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Gender-related association between the −93T→G/D9N haplotype of the lipoprotein lipase gene and elevated lipid levels in familial combined hyperlipidemia

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Abstract

Familial combined hyperlipidemia (FCHL) is a frequent cause of premature coronary artery disease. Affected family members are characterized by different combinations of elevated cholesterol and/or triglyceride levels. A reduction in lipoprotein lipase (LPL) activity has been observed in a subgroup of FCHL patients. Recently, we have demonstrated an increased frequency of mutations in the LPL gene in Dutch FCHL patients compared to normolipidemic controls. In the present study, we have applied a pedigree-based maximum likelihood method to study the effect of LPL mutations on the phenotypic expression of FCHL in families. In 40 FCHL probandi, three different previously reported mutations in the LPL gene were identified resulting in amino acid changes, D9N, N291S, and S447X. The D9N mutation in exon 2 appeared to be in strong linkage disequilibrium with a T→G substitution at position −93 in the promoter region of the LPL gene. We present data that the −93T→G/D9N haplotype is associated with significantly higher levels of LDL and VLDL cholesterol, and VLDL triglycerides. Interestingly, the effect was only observed in male carriers. In line with our previous observations, these results further sustain that the LPL gene is a susceptibility gene for dyslipidemia which explains part of the variability in the phenotype observed among FCHL family members.

Keywords: Familial combined hyperlipidemia; Lipoprotein lipase; Family study; Mutation analysis; Genetic predisposition

1. Introduction

Familial combined hyperlipidemia (FCHL) is a common lipid trait found among survivors of premature myocardial infarction. In the general population, FCHL occurs with an estimated frequency of 0.3–2% [1,2]. Goldstein et al. [1] and Rose et al. [2] were the first to show that the FCHL syndrome was distinct from familial hypercholesterolemia and familial hypertriglyceridemia.

In FCHL families, affected relatives exhibit different combinations of elevated plasma cholesterol and elevated triglyceride levels. In addition, characteristics such as increased very low density lipoprotein (VLDL) production, increased plasma apolipoprotein (APO) B100, predominance of small dense low density lipoprotein (LDL), and insulin resistance have been de-

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scribed [3–6]. Based on the pattern of inheritance of elevated levels of either VLDL, LDL or both, FCHL was suggested to be an autosomal dominant disorder [1]. In addition, complex segregation analysis provided evidence for a major gene acting primarily on triglycerides [7].

Babirak et al. [8] showed that a subgroup of FCHL patients had reduced lipoprotein lipase (LPL) activity. LPL is the rate-limiting enzyme for hydrolysis of the triglyceride core in circulating chylomicrons, and VLDL [9]. The human LPL gene has been localized on chromosome 8p22 and spans approximately 30 kb. The gene consists of 10 exons encoding a mature protein of 448 amino acids [10–12]. Several mutations leading to amino acid substitutions have been reported in patients with LPL deficiency. LPL deficiency is a rare autosomal recessive disorder characterized by a massive accumulation of chylomicrons in plasma [9]. Mild hyperlipidemia and variable lipid phenotypes, resembling the lipid phenotype in FCHL, have been observed in heterozygous carriers of rare LPL mutations [13–16].

In several studies among patients with dysbetalipoproteinemia, combined hyperlipidemia, and hypertriglyceridemia the role of LPL in the development of these, more common, types of hyperlipidemia has been investigated. A number of missense mutations in the LPL gene were found to be associated with elevated lipid levels in these patients [17–22]. Since these mutations were also detected with a relatively high frequency in apparently healthy control populations [19,20], this indicates that heterozygosity for these LPL mutations requires the presence of additional genetic and/or environmental factors for the expression of hyperlipidemia. For instance, Ma et al. [23] showed that partial LPL deficiency in combination with an APOE*2 allele can lead to severe chylomicronemia during pregnancy. In addition, an interaction of the N291S mutation with body mass index (BMI) resulting in a more severe hyperlipidemic phenotype has also been observed [18].

So far, most studies have been performed using unrelated FCHL patients. In order to further investigate the contribution of LPL to the expression and variability of the FCHL phenotype we have analyzed families. Therefore, we have screened the coding sequence and promoter region of the LPL gene for mutations in 40 FCHL probandi. Subsequently, using a pedigree-based maximum likelihood method, it became possible to investigate the phenotypic expression of the LPL mutation among family members. In this study, we have focussed on a haplotype containing a mutation in the promoter region, the -93 T$\rightarrow$G, as well as an amino acid substitution, D9N, in exon 2.

2. Methods

2.1. Subjects

FCHL probandi were selected from patients attending the University Lipid Research Clinics at the Universities of Nijmegen and Amsterdam, fulfilling the following criteria: (i) elevated levels of both total cholesterol and triglycerides (at first measurement; cholesterol and/or triglyceride levels above the 90th percentile using the age- and sex-related percentile levels of the Prospective Cardiovascular Münster (PROCAM) study [24], (ii) a personal or family history of premature cardiovascular disease, and (iii) at least one first degree relative with elevated total cholesterol and/or triglycerides levels. None of the FCHL probandi had specific clinical signs, like tendon xanthomata, and none were homozygous for the APOE*2 allele. For all probandi, a secondary cause of hyperlipidaemia was excluded by standard laboratory tests. The study protocol was approved by the ethical committee of the universities of Amsterdam, Leiden and Nijmegen and all patients gave informed consent.

2.2. Measurement of lipids, lipoproteins and apolipoproteins

EDTA blood samples were obtained from the probandi and family members after overnight fasting. No lipid lowering drugs were administered to the subjects for 6 weeks prior to the onset of the study. Plasma was separated from cells by centrifugation at $500 \times g$ for 10 min at room temperature, and used immediately for lipid and lipoprotein analysis. VLDL ($d < 1.006$ g/ml) was isolated by ultracentrifugation for 16 h at 36000 rpm in an fixed-angle TFT 45.6 rotor (Kontron, Zurich) [25]. Plasma and lipoprotein cholesterol and triglyceride concentrations were determined by enzymatic, commercially available reagents (No. 237574; Boehringer-Mannheim, FRG; Sera-pak, No. 6639; Tournai, Belgium). HDL-cholesterol was determined in whole plasma using the polyethylene glycol 6000 method [26]. LDL cholesterol was subsequently calculated by subtracting HDL- and VLDL cholesterol from total cholesterol. The apolipoproteins apo A1 and apo B were determined by rate immunonephelometry as previously described for apo B [27].

2.3. DNA analysis of the LPL gene.

Genomic DNA was isolated from leucocytes according to Miller et al. [28]. All exons, intron/exon boundaries and the promoter region, between positions -12 and -475, of the LPL gene were amplified by polymerase chain reaction (PCR) and products were screened for mutations using denaturing gradient gel

electrophoresis (DGGE) as has been described previously [20]. PCR fragments showing changes in the band pattern with DGGE, were sequenced to determine the nucleotide change as described previously [20]. Identification of carriers of the −93 T→G promoter mutation was performed by PCR using the primers: 5′-GGC AGG GTT GTT CCT CAT TAC TGT T-3′ (sense) and 5′-GAC ACT GTT TTC ACG CCA AGG CTG C-3′ (anti-sense) [10]. The reaction mixture included 15 pmol of each primer, 0.5 μg genomic DNA, 0.2 mM of each dNTP, 10 mM Tris–HCl; pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% (w/v) gelatin, 0.1% Triton X-100, 1 unit *Taq* polymerase (Amplitaq, Perkin Elmer), in a total volume of 50 μl. Amplification was performed for 32 cycles of 30 s at 94°C, 30 s at 50°C and 1 min at 72°C, with an initial denaturation period of 3 min. Some 20 μl of PCR products were digested with the restriction enzyme *Ava*II according to recommendations of the supplier (Pharmacia). Thereafter, fragments were separated on a 4% MP agarose gel (Boehringer Mannheim, FRG) and stained with ethidium bromide. In the case of the mutant allele an *Ava*II restriction site is abolished and therefore, digestion of the PCR product will reveal one fragment of 189 bp for the mutant allele, and two fragments of 137 and 52 bp for the normal allele.

To identify carriers for the D9N mutation among relatives of the FCHL patients, PCR analysis was performed with primers: 5′-CTC ATA TCC AAT TTT TCC TT-3′ (sense); 5′-GGG GTC AGG GCA AAT TTA CTT TC*A* ATG-3′ (anti-sense). The 3′ primer was designed with a nucleotide mismatch (underlined) as compared to the wild type LPL sequence [12] to abolish a second *Taq*I site. The 3′-primer was elongated with a 20 basepair AT-tail for optimal visualization of the digested fragments. After amplification, the PCR-product was digested with *Taq*I, and subsequently fragments were separated on a 4% agarose gel. In case of the D9N mutation, two fragments of 74 and 40 bp could be detected.

To identify carriers for the S447X mutation, PCR analysis was performed using primers: 5′-CAT CCA TTT TCT TCC ACA GGG-3′ (sense); 5′-GCC CAG AAT GCT CAC CAG ACT-3′ (anti-sense). After amplification, the PCR-product was digested with *Hin*fI, and subsequently fragments were separated on a 4% agarose gel. The 3′-primer was elongated with a 20 basepair AT-tail for optimal visualisation of the digested fragment. In case of the S447X, two fragments could be detected. Identification of the N291S mutation was performed as described previously [29].

2.4. Statistical analysis

To test for the statistical significance of the effect of the haplotype containing the −93 T→G and D9N mutations on lipoprotein traits using all carriers in multiple families, a pedigree-based maximum likelihood method, developed by Lange et al. [30,31], was used as has been described previously [29]. The Fisher package [30] was used for the genetic modeling. The most general (full) model allowed for the effects of (i) age, (ii) gender, (iii) N291S, and (iv) −93T→G/D9N haplotype. Age was modeled as a regression, whereas based on gender and carrier status of N291S or −93T→G/D9N haplotype, eight categories ($2 \times 2 \times 2$) could be discriminated. These eight categories could be reduced to six, as there were no individuals (neither males nor females) carrying both mutations. For each of those six categories a different mean was estimated in the full model. By setting the age regression to zero or by constraining estimated means to be equal across gender or across carrier status of N291S or the −93T→G/D9N haplotype in the specification of the submodels, it was possible to test for each of those effects separately. A significant decrease in log-likelihood of the submodel compared to the full model, as apparent from the chi-square difference test, indicates that a model which allows for the effect shows a better fit, i.e. the specific effect is significant. In case a significant effect of the −93T→G/D9N haplotype was observed, it was tested whether the model could be simplified further (i.e. whether more estimated means could be set equal) without a significant loss in fit, in order to find out whether the effect of this mutation was restricted to either males or females.

In the model fitting to the pedigree data with the Fisher package, ascertainment correction was carried out by conditioning on the probandi. However, for some probandi, data on some lipoprotein traits were not available, which precluded application of the ascertainment correction and thereby the use of these pedigrees for further analyses for these specific traits. For each lipoprotein trait an optimal number of pedigrees (and an optimal number of individuals within these pedigrees) was used in the analyses. The data from the probandi were omitted from statistical calculations (e.g. the estimation of the means) to avoid possible ascertainment bias. Because the distribution of triglyceride was skewed, these data were transformed by natural logarithm in order to obtain a normal distribution.

3. Results

Screening of 40 Dutch FCHL probandi for mutations in the LPL gene using DGGE-analysis [20] showed aberrant patterns for exons 2, 6 and 9. Sequence analysis revealed an A→G substitution in exon 6, changing an asparagine to a serine in codon 291 (N291S). The results found for N291S have been published previously [29]. A C→G conversion in exon 9,

Table 1
Descriptive statistics (means ± S.E.) of BMI, plasma and lipoprotein triglyceride and cholesterol concentrations and apolipoprotein levels

Variable	Non-affected family members		Carriers of −93T→G/D9N haplotype		Carriers of N291S	
	Male	Female	Male	Female	Male	Female
N	32	40	19	18	4	8
Group	1	2	3	4	5	6
Age	42 ± 3.28	41 ± 2.52	41 ± 3.61	41 ± 4.88	29 ± 2.97	54 ± 5.10
BMI	24.5 ± 0.72	22.9 ± 0.67	25.3 ± 0.95	23.9 ± 0.90	21.9 ± 1.70	24.0 ± 1.57
Plasma cholesterol	5.30 ± 0.27	5.57 ± 0.24	6.29 ± 0.95	5.35 ± 0.35	4.88 ± 0.65	5.93 ± 0.50
Plasma triglycerides	1.18 ± 1.14	1.09 ± 1.13	1.73 ± 1.19	1.00 ± 1.19	1.21 ± 1.36	1.68 ± 1.27
VLDL cholesterol	0.43 ± 0.11	0.35 ± 0.10	0.80 ± 0.16	0.35 ± 0.18	0.79 ± 0.25	0.82 ± 0.20
VLDL triglycerides	0.77 ± 0.25	0.54 ± 0.24	1.60 ± 0.36	0.60 ± 0.41	1.73 ± 0.59	1.34 ± 0.45
LDL cholesterol	3.61 ± 0.22	3.52 ± 0.19	4.15 ± 0.30	3.53 ± 0.30	2.73 ± 0.63	3.92 ± 0.41
HDL cholesterol	1.30 ± 0.07	1.61 ± 0.07	1.21 ± 0.09	1.58 ± 0.09	1.15 ± 0.15	1.22 ± 0.12
Apo B100	122 ± 8	117 ± 7	138 ± 11	119 ± 12	126 ± 19	146 ± 14
Apo A1	138 ± 5	161 ± 4	139 ± 6	149 ± 8	127 ± 10	145 ± 8

Means and standard errors were estimated from the most general model using maximum likelihood estimate procedures implemented in the Fisher program. Probandi were excluded for these quantitative analysis. All levels are given in mmol/l except BMI which is given in kg/m^2 and apolipoprotein levels which are given in mg/dl.

codon 447, changes a serine to a stop codon (S447X). In exon 2, a G→A substitution, was found predicting an amino acid change of aspartic acid to an asparagine (D9N). Screening the promoter region revealed a point mutation at position −93, T→G. All carriers of this mutation were also carrying the D9N mutation in exon 2 indicating that these two mutations were in complete linkage disequilibrium. Therefore, we have denoted these mutations as the −93 T→G/D9N haplotype.

The S447X mutation was found in six out of 40 FCHL probandi giving an allele frequency of 0.08. Screening family members for this mutation and analyzing the effect of carrier status on lipid and lipoprotein levels showed no significant difference between individuals heterozygous for the S447X mutation and those homozygous for the common allele (data not shown).

The D9N mutation was detected in two out of 40 FCHL families (0.025) compared with five out of 110 individuals (0.023) in a control population. In one of these two families, the N291S mutation was also detected. By combining these two families with three additional families, previously characterized in another Dutch study [19], sufficient individuals carrying the −93 T→G/D9N haplotype could be obtained for statistical analysis. In total, five families, encompassing 122 relatives of which 38 carriers of the −93T→G/D9N haplotype and 12 carriers of the N291S mutation, were included in the statistical analysis (Fig. 1). The effect of the −93T→G/D9N haplotype and the N291S mutation on plasma lipid levels in family members of FCHL patients was analyzed by a pedigree-based maximum likelihood method.

The most general (full) model allowed for the effects of age, gender, and carrier status for the −93T→G/D9N haplotype and the N291S mutation by estimating a regression for age and separate means for the six different categories, which were defined by gender and carrier status of N291S and the −93T→G/D9N haplotype. Table 1 shows the estimated means and their standard errors of these six categories for BMI and measured lipid parameters. Table 2 shows log-likelihoods of the full model and the four submodels which provide a test for each of the abovementioned effects (age, gender, N291S and −93T→G/D9N haplotype) on BMI and lipid variables. No significant effect of either of the mutations is found on BMI, which means that BMI cannot explain possible differences between carriers and non-carriers. Therefore, BMI was excluded as a parameter in all further models. As shown in Table 2, age has a significant effect on BMI and all lipid traits except HDL cholesterol and apo A1 levels. Gender has a significant effect on serum cholesterol, VLDL- and HDL-cholesterol, serum triglycerides and VLDL triglycerides, and apo A1 levels. In these families, the N291S mutation had only significant effects on VLDL and HDL cholesterol compared with a previous study, probably due to the low number of carriers ($n = 12$, 4M/8F).

Conversely, the −93T→G/D9N haplotype does have significant effects on plasma cholesterol, VLDL-, and LDL cholesterol and plasma triglycerides, and VLDL triglycerides explaining approximately 6% of the variance in these variables. Data given in Table 1 suggested that only the male carriers had higher lipid levels. Therefore further submodels were tested to find out whether the effect of the −93T→G/D9N haplotype was gender dependent. Table 3 shows the log-likelihoods of the full model, one mean model that estimated the same mean for each of the six groups

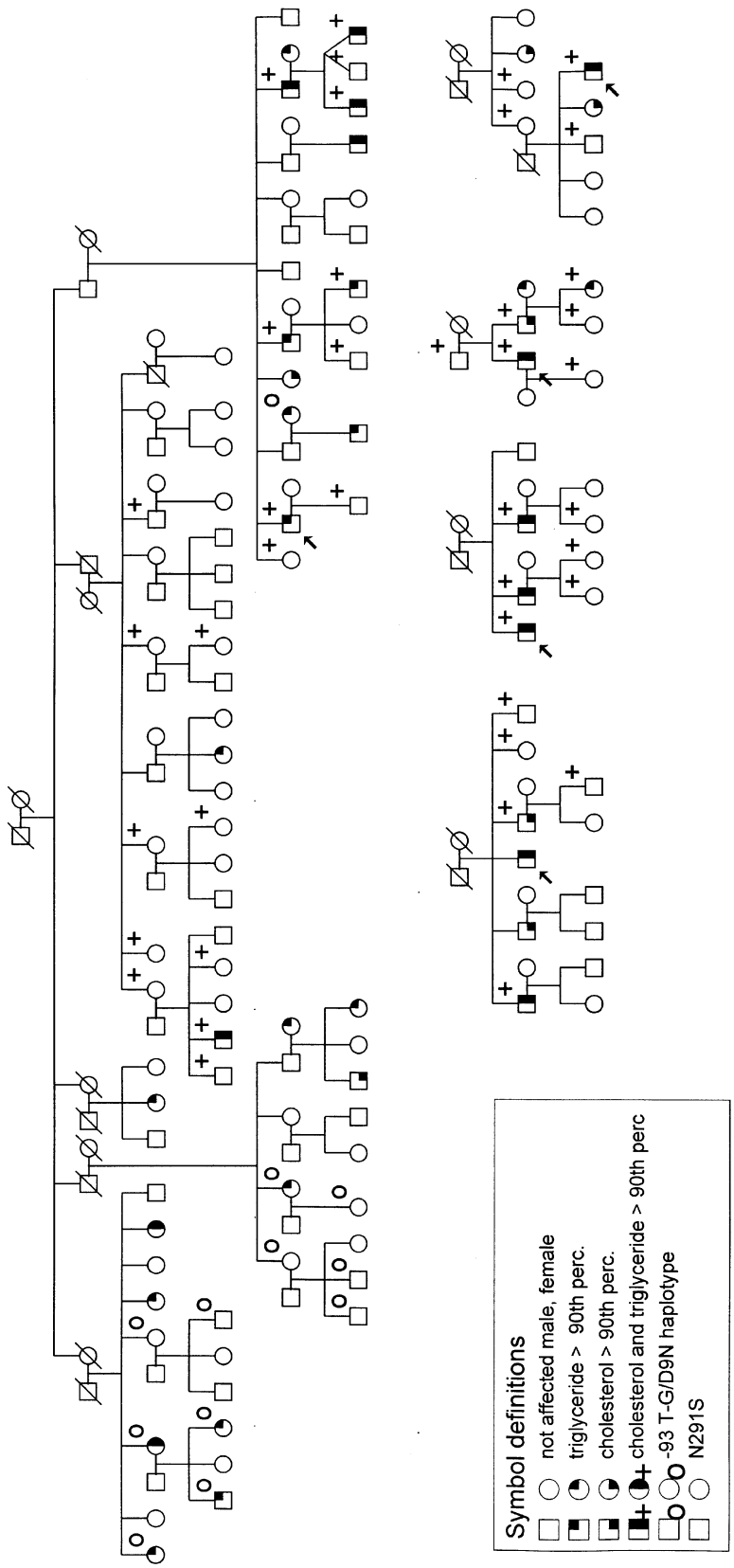

Fig. 1. Pedigrees of FCHL families. Open symbols indicate the non-carrier relatives (○ = female; □ = male). Heterozygous carriers for the −93T→G/D9N haplotype are indicated with a plus (+) above the symbols. Heterozygous carriers for the N291S mutation are indicated with a circle (o) above the symbols. Arrows indicate the proband.

Table 2
Log-likelihood for five different models testing the effects of age, gender, and carrier status for N291S or −93T→G/D9N haplotype for the quantitative traits and BMI in FCHL pedigrees

	Individuals[a] (*N*)	Pedigrees[a] (*N*)	Model 1 Full	2 Age	3 Gender	4 N291S	5 −93T→G/D9N
BMI	118	5	−186.01	−197.70[b]	−188.66	−186.16	−187.11
Plasma cholesterol	122	5	−66.47	−87.01[b]	−71.92[c]	−66.80	−73.38[b]
Plasma triglycerides	122	5	111.39	104.97[b]	106.94[c]	110.32	108.08[c]
VLDL cholesterol	102	2	28.46	22.77[b]	24.09[c]	24.95[c]	23.84[c]
VLDL triglycerides	102	2	−61.88	−65.14[c]	−65.80[c]	−64.54	−65.52[c]
LDL cholesterol	112	4	−44.51	−61.38[b]	−47.55	−44.62	−47.51[c]
HDL cholesterol	122	5	97.18	96.71	80.07[b]	91.69[d]	96.44
Apo B100	103	3	64.20	53.65[d]	62.52	62.79	62.07
Apo A1	106	3	119.94	119.77	108.12[b]	117.42	118.07

[a] Number of individuals or pedigrees used for the analysis. Log-likelihoods for five models. Model definition; full: most general model allowing for: (i) age regression, (ii) gender-difference, (iii) difference between carriers and non-carriers of N291S and (iv) −93T→G/D9N. The other (sub)models provide a test for each of the effects separately. Testing procedure: each model is tested against the full model. A significant decrease in log-likelihood of the submodel compared to the full model indicates a significant effect of the tested parameter. When twice the difference in log-likelihood of these models is higher than the χ^2 corresponding to d.f. = 1, d.f. = 2 or d.f. = 3 depending on the tested submodel, this indicates a significant age difference as indicated by [c] $p<0.05$, [d] $p<0.01$, [b] $p<0.001$. Model 2: d.f. = 1; model 3: d.f. = 3; model 4: d.f. = 2; model 5: d.f. = 2.

categorized according to their sex and carrier status and the model for gender-dependent effect of the −93T→G/D9N haplotype. In the best-fitting model (i.e. not significantly different from the full model) the estimated mean values of the female carriers of the −93T→G/D9N haplotype could be set equal to the means of non-carrier males and females. However, the mean for male carriers had to be estimated separately to show a satisfactory fit, as soon as its mean was set equal to the other groups in the one mean model it showed a significant deterioration in fit. These results give evidence for a gender-genotype interaction for the −93T→G/D9N haplotype in the FCHL families studied, resulting in elevated levels of plasma cholesterol, VLDL- and LDL cholesterol, and plasma triglycerides, and VLDL triglycerides (but not for HDL, apo A1 and apo B100) only in male carriers of the −93T→G/D9N haplotype.

4. Discussion

Previous studies among FCHL patients have shown that decreased LPL activity can be detected in about one third of the cases [9]. Therefore, we screened Dutch FCHL patients for mutations in the LPL gene that might explain their hyperlipidemic phenotype. Three previously reported amino acid changes were detected: D9N, N291S and S447X [23,32,33].

The S447X mutation occurs with a high frequency (0.11) in the general population [33,34] and does not impair the capacity of LPL to hydrolyze long-chain fatty acids in vitro [35]. This LPL truncation variant was reported to be present at a lower frequency in hypertriglyceridemic subjects and was suggested to be protective against hypertriglyceridemia [34]. Recently, a significant association with higher postheparin LPL activity, higher HDL cholesterol and lower triglyceride levels was found among coronary artery disease (CAD) patients carrying the S447X mutation (heterozygotes and homozygotes) [36–38]. Similar results were found in healthy, normolipidemic individuals [39]. We did not detect any association with one of these lipid levels in our FCHL families. These opposing results could be due to the effect of additional genetic factors involved in FCHL which also influence these traits but in an opposite way.

Several studies in large numbers of subjects have shown that both the N291S and D9N mutations occur with a higher frequency among combined hyperlipidemic patients when compared with a control population [19,20]. In vitro mutagenesis studies indicated that the N291S mutation resulted in a decrease in the catalytic function of the LPL protein to 69% of the normal activity [40]. Although the results found for in vitro expression studies using the D9N construct indicated lower levels of LPL activity and mass compared with the wild-type construct, the specific activity was within the normal range [19,21]. Interestingly, we found that the D9N mutation is in strong linkage disequilibrium with a mutation in the promoter region, −93 T→G as has also been shown previously by others [41]. Expression studies with this promoter mutation [41] showed an increase in expression levels, suggesting that for this allele, the substitution at position −93 seems to be the functional mutation rather than D9N. Because it is not clear whether both mutations influence the enzyme activity or that only one of these substitutions has a functional effect we have designated this allele as the −93T→G/D9N haplotype. Further investigation will

Table 3
Log-likelihood for submodels testing the gender-related association between the −93T→G/D9N haplotype and quantitative traits in FCHL pedigrees

	Individuals[a]	Pedigrees[a]	Full model	One mean		Gender-dependent effect of −93T→G/D9N haplotype	
	(*N*)	(*N*)	Log-likelihood	Type[b]	Log-likelihood	Type[b]	Log-likelihood
Plasma cholesterol	122	5	−66.47	1 = 2 = 3 = 4 = 5 = 6	−73.44[c]	1 = 2 = 4 = 5 = 6	−68.08
Plasma triglycerides	122	5	111.39	1 = 2 = 3 = 4 = 5 = 6	105.21[c]	1 = 2 = 4 = 5 = 6	109.95
VLDL cholesterol	102	2	28.46	1 = 2 = 3 = 4, 5 = 6	19.31[d]	1 = 2 = 4, 5 = 6	28.00
VLDL triglycerides	102	2	−61.88	1 = 2 = 3 = 4 = 5 = 6	−69.45[d]	1 = 2 = 4 = 5 = 6	−65.02
LDL cholesterol	112	4	−44.51	1 = 2 = 3 = 4 = 5 = 6	−48.73[c]	1 = 2 = 4 = 5 = 6	−44.66

[a] Number of individuals or pedigrees used for the analysis.
[b] For annotation of group numbers see Table 1. For VLDL cholesterol, means of groups 5 and 6 were estimated separately from groups 1, 2, 3 and 4 to allow for the significant effect of N291S. Log-likelihoods for the models. Model definition; full: most general model allowing for: (i) age regression, (ii) gender-difference, (iii) difference between carriers and non-carriers of N291S and (iv) −93T→G/D9N. The submodels provide a test for the gender-dependent effects of the −93T→G/D9N haplotype. Testing procedure: each model is tested against the full model. A significant decrease in log-likelihood of the submodel compared to the full model indicates a loss in fit. When twice the difference in log-likelihood of the submodel compared to the full model indicates a significant loss in fit as indicated by [c] $p<0.05$, [d] $p<0.01$. Model one mean: d.f. = 5; model 1 = 2 = 4 = 5 = 6: d.f. = 4; model 1 = 2 = 4, 5 = 6: d.f. = 3.

be necessary to unravel the functional mutation within this haplotype.

In a previous study [29], using FCHL families, we have shown that carriers of the LPL N291S mutation have significantly elevated plasma and VLDL triglyceride, VLDL cholesterol levels and decreased HDL cholesterol levels compared with their noncarrier relatives. In the current study we have investigated five additional families of probandi carrying the −93T→G/D9N haplotype and analyzed the effect of carrier status on the variability in phenotypic expression of this common disorder. Using a pedigree-based maximum likelihood method we were able to show that only male carriers of the −93T→G/D9N haplotype have elevated levels of plasma cholesterol, VLDL- and LDL cholesterol, plasma triglycerides, and VLDL triglycerides, indicating gene-gender interacting effects for this allele.

Similar results were found in another study of LPL polymorphisms and plasma triglyceride levels within families in which associations with elevated lipid levels were only found for the fathers [42]. Since the effect associated with the −93T→G/D9N haplotype is smaller in females than in males this implies a possible modulation by hormonal or other gender-specific factors. This indicates that this haplotype has a context-dependent effect, i.e. it only has effects on LPL activity in specific conditions. Gender-specific effects on various lipid and lipoprotein levels are not uncommon. On several occasions variants of apolipoprotein genes have been reported to display larger effects on these traits in either males or females [43–45]. However, in a previous study we have shown that this gender-specific effect on lipid levels is not found for the N291S mutation [29].

We are aware of only one other study in which the D9N mutation has been studied in FCHL families [36]. In that study, the presence of the D9N mutation was associated with hypertriglyceridemia and reduced HDL-cholesterol levels. No association with hypercholesterolemia was found within the two families studied. Similar results were found for a study on healthy men [41]. A case-control study [19] in which the effect of carrier status for the D9N mutation has been studied in hyperlipidemic patients revealed associations with higher plasma triglyceride as well as cholesterol levels. A number of studies have analyzed the effect of carrier status among patients with premature CAD. These studies [46,47] revealed that carriers had not only higher levels of several plasma lipid traits but also an increased progression of coronary atherosclerosis.

The studies [48] examining the −93T→G/D9N haplotype and N291S within FCHL families did not indicate that these mutations represent the major gene causing the affected FCHL phenotype. In addition, linkage analysis using intragenic markers did not give evidence for linkage between FCHL and the LPL gene [49]. In contrast, our data show that both mutations were associated with significantly elevated lipid levels. This indicates that in addition to the LPL mutations,

other genetic predisposing factors have to be present within our FCHL families to explain the FCHL phenotype.

Taken together, our results found for the −93T→G/D9N haplotype and N291S mutations strongly suggest that the LPL gene is a susceptibility gene for several types of hyperlipidemia, and that heterozygosity for these LPL mutations predisposes to the development of a hyperlipidemic state and subsequently atherosclerosis.

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References

[1] Goldstein JL, Schrott HG, Hazzard WR, Bierman EL, Motulsky AG. Hyperlipidemia in coronary heart disease. II. Genetic analysis of lipid levels in 176 families and delineation of a new inherited disorder, combined hyperlipidemia. J Clin Invest 1973;52:1544.

[2] Rose HG, Kranz P, Weinstock M, Juliano J, Haft JI. Inheritance of combined hyperlipoproteinemia: evidence for a new lipoprotein phenotype. Am J Med 1973;54:148.

[3] Kwiterovich PO, White S, Forte T, Bachorik PS, Smith H, Sniderman A. Hyperapobetalipoproteinemia in a kindred with familial combined hyperlipidemia and familial hypercholesterolemia. Arteriosclerosis 1987;7:211.

[4] Hunt SC, Wu LL, Hopkins PN, Stults BM, Kuida H, Ramirez ME, Lalouel J-M, Williams RR. Apolipoprotein, low density lipoprotein subfraction, and insulin associations with familial combined hyperlipidemia. Arteriosclerosis 1989;9:335.

[5] Austin MA, Brunzell JD, Fitch WL, Krauss RM. Inheritance of low density lipoprotein subclass patterns in familial combined hyperlipidemia. Arteriosclerosis 1990;10:520.

[6] Stalenhoef AFH, Demacker PNM, Lutterman JA, van't Laar A. Plasma lipoproteins, apolipoproteins, and triglyceride metabolism in familial hypertriglyceridemia. Arteriosclerosis 1986;6:387.

[7] Cullen P, Farren B, Scott J, Farrall M. Complex segregation analysis provides evidence for a major gene acting on serum triglyceride levels in 55 British families with familial combined hyperlipidemia. Arterioscler Thromb 1994;14:1233.

[8] Babirak SP, Brown BG, Brunzell JD. Familial combined hyperlipidemia and abnormal lipoprotein lipase. Arterioscler Thromb 1992;12:1176.

[9] Brunzell JD. Familial lipoprotein lipase deficiency and other causes of the chylomicronemia syndrome. In: Stanbury JB, Wyngaarden JB, Frederickson DS, Goldstein JL, Brown MS editors. The Metabolic Basis of Inherited Disease, 6th ed. New York: McGraw-Hill, 1994:59–1913.

[10] Deeb SS, Peng RL. Structure of the human lipoprotein lipase gene [published erratum appears in Biochemistry 1989 Aug 8;28(16):6786]. Biochemistry 1989;28:4131.

[11] Sparkes RS, Zollman S, Klisak I, Kirchgessner TG, Komaromy MC, Mohandas T, Schotz MC, Lusis AJ. Human genes involved in lipolysis of plasma lipoproteins: mapping of loci for lipoprotein lipase to 8p22 and hepatic lipase to 15q21. Genomics 1987;1:138.

[12] Wion KL, Kichgessner TG, Lusis AJ, Schotz MC, Lawn RM. Human lipoprotein lipase complementary DNA sequence. Science 1987;235:1638.

[13] Miesenbock G, Holz B, Foger B, Brandstatter E, Paulwebber B, Sandhofer F, Patch JR. Heterozygous lipoprotein lipase deficiency due to a missense mutation as the cause of impaired triglyceride tolerance with multiple lipoprotein abnormalities. J Clin Invest 1993;91:448.

[14] Babirak SP, Iverius P-H, Fujimoto WY, Brunzell JD. Detection and characterization of the heterozygote state for lipoprotein lipase deficiency. Arteriosclerosis 1989;9:326.

[15] Wilson DE, Emi M, Iverius P-H, Hata A, Wu LL, Hillas E, Williams RR, Lalouel J-M. Phenotypic expression of heterozygous lipoprotein lipase deficiency in the extended pedigree of a proband homozygous for a missense mutation. J Clin Invest 1990;86:735.

[16] Chamberlain JC, Thorn JA, Oka K, Galton DJ, Stocks J. DNA polymorphisms at the lipoprotein lipase gene: association in normal and hypertriglyceridamic subjects. Atherosclerosis 1989;79:85.

[17] Minnich A, Kessling A, Roy M, Giry C, DeLangavant G, Lavigne J, Lussier-Cacan S, Davignon J. Prevalence of alleles encoding defective lipoprotein lipase in hypertriglyceridemic patients of French Canadian descent. J Lipid Res 1995;36:117.

[18] Fisher RM, Mailly F, Peacock RE, Hamsten A, Seed M, Yudkin JS, Beisiegel U, Feussner G, Miller G, Humphries SE, Talmud PJ. Interaction of the lipoprotein lipase asparagine 291→serine mutation with body mass index determines elevated plasma triacylglycerol concentrations: A study in hyperlipidemic subjects, myocardial infarction survivors, and healthy adults. J Lipid Res 1995;36:2104.

[19] Mailly F, Tugrul Y, Reymer PWA, Bruin T, Seed M, Groenemeyer BF, Asplund-Carlson A, Vallance D, Winder AF, Miller GJ, Kastelein JJP, Hamsten A, Olivecrona G, Humphries SE, Talmud PJ. A common variant in the gene for lipoprotein lipase (Asp9→Asn): Functional implications and prevalence in normal and hyperlipidemic subjects. Arterioscler Thromb 1995;15:468.

[20] Reymer PWA, Groenemeyer BE, Gagne E, Miao L, Appelman EEG, Seidel JC, Kromhout D, Bijvoet SM, Van de Oever K, Bruin T, Hayden MR, Kastelein JJP. A frequently occurring mutation in the lipoprotein lipase gene (Asn291Ser) contributes to the expression of familial combined hyperlipidemia. Hum Mol Genet 1995;4:1543.

[21] Gagne E, Genest J Jr., Zhang H, Clarke LA, Hayden MR. Analysis of DNA changes in the LPL gene in patients with familial combined hyperlipidemia. Arterioscler Thromb 1994;14:1250.

[22] Nevin DN, Brunzell JD, Deeb SS. The LPL gene in individuals with familial combined hyperlipidemia and decreased LPL activity. Arterioscler Thromb 1994;14:869.

[23] Ma Y, Ooi TC, Liu M-S, Zhang H, McPherson R, Edwards AL, Forsythe IJ, Frohlich J, Brunzell JD, Hayden MR. High frequency of mutations in the human lipoprotein lipase gene in pregnancy-induced chylomicronemia: possible association with apolipoprotein E2 isoform. J Lipid Res 1994;35:1066.

[24] Assmann G, Schulte H. Results and conclusions of the prospective cardivascular Munster (PROCAM) study. In: Assmann G, editor. Lipid Metabolism Disorders and Cardiovascular Disease. Munich: MMV-Medizin, 1993:19.

[25] Demacker PNM, Vos-Janssen HE, Jansen AP, van't Laar A. Evaluation of the dual-precipitation method by comparison with the ultracentrifugation method for measurement of lipoproteins in serum. Clin Chem 1977;23:1238.

[26] Demacker PNM, Hijmans AG, Vos-Janssen HE, van't Laar A, Jansen AP. A study of the use of polyethylene glycol in estimating cholesterol in high-density lipoprotein. Clin Chem 1980;26:1775.

[27] Bredie SJH, De Bruin TWA, Demacker PNM, Kastelein JJP, Stalenhoef AFH. Comparison of Gemfibrozil versus simvastatin in familial combined hyperlipidemia and effect on apolipoprotein-B-containing lipoproteins, low-density lipoprotein subfractions profile, and low-density lipoprotein oxidizability. Am J Cardiol 1995;75:348.

[28] Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from nucleated cells. Nucleic Acids Res 1988;16:1215.

[29] Hoffer MJV, Bredie SJH, Boomsma DI, Reymer PWA, Kastelein JJP, De Knijff P, Demacker PNM, Stalenhoef AFH, Havekes LM, Frants RR. The lipoprotein lipase (Asn291→Ser) mutation is associated with elevated lipid levels in families with familial combined hyperlipidaemia. Atherosclerosis 1996;119:159.

[30] Lange K, Weeks D, Boehnke M. Programs for Pedigree Analysis: MENDEL, FISHER, and dGENE [letter]. Genet Epidemiol 1988;5:471.

[31] Lange K, Westlake J, Spence MA. Extensions to pedigree analysis. III. Variance components by the scoring method. Ann Hum Genet 1976;39:485.

[32] Lohse B, Beg OU, Brunzell JD, Santamarina-Fojo S, Brewer HB. Familial chylomicronemia: identification of a unique patient homozygous for two seperate mutations in the LPL gene. Circulation 1991;84(suppl II):376.

[33] Hata A, Robertson M, Emi M, Lalouel JM. Direct detection and automated sequencing of individual alleles after electrophoretic strand separation: identification of a common nonsense mutation in exon 9 of the human lipoprotein lipase gene. Nucleic Acids Res 1990;18:5407.

[34] Stocks J, Thorn JA, Galton DJ. Lipoprotein lipase genotypes for a common premature termination codon mutation detected by PCR-mediated site-directed mutagenesis and restriction digestion. J Lipid Res 1992;33:853.

[35] Faustinella F, Chang A, Van Biervliet JP, Rosseneu M, Vinaimont N, Smith LC, Chen S-H, Chan L. Catalytic triad residue mutation (Asp1566Gly) causing familial lipoprotein lipase deficiency. J Biol Chem 1991;266:14418.

[36] Groenemeijer BE, Hallman MD, Reymer PWA, Gagné E, Kuivenhoven JA, Bruin T, Jansen H, Lie KI, Bruschke AVG, Boerwinkle E, Hayden MR, Kastelein JJP. A genetic variant, showing a positive interaction with β-blocking agents, with a beneficial influence on lipoprotein lipase activity, HDL-cholesterol and triglyceride levels in CAD patients: the Ser^{447}-stop substitution in the lipoprotein lipase gene. Circulation 1996;95:2169.

[37] Jemaa R, Fumeron F, Poirier O, Lecerf L, Evans A, Arveiler D, Luc G, Cambou JP, Bard JM, Fruchart JC, Apfelbaum M, Cambien F, Tiret L. Lipoprotein lipase gene polymorphisms: Associations with myocardial infarction and lipoprotein levels, the ECTIM study. J Lipid Res 1995;36:2141.

[38] Mattu RK, Needham EWA, Morgan R, Rees A, Hackshaw AK, Stocks J, Welwood PC, Galton DJ. DNA variants at the LPL gene locus associate with angiographically defined severity of atherosclerosis and serum lipoprotein levels in Welsh population. Arterioscler Thromb 1994;14:1090.

[39] Kuivenhoven JA, Groenemeyer BE, Boer JMA, Reymer PWA, Berghuis R, Bruin T, Jansen H, Seidell JC, Kastelein JJP. The Ser^{447}stop mutation in lipoprotein lipase is associated with elevated HDL-cholesterol levels in normolipidemic males. Arterioscler Thromb Vasc Biol 1997 (in press).

[40] Reymer PWA, Gagné E, Groenemeyer BE, Zhang H, Forsyth I, Jansen H, Seidell JC, Kromhout D, Lie KE, Kastelein J, Hayden MR. A lipoprotein lipase mutation (Asn291Ser) is associated with reduced HDL cholesterol levels in premature atherosclerosis. Nature Genet 1995;10:28.

[41] Hall S, Chu G, Miller G, Cruickshank K, Coopre JA, Humphries SE, Talmud PJ. A common mutation in the lipoprotein lipase gene promoter, −93T/G, is associated with lower plasma triglyceride levels and increased promoter activity in vitro. Arterioscler Thromb Vasc Biol 1997;17:1969.

[42] Georges J, Regis-Bailly A, Salah D, Rakotovao R, Siest G, Visvikis S, Tiret L. Family study of lipoprotein lipase gene polymorphisms and plasma triglyceride levels. Genet Epidemiol 1996;13:179.

[43] Kessling A, Ouelette S, Bouffard O, Chamberland A, Betard C, Xhignesse M, Lussier-Cacan S, Davignon D. Patterns of association between genetic variability in apolipoprotein (apo) B, apo A1-CIII-AIV, and cholesterol ester transfer protein gene regions and quantitative variation in lipid and lipoprotein traits: influence of gender and exogenous hormones. Am J Hum Genet 1991;50:92.

[44] Reilly SL, Ferrell RE, Kottke BA, Kamboh MI, Sing CF. The gender-specific apolipoprotein E genotype influence on the distribution of lipids and apolipoproteins in the population of Rochester, MN. I. Pleiotropic effects on means and variances [published erratum appears in Am J Hum Genet 1992 Oct;51(4):942]. Am J Hum Genet 1991;49:1155.

[45] Guerra R, Wang J, Grundy SM, Cohen JC. A hepatic lipase (LIPC) allele associated with high plasma concentrations of high density lipoprotein cholesterol. Proc Natl Acad Sci USA 1997;94:4532.

[46] Mailly F, Fisher R, Nicaud V, Luong L, Evans A, Marques-Vidal P, Luc G, Arveiler D, Bard J, Poirier O, Talmud P, Humphries S. Association between the LPL-D9N mutation in the lipoprotein lipase gene and plasma lipid traits in myocardial infarction survivors from the ECTIM study. Atherosclerosis 1996;122:21.

[47] Jukema JW, van Boven AJ, Groenemeijer BE, Zwinderman AH, Reiber JHC, Bruschke AVG, Henneman JA, Molhoek GP, Bruin T, Jansen H, Gagné E, Hayden MR, Kastelein JJP. The Asp_9 Asn mutation in the lipoprotein lipase gene is associated with increased progression of coronary atherosclerosis. Circulation 1997;94:1913.

[48] De Bruin TWA, Mailly F, Van Barlingen HHJJ, Fisher R, Castro Cabezas M, Talmud P, Dallinga-Thie GM, Humphries SE. Lipoprotein lipase gene mutations D9N and N291S in four pedigrees with familial combined hyperlipidaemia. Eur J Clin Invest 1996;26:631.

[49] Pajukanta P, Porkka KVK, Antikainene M, Taskinen MR, Perola M, Murtomaki-Repo S, Ehnholm S, Nuoti I, Suurinkeroinene L, Lahdenkari AT, Syvanen AC, Viikari JSA, Ehnholm C, Peltonen L. No evidence of linkage between familial combined hyperlipidemia and genes encoding lipolytic enzymes in Finnish families. Arterioscler Thromb Vasc Biol 1997;17:841.