotic lesion | 6.8 | 5.1 | 6.8 | 5.8 | 7.1 | 5.6 | 3.2 |
| Total occlusion | 13.6 | 21.4* | 13.7 | 14.2 | 13.9 | 13.8 | 6.5 |
| Type C lesion | 25.5 | 32.7 | 25.7 | 25.2 | 26.1 | 24.5 | 12.9 |
| LAD proximal | 22.3 | 18.4 | 22.0 | 24.5 | 22.3 | 21.9 | 25.8 |
| RCX | 26.9 | 30.6 | 27.1 | 26.5 | 27.2 | 26.8 | 12.9 |
| Multivessel disease | 46.2 | 48.0 | 46.3 | 45.5 | 47.6 | 42.0 | 22.6* |
| Stable angina | 33.1 | 28.6 | 33.1 | 31.6 | 32.5 | 34.9 | 38.7 |
| Residual stenosis >20% | 11.6 | 10.2 | 11.7 | 9.2 | 11.3 | 12.2 | 3.2 |
| Stenting | 74.5 | 72.4 | 74.6 | 71.6 | 74.5 | 75.1 | 67.7 |
| Glycoprotein IIb/IIIa antagonist | 26.4 | 25.5 | 26.3 | 28.4 | 26.5 | 25.9 | 22.6 |

**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*

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 (relative risk (RR) 0.6, 95% confidence interval (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 3).

**Table 3. Univariate Analysis of LPL Polymorphisms in Association with TVR and the Distributions of the Polymorphisms**

| Polymorphisms | Number of patients | TVR (%) | Model used | P-value |
|---|---|---|---|---|
| Asp9Asn | 3,031 | | Dominant | 0.77 |
| GG | | 9.7 | | |
| GA/AA | | 10.4 | | |
| Asn291Ser | 3,021 | | Dominant | 0.62 |
| AA | | 9.9 | | |
| AG/GG | | 8.5 | | |
| Ser447Ter | 3,054 | | Dominant | 0.004 |
| CC | | 10.5 | | |
| CG | | 7.0 | | |
| GG | | 0 | | |

*TVR, target vessel revascularization; LPL, lipoprotein lipase, Asp, aspartic acid; Asn, asparagine; Ser, serine; Ter, stop*

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 genotype (such as age, gender, diabetes, stenting, residual stenosis>20%, current smoking, total occlusion, lipid lowering medication, beta-blocker use, multivessel disease and previous CABG).[13] This backward stepwise selection yielded similar results (RR 0.6, 95%CI: 0.44-0.86) (Table 4).

**Table 4. Multivariable Cox Regression of Clinical Variables and LPL Ser447Ter Polymorphism Associated with TVR**

| | | 95% CI | | |
|---|---|---|---|---|
| | RR | Low | High | p-value |
| Diabetes | 1.52 | 1.14 | 2.01 | 0.004 |
| Current smoker | 0.73 | 0.54 | 0.97 | 0.03 |
| Stenting | 0.76 | 0.58 | 1.00 | 0.05 |
| Total occlusion | 1.51 | 1.12 | 2.02 | 0.006 |
| Residual stenosis>20% | 1.35 | 0.97 | 1.89 | 0.08 |
| LPL 447Ter | 0.62 | 0.44 | 0.86 | 0.005 |

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

Furthermore, because the severity of the stenosis before angioplasty as well as (especially) the severity of the stenosis immediately after 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-per cent stenosis values did not differ significantly between the three genotypes of the 447 polymorphism, (p=0.75 and p=0.83, respectively).

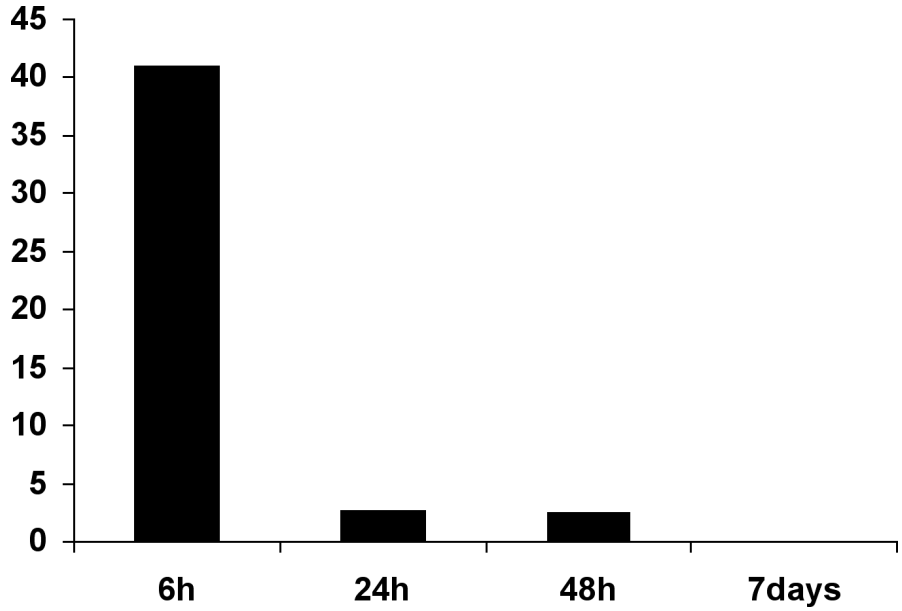
The polymorphisms were also combined into haplotypes for further analysis. Seven out of eight possible haplotypes were indeed observed (data not shown). The 9G/291A/447C haplotype was most common in both TVR cases and con trols (89.7% and 85.1%, respectively). A smallest RR was seen with respect to the 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 of lipid levels in this subgroup analysis had no influence on the association of the different polymor phisms and TVR.

Subsequently, we evaluated if LPL gene expression played a role in the devel opment 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 mmol/L.
The mRNA encoding LPL, isolated from the cuffed right femoral artery and un treated left femoral artery, was quantified at various time points after cuff place ment (Figure 1). The mRNA encoding LPL showed peak expression 6 h after cuff placement, where it showed a 40-fold increase compared with the normal artery. The LPL mRNA levels were back to baseline after 24 h of the induction of the restenotic process. Sham-operated vessels (femoral artery prepared free, but without cuff placement) showed essentially the same results as non-operated vessels.

**Figure 1. LPL mRNA expression in cuffed femoral artery (as compared to HPRT mRNA expression, cuff vs normal artery)**




# *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.[3] This polymorphism has been shown in most but not all studies to modulate

lipid levels, as well as LPL.[2;4;19-25] In fact, it was shown that this polymorphism is associated with decreased triglycerides, increased HDL cholesterol, and a de creased risk of coronary artery disease.[4;26;27] Furthermore, a recent meta-analy sis confirmed that the Ser447Ter variant has an effect on the lipoprotein pro file, by decreasing plasma triglycerides and increasing HDL cholesterol.[4] 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.[2;4;21;28;29]

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, which may influence overall catalytic activity, whereas the Asn291Ser sub stitution is located in a heparin-binding cluster and may thus affect the interac tion of LPL and 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, possibly forming the basis of the observed association.[4;7]

In our study, we did not find a significant association between the LPL447 poly morphism and HDL and LDL cholesterol and triglycerides levels (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 increas es monocyte adherence via a mechanism that requires interaction between the C-terminal domain of the LPL, heparin sulfate proteoglycans and integrins.[30-32] However, Zhang et al. showed that the affinity of LPL Ser447Ter for heparin sul fate proteoglycans was not different from wild-type LPL.[29] Because LPL is also expressed in smooth muscle cells in the arterial media [33], 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[34], 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 endotheli al nitric oxide synthase (eNOS) activity, the key enzyme in basal endothelial cell NO production. Nitric oxide dilates coronary arteries and promotes blood flow by inhibiting smooth muscle contraction, platelet aggregation and platelet adhe sion to endothelial cells by a cyclic guanosine monophophate (cGMP)- mediated mechanism.[35;36] In fact, LPL has been reported to increase NOS 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.[1;37] Therefore, mutated levels of LPL, as observed with the Ser447Ter polymorphism, may be beneficial for endothelial function, an important contributor involved in restenosis.[38]

In addition, Ziouzenkova et al. found a link between LPL and peroxisome pro liferators-activated receptor (PPAR) activation, suggesting that impaired LPL enzymatic activity might decrease endogenous PPAR-alpha activation and its subsequent downstream effects, including anti-inflammatory responses.[39] In flammation has been previously reported as a very important component of re stenosis.[40-42]

Clee et al. found a decrease in blood pressure independent of the lower level of triglycerides in patients with LPL Ser447Ter polymorphism.[43] Another study also showed that LPL Ser447Ter polymorphism was associated with lower sys tolic blood pressure and pulse pressure levels in women.[34] Because inflamma tion[40-42] and elevated blood pressure[44] have been implicated in increased risk for restenosis, any positive effect of this polymorphism on arterial tone, inflam matory status, and elevated blood pressure may ultimately translate into lesser risk for restenosis.

Because we hypothesize a potential interaction between LPL activity by geno type and inflammatory activity, we also investigated several inflammatory mark ers, which could be of influence on the development of restenosis and their ef fect 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 1,000 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 poly morphisms.

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 with control arteries at 6 h 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, owing to a number of reasons. Circulating LPL protein levels, were not assessed here, as basal (pre-PCI) plasma measurements of the gene product are not likely to re flect the genetically determined differences in reaction to a trauma such as PCI. Moreover, local differences in LPL sensitive reactions, such as those 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 early 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 polymor phisms 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 with PCI, it results in intimal injury. It is not certain to what extent these apparently different ways of vascular injury differ in their reaction regard ing 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 character istic 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: 2,914 for T/T, 109 for T/G, and 5 for G/G, as opposed to the expected num ber of patients: 2,910 for T/T, 117 for T/G, and 1 for G/G. This discrepancy could be explained by the fact that it is possible that this polymorphism is associated with one or more inclusion/exclusion criteria; however, we cannot confirm this with our data, as all participants fulfilled those criteria. The most important inclusion criterion was that all participants were scheduled for PCI, and obvi ously the classic risk factors of cardiovascular disease will be enriched in such a population. The -93T/G polymorphism is sometimes found to be especially as

sociated with HDL2 cholesterol, and ApoA-I levels with lower levels in -93G/G carriers.[45] Based on this assumption, one might expect more heterozygote or homozygote carriers, and the latter were indeed slightly more present than ex pected.

Drug-eluting stents are now more widely used. In our study, we do not have any data on these stents, as 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 drug-eluting stents are probably different from the process of restenosis after bare-metal stent placement or plain balloon angioplasty. There fore, new studies have to be set up to investigate genes involved in the process of restenosis after drug-eluting stents.

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*

We have demonstrated, that LPL is significantly associated with TVR. The LPL C/G polymorphism, which results in a 447 Serine→Stop (X) mutation, ap pears to be an important independent protective factor in this. Furthermore, LPL-mRNA was highly up-regulated in the first six h 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.

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