

Molecular Genetic Study of Finns With Hypoalphalipoproteinemia and Hyperalphalipoproteinemia

A Novel Gly²³⁰Arg Mutation (LCAT_{Fin}) of Lecithin:Cholesterol Acyltransferase (LCAT) Accounts for 5% of Cases With Very Low Serum HDL Cholesterol Levels

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Abstract—In an attempt to identify genetic factors underlying extreme alterations of serum HDL cholesterol (HDL-C) concentrations, we examined two probands with HDL-C levels <0.2 mmol/L and subsequently screened two large cohorts of smoking men, one with very low (0.2 to 0.7 mmol/L, n=156) and the other with elevated (1.9 to 3.6 mmol/L, n=160) HDL-C levels, for the newly detected mutations as well as some other mutations proposed to affect HDL-C levels. One of the probands had corneal opacities, microalbuminuria, hypertriglyceridemia, and reduced LDL apoprotein B concentration; the other had anemia and presented with stomatocytosis in his peripheral blood. The first proband was found to be homozygous for a novel LCAT Gly²³⁰Arg (LCAT_{Fin}) mutation, and the second was homozygous for an Arg³⁹⁹Cys mutation we described previously. Transient expression of the mutant LCAT_{Fin} cDNA in COS cells disclosed markedly diminished LCAT enzyme activity. In the low-HDL-C group of men (n=156), 8 carriers of LCAT_{Fin} and 1 carrier of the LCAT Arg³⁹⁹Cys were identified. In addition, the frequency of the lipoprotein lipase (LPL) Asn²⁹¹Ser mutation was significantly ($P<.05$) higher in the low-HDL-C group (4.8%) than in the high-HDL-C group (1.6%). In addition, we identified 1 carrier of the intron 14G→A mutation of cholesterol ester transfer protein (CETP) in the high-HDL-C group and subsequently demonstrated cosegregation of the mutant allele with elevated HDL-C levels in the proband's family. In conclusion, we have identified a novel LCAT gene Gly²³⁰Arg mutation (LCAT_{Fin}), which, together with the LPL Asn²⁹¹Ser mutation, represents a relatively common genetic cause of diminishing HDL-C levels, at least among Finns. This article also reports occurrence of a CETP mutation in subjects having non-Japanese roots. (*Arterioscler Thromb Vasc Biol.* 1998;18:591-598.)

Key Words: HDL cholesterol ■ LCAT deficiency ■ lipoprotein lipase ■ cholesterol ester transfer protein ■ mutation

Low serum HDL-C concentration is associated with increased risk for CAD.¹ According to twin and pedigree analyses, 30% to 55% of HDL-C concentration variability is determined by genes.² A number of rare mutations of both the apo A-I and LCAT genes have been reported to cause extremely low HDL-C levels in some families (for review, see Reference 3). Data on more prevalent gene mutations that diminish HDL-C concentrations and influence risk of CAD at the population level are relatively scanty, although a common mutation in the LPL gene, resulting in a substitution of serine for asparagine at codon 291 and occurring at a frequency of 2% to 4% in white populations, was shown to associate with diminished serum HDL-C levels in two studies.^{4,5}

One of the best-understood genetic causes for low HDL-C is LCAT deficiency, a recessively inherited disorder with impaired peripheral esterification of cholesterol.⁶ Phenotypi-

cally, LCAT deficiency has been classified into FED and classic familial LCAT deficiency. FED is characterized by massive corneal opacities, marked reduction of serum HDL-C level, and selective inability of LCAT to esterify HDL cholesterol (α -LCAT activity).⁷ Typical findings in classic familial LCAT deficiency include corneal opacities, normochromic anemia, and proteinuria; the serum HDL-C level is very low, and LCAT activities on both HDL and apolipoprotein B-containing lipoproteins (α - and β -activities, respectively) are virtually absent. Molecular pathogenesis of LCAT deficiency and FED was recently reviewed by Kuivenhoven and coworkers.⁸ We previously reported the occurrence of the Arg³⁹⁹Cys mutation of LCAT in a few Finnish families.⁹ Transient expression of the Arg³⁹⁹Cys cDNA suggested that this mutation affects secretion or activity of LCAT but would not totally abolish them. Because of the lack of patients

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Selected Abbreviations and Acronyms

apo	=	apolipoprotein
CAD	=	coronary artery disease
CETP	=	cholesterol ester transfer protein
FED	=	fish-eye disease
HDL-C	=	HDL cholesterol
LCAT	=	lecithin:cholesterol acyltransferase
LPL	=	lipoprotein lipase
PCR	=	polymerase chain reaction
SSCP	=	single-strand conformational polymorphism

homozygous for the LCAT Arg³⁹⁹Cys mutation, phenotypic manifestations of the Arg³⁹⁹Cys could not be further investigated during these early studies. Apo A-I deficiency is another rare cause for HDL deficiency.² Recently, we identified a unique apo A-I_{Fin} mutation substituting arginine for leucine at position 159 of the mature apo A-I protein, causing a dominantly negative phenotype in serum HDL-C levels.¹⁰

Two presuppositions were considered during the design of the present study, aimed at identification of DNA alterations that have profound effects on HDL-C levels. First, Finns were judged to represent an attractive candidate in the search for commonly occurring gene alterations affecting HDL-C levels, because of the genetically homogeneous nature of this population.^{11,12} In harmony with this assumption, we have previously shown that two founder mutations of the LDL receptor account for two thirds of the mutations underlying familial hypercholesterolemia in Finland.^{13,14} Second, selection of large numbers of individuals showing extreme deviations from the population mean of the HDL-C level was considered to facilitate detection of mutations that have significant impact on HDL-C levels. We commenced our studies by characterizing the mutation(s) responsible for greatly diminished serum HDL-C levels in two independent probands whose parents were known to be of Finnish origin.

Methods

Probands With Greatly Diminished Serum HDL-C Levels

Family 1

Proband 1 was a 28-year-old, apparently healthy man from the Mikkeli area, central eastern Finland. He was found to have a very low serum HDL-C level (0.19 mmol/L) on a routine medical checkup. He did not have any disease history and did not use any regular medication. His father had died suddenly of unknown causes at the age of 60 years, and his mother had died of a chronic liver disease. The proband had six siblings, all of whom were healthy. There was no history of CAD in the family.

Family 2

Proband 2 was a 25-year-old, healthy man from the Turku area, southwestern Finland. In a routine medical checkup at work, he was found to have an extremely low serum HDL-C concentration (0.09 mmol/L) and elevated fasting serum triglycerides (6 to 12 mmol/L). His medical history was unremarkable, and he was not on any drug treatment. The mother (52 years old), father (57 years old), and sister (31 years old) of the proband did not suffer from any illnesses, nor was there any history of CAD in the family.

Groups of Individuals With Low and High Serum HDL-C Levels

Two groups of individuals were selected from the original cohort of the Alpha-Tocopherol, Beta-Carotene (ATBC) Cancer Prevention Study.¹⁵ A total of 29 133 male smokers, 50 to 69 years old and living in the southwestern part of Finland, were originally recruited for this large-scale cancer prevention study comparing α -tocopherol, β -carotene, and the two combined against placebo during a 6-year follow-up period. Serum cholesterol and HDL-C levels of each participant were measured twice: at the entry to the study and 3 years later. In a later phase, whole-blood samples blotted on a filter paper were collected, and these were available from 70% of the original participants. For the present investigation, a total of 156 whole-blood samples from subjects with lowest available HDL-C levels (0.20 to 0.70 mmol/L) and 160 samples from those with highest available HDL-C levels (1.90 to 3.60 mmol/L) were picked up. In each case, the criterion for the established HDL-C level had to be fulfilled both at entry and at the 3-year follow-up visit.

DNA Sample Preparation

DNA was isolated from peripheral venous blood by standard techniques. To isolate material for PCR from the dried blood spots, the filter papers were eluted with 1 mL of the PCR buffer (Dyna-zyme, Finnzymes) for 16 hours at room temperature. An aliquot (40 to 50 μ L) of the mixture was then boiled for 5 minutes to break the cells, followed by cooling on ice for 10 minutes. Samples were briefly centrifuged, and supernatants (30 μ L) were used as a template for PCR assay. Eluted stocks were stored at -20°C .

Assay for LCAT Mutations

The possibility that either of the two LCAT mutations (LCAT Arg³⁹⁹Cys or insertion of C in exon 1) we identified previously would have caused diminished HDL-C levels in the probands was explored with duplex PCR for simultaneous detection of these two mutations as described before.⁹ In brief, PCR was performed with primers A (5-AGCCACAGCCTGTGCACCT-3) and B (5-AGGGCTTACG GTAGCAAAGGA-3), together with a mismatch primer C (5-TGGCTCCTCAATGTGCTCTTCCCCTC-3) and primer D (5-AGGGTCTGGCGTGGTGATCA-3). Primers A and B amplify a 191-bp fragment of exon 6 and primers C and D a 120-bp fragment of exon 1. In exon 6, the C-to-T mutation destroys a normally existing *Aci* I cleavage site, and the mismatch in primer C¹⁶ eliminates an *Aci* I cleavage site from the normal exon 1 sequence but leaves it intact in the mutated one. PCR was performed for 32 cycles for 1 minute each at 95 $^{\circ}\text{C}$, 58 $^{\circ}\text{C}$, and 72 $^{\circ}\text{C}$. PCR products were digested with *Aci* I (New England Biolabs), size-fractionated on a 12% polyacrylamide gel, and stained with ethidium bromide for visualization.

SSCP and Sequence Analysis of the LCAT Gene

All six exons of the LCAT gene of proband 2 were analyzed by the SSCP method (reviewed in Reference 17) with primers reported previously.⁹ The fragment of exon 6 presenting a shift in the SSCP analysis was amplified by PCR with primers 5'-CCACCTTGCTC CATATCCA-3' and 5'-AGAAGCGTTGGAAGTCACGGC-3', and the amplified DNA fragments were directly sequenced (antisense sequencing primer, 5'-ACGCCATGCGAGAGGGGAAAC-3') with a commercial sequencing kit (Sequenase 2.0, United States Biochemical Corp). A solid-phase sequencing method with streptavidin-coated magnetic beads (Dynabeads M 280 Streptavidin, DYNAL) was used to generate single-stranded DNA. Strong DNA secondary structures in the G-C-rich area of exon 6 and compressions in the sequencing gel were resolved by use of 7-deaza-dGTP nucleotides (United States Biochemical Corp) in sequencing reactions.

Restriction Enzyme Analysis of LCAT_{Fin}

Results from sequencing analysis were confirmed by restriction enzyme analysis. A 190-bp fragment of exon 6 was amplified with primers 5'-CCACCTTGCTCCATATCCA-3' and 5'-AGAAGCGT TGAAGTCACGGC-3' and digested with the restriction enzyme

Hha I (New England Biolabs). The digested fragments were size-fractionated on a 12% polyacrylamide gel and stained with ethidium bromide for visualization (Fig 2).

In Vitro Mutagenesis of LCAT cDNA

The G-to-C point mutation at nucleotide 4469 (nucleotide numbering according to McLean et al¹⁸) of the human LCAT gene was introduced to a full-length LCAT cDNA⁹ by the megaprimer-PCR method.¹⁹ In brief, an oligonucleotide carrying the desired mutation (5'-GTGACAACCAGCGCATCCCCAT-3', with the mismatched base underlined) was first synthesized. A conventional PCR was carried out using the wild-type LCAT cDNA construct in pGEM3 vector as a template and SP6 primer (Promega) as a downstream primer. The resulting 701-bp-long fragment was then used as megaprimer to amplify an 892-bp-long region from the wild-type LCAT cDNA with the aid of an upstream primer, 5'-GCTGGTG GAGGAGATGCACG-3'. The resulting fragment was purified from agarose gel, digested with the restriction enzymes *Bam*HI and *Msc* I, and ligated to *Bam*HI-*Msc* I sites of the wild-type LCAT cDNA in pSVL SV40 vector. DNA sequences of the mutated construct corresponding to the LCAT_{Fin} mutation (Gly²³⁰Arg) were confirmed by dideoxynucleotide sequencing as described above.

Transient Transfection of COS Cells

Transfection of COS-7 cells (African green monkey kidney cells, ATCC CRL 1651) was performed as previously described.⁹ Briefly, cells (4×10^5 cells per 60-mm dish) were plated 18 hours before transfection. Before the addition of the DNA, the media were changed to serum-free OPTIMEM 1 medium (GIBCO). Cells were transfected with 5 μ g of either LCAT_{Fin}-cDNA construct, wild-type LCAT-cDNA construct, or pSVL SV40 vector DNA (Pharmacia) by the lipofectamine procedure (GIBCO). Four separate transfections were carried out for each construct. The media were harvested 48 hours after transfection and stored at -70°C . Cells were washed with PBS, harvested in 0.25 mol/L Tris-HCl, pH 7.5, and stored at -70°C .

Determination of LCAT Activity

Cholesterol esterification in serum (pCER) was measured according to Dobiasova and Schutzova²⁰ with a cold-labeling method. Values were expressed as percent [³H]cholesterol esterified during a 3-hour incubation. All experiments were performed in duplicate. LCAT α -activity in the serum or cell medium of the transfected COS cells was measured according to the method of Chen and Albers²¹ with exogenous proteoliposomes as a substrate. Freshly made proteoliposome substrates contained apo A-I, [³H]1,2-cholesterol (Amersham), and egg lecithin (Sigma Chemical Co) (molar ratios, 1:11:250).²² Aliquots of serum (10 μ L) or cell-culture medium (10 and 20 μ L) were used to initiate each reaction, and reactions were carried out for 30 minutes at 37°C . The labeled cholesteryl esters were separated from free cholesterol by thin-layer chromatography. All LCAT activity experiments from the media of transfected COS cells were performed in quadruplicate. The LCAT activity in media of the COS cells transfected with the pSVL SV40 vector alone was subtracted from the activities recorded for the other transfections. Transfection efficacy was controlled by slot blotting intracellular DNA from each cell culture dish to nylon filters.²³ Filters were hybridized with a ³²P-labeled pSVL SV40 plasmid, and the radioactivity of each slot was counted by a liquid scintillation counter. The level of LCAT activity was normalized to the amount of transferred plasmid DNA.

Assays for Specific Mutations and Polymorphisms

A PCR-based assay using the principle of a mismatch primer in the detection of a point mutation¹⁶ was developed for a rapid screening of the LCAT Gly²³⁰Arg mutation. PCR was performed with the primer 5'-AGAAGCGTTGGAAGTCACGGC-3' together with the primer 5'-GCTCCATATCCACAGGTGACAACCTG-3' carrying the mismatch nucleotide (underlined) amplifying a 183-bp fragment of exon 6. The mismatch in the primer introduces an *Fsp* I cleavage

site into the mutant sequence. PCR was performed for 30 cycles at 95°C , 60°C , and 72°C , 1 minute each. The amplified products were digested with *Fsp* I (New England Biolabs), size-fractionated on a 12% polyacrylamide gel, and stained with ethidium bromide for visualization. The apo A-I_{Fin} (Leu¹⁵⁹Arg) mutation was identified by PCR amplification of exon 4 of the apo A-I gene, followed by digestion of the amplification products with the restriction enzyme *Fsp* I as previously described.¹⁰ The Asn²⁹¹Ser mutation of the LPL gene was detected by PCR amplification using a mismatch primer introducing an *Rsa* I cleavage site into the mutant PCR product, followed by restriction enzyme digestion and electrophoresis of the resulting fragments as described.⁴ The intron 14 (G \rightarrow A) and exon 15 point mutations of the CETP gene were detected by use of PCR and the mismatch primer techniques as reported.²⁴

Lipid and Lipoprotein Analysis

Serum lipoproteins were separated by ultracentrifugation into density classes as described.²⁵ Serum total cholesterol, free cholesterol, triglycerides, and phospholipids were analyzed with commercial kits (La Roche, Boehringer Diagnostica, Wako Chemicals). Apo A-I, A-II, and B concentrations were determined by immunochemical assays (Orion Diagnostica).

Statistical Analysis

Lipid values were compared by Student's *t* test. Allele frequencies in the different study groups were compared by χ^2 test.

Results

Clinical Data and Serum Lipoproteins of the Two Proband and Their Family Members

Clinical examination of proband 1 (male, 28 years old) did not reveal corneal opacities, lipid arcus, skin xanthomatosis, or extensor tendon xanthomas. He was anemic, with a blood hemoglobin level of 11.0 g/dL, and presented with many stomatocytes in his peripheral blood; unfortunately, samples from the relatives were not available for a peripheral blood smear. Proband 2 (male, 25 years old) had corneal opacifications; he was likewise anemic and presented with target cells as well as a few stomatocytes in his peripheral blood. Serum creatinine levels were normal in both probands, but proband 2 had mild microalbuminuria. There was no clinical evidence of CAD (exercise-induced angina, past history of myocardial infarction, history of coronary bypass operation, or ECG abnormalities suggestive of ischemia) in the probands or any of their family members included in the present study.

Proband 1 had low serum total, LDL, and HDL cholesterol concentrations, and cholesteryl ester percentages were reduced by 20% to 50% in serum and all other lipoprotein classes except the LDL species, which had normal cholesteryl ester percentages (Table 1). Four siblings (29 to 47 years old) of proband 1, all subsequently demonstrated to be heterozygous carriers of the Arg³⁹⁹Cys mutation of the LCAT gene (see below), were available for genetic and lipoprotein studies. They had diminished serum HDL-C concentrations (0.85 ± 0.08 mmol/L; range, 0.71 to 1.06 mmol/L) compared with the control subjects (1.34 ± 0.04 mmol/L), but otherwise their lipid profiles were normal (Table 1).

Proband 2 had normal serum cholesterol but low HDL-C and LDL cholesterol concentrations; in fact, almost half of his serum cholesterol was transported in the VLDL density fraction (Table 1). He was also severely hypertriglyceridemic (Table 1), with serum triglyceride levels occasionally mea-

TABLE 1. Lipids, Lipoproteins, and Apolipoproteins (mean±SEM) in the Two Families With LCAT Mutations and in a Group of Control Subjects

Variable	Arg ³⁹⁹ Cys Mutation		Gly ²³⁰ Arg Mutation		Control Subjects
	Proband 1	Four Heterozygous Carriers	Proband 2	Three Heterozygous Carriers	
Cholesterol, mmol/L (esters, %)					
Serum	2.03 (58)	4.36±0.24 (74.5±0.4)	4.04 (15)	5.49±0.25 (73.3±0.6)	5.80±0.13 (73.3)
VLDL	0.52 (35)	0.47±0.11 (52.6±2.0)	1.73 (26)	0.99±0.31 (52.5±1.4)	0.44±0.05 (52.0)
IDL	0.03 (33)	0.08±0.02 (64.6±2.1)	0.13 (0)	0.28±0.02 (65.6±2.9)	0.21±0.02 (67.1)
LDL	1.28 (68)	2.70±0.33 (75.0±1.4)	1.80 (0)	2.97±0.12 (76.0±2.0)	3.23±0.11 (72.4)
HDL	0.19 (68)	0.85±0.08 (85.7±0.7)	0.16 (0)	0.98±0.11 (82.5±1.2)	1.34±0.04 (82.1)
Triglycerides, mmol/L					
Serum	1.75	1.10±0.21	4.83	2.64±0.60	1.40±0.10
VLDL	1.40	0.65±0.16	4.46	1.85±0.59	0.80±0.08
IDL	0.03	0.06±0.01	0.09	0.15±0.0	0.10±0.00
LDL	0.23	0.23±0.03	0.20	0.40±0.03	0.26±0.01
HDL	0.09	0.16±0.02	0.09	0.25±0.04	0.17±0.26
Phospholipids, mg/dL					
Serum	122.0	177.4±8.1	301.0	259.0±13.3	248.3±5.1
VLDL	35.2	19.1±4.7	110.6	54.6±16.7	24.0±2.5
IDL	2.1	3.3±0.5	11.3	11.0±0.48	7.2±0.5
LDL	43.6	66.8±7.7	130.7	83.8±1.78	84.9±2.6
HDL	38.0	74.4±4.4	38.1	102.4±8.92	105.6±3.2
Apo A-I, mg/dL	ND	ND	34.1	128.5±1.79	134.8±4.8
Apo A-II, mg/dL	ND	ND	5.8	28.7±5.14	31.3±1.3
LDL apo B, mg/dL	ND	ND	6.9	79.3±6.6	72.1±3.4

ND indicates not determined. Control subjects (n=50) were healthy women, >50 years old, from southern Finland.

suring up to 12.0 mmol/L. Cholesteryl ester percentages were low in all lipoprotein classes, and in IDL, LDL, and HDL fractions, all measurable cholesterol was in the free form. Serum apo A-I concentration was diminished to ≈25% and apo A-II to 15% of the corresponding levels in the control subjects. A striking feature in proband 2 was the extremely low apo B concentration (<10 mg/dL) in the LDL density fraction (Table 1). The parents and sister of the proband, all subsequently shown to be heterozygous carriers of the Gly²³⁰Arg mutation of the LCAT gene (see below), had slightly diminished serum HDL-C levels, in addition to somewhat elevated serum triglyceride and VLDL cholesterol concentrations, compared with the control subjects (Table 1).

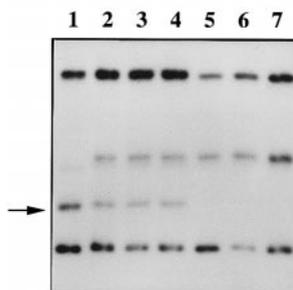


Figure 1. SSCP analysis of a 190-bp fragment of LCAT gene exon 6 of proband 2 (lane 1), his family members (lanes 2 through 4), and three apparently healthy control subjects (lanes 5 through 7). Mutant DNA fragment is indicated by arrow.

Identification of the LCAT Gene Mutations

DNA samples from both probands 1 and 2 were screened for the presence of the LCAT Arg³⁹⁹Cys and exon 1 C insertion mutations that we had previously identified in Finland.⁹ Our duplex PCR assay indicated that proband 1 was homozygous for the Arg³⁹⁹Cys mutation, and all of his four siblings were heterozygous carriers of the mutation. In contrast, proband 2 was not a carrier of either of these two mutations. SSCP analysis of all the exons of his LCAT gene showed a shift in exon 6 (Fig 1), which was subsequently sequenced. Sequence analysis of the proband's DNA sample revealed an apparent homozygosity for a novel G-to-C mutation at nucleotide position 4469, predicted to substitute arginine for glycine at residue 230 of the mature LCAT protein (Fig 2). The G-to-C substitution creates an *Hha* I cleavage site, and the results from the sequence analysis could thus be confirmed by

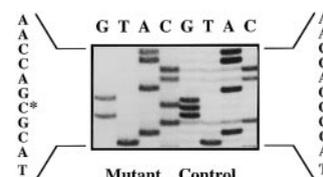


Figure 2. DNA sequence analysis of exon 6 of LCAT gene from proband 2 and a control subject. G-to-C substitution at position 4469 (nucleotide numbering according to McLean et al¹⁸ is indicated by asterisk).

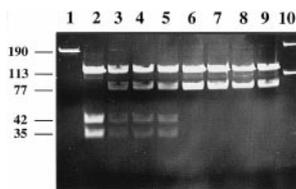


Figure 3. PCR digestion assay of the LCAT_{Fin} mutation. Cleavage of PCR-amplified 190-bp fragment of exon 6 (lane 1, undigested fragment) with *Hha* I normally results in 113- and 77-bp fragments. In the presence of the LCAT_{Fin} mutation creating a new *Hha* I restriction site, the 77-bp fragment is further cleaved into 42- and 35-bp fragments. Data on analysis of samples from proband 2 (lane 2), his family members (lanes 3 through 5), and four control subjects (lanes 6 through 9) are shown. Lane 10 shows molecular size marker (100 and 200 bp).

restriction digestion analysis (Fig 3). Cleavage of the PCR-amplified 190-bp fragment of exon 6 with *Hha* I normally results in 113- and 77-bp fragments. When the G-to-C substitution is present, the 77-bp fragment is further cleaved into 42- and 35-bp fragments. Analysis of the mutant digestion products confirmed that the proband was homozygous for the LCAT_{Fin} mutation, and the proband's parents and sister were all heterozygous carriers of this mutation (Fig 3).

LCAT Activity Assays

Serum cholesterol esterification was determined from samples from both probands and one healthy control subject. The serum cholesterol esterification percentage of proband 1 was reduced to 22% of that of the control serum (2.6% versus 11.7% per 3 hours, mean of two experiments), whereas the corresponding cholesterol esterification of proband 2 was almost completely abolished (0.3% versus 7.4% per 3 hours, mean of two experiments).

To further characterize the substrate specificity of the Arg³⁹⁹Cys-mutated LCAT enzyme, LCAT α -activities were determined in serum samples from proband 1, four heterozygous carriers of the Arg³⁹⁹Cys mutation, and a healthy control subject by use of artificial proteoliposomes. The proband, homozygous for the LCAT Arg³⁹⁹Cys mutation, had LCAT activity (3.6%) \approx 25% of that of the four heterozygous carriers (14.1 \pm 0.7%, mean \pm SEM) and 20% of that of the control individual (17%). On the contrary, the LCAT α -ac-

TABLE 2. Summary of LCAT α -Activity Associated With the LCAT_{Fin} Mutant Enzyme

Sample	n	LCAT α -Activity
Serum, nmol \cdot mL ⁻¹ \cdot h ⁻¹		
Control subject	2	105.0 \pm 7.5
Proband 2	4	6.1 \pm 0.5
Media of COS cells, pmol \cdot mL ⁻¹ \cdot h ⁻¹		
Wild-type LCAT	4	208.0 \pm 22
LCAT _{Fin}	4	55.0 \pm 27

LCAT activity, measured by use of exogenous proteoliposomes, was assayed in serum samples from proband 2 (homozygous for the LCAT_{Fin} mutation) and a control subject and from the media of COS cells transfected with either the wild-type or LCAT_{Fin}-mutated LCAT cDNAs.

tivity in the serum of proband 2 was almost totally abolished (Table 2).

In Vitro Activity of Transiently Expressed LCAT_{Fin}

Our earlier study showed that transient expression in the COS cells of the LCAT cDNA containing the Arg³⁹⁹Cys mutation resulted in an enzyme activity <15% of that of wild-type LCAT.⁹ In the present study, similar experiments, run in quadruplicate, were carried out with an LCAT cDNA construct corresponding to the LCAT_{Fin} mutation. Results of these transfection experiments, showing that the LCAT_{Fin} mutation results in LCAT activity 25% of that of the wild-type LCAT activity, are summarized in Table 2.

Frequencies of the Mutations and Polymorphisms in Subjects Selected for Their HDL-C Levels

Screening for the two LCAT point mutations among 156 smoking men with very low serum HDL-C levels (0.2 to 0.7 mmol/L) disclosed 1 additional heterozygous carrier of the LCAT Arg³⁹⁹Cys mutation and 8 heterozygous carriers of the LCAT_{Fin} mutation (Table 3). In contrast, the exon 1 C insertion mutation of the LCAT gene or apo A-I_{Fin} mutation were not found in any of the subjects in the low-HDL-C group. The LCAT_{Fin} mutation alone thus accounted for 5% of the causes of diminished HDL-C concentration in these Finns. In addition, the nine LCAT mutation carriers had lower mean (\pm SEM) HDL-C levels than the noncarriers (n=147) of the low-HDL-C group (0.55 \pm 0.02 versus 0.63 \pm 0.01 mmol/L, P <.01). No carriers of the LCAT_{Fin} mutation were detected in the high-HDL-C group of subjects (n=160) (Table 3).

We also carried out a preliminary study on a possible association of the LCAT_{Fin} mutation with risk of CAD. To this end, we screened for its presence in 77 clinically verified cases of CAD examined previously by us²⁶ as well as in 111 angiographically established cases of CAD,²⁷ but we failed to identify a single carrier of the LCAT_{Fin} gene in these two groups of patients. All 188 patients with CAD were living

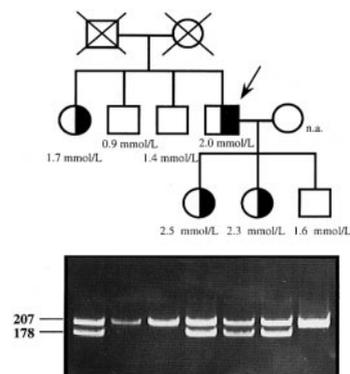


Figure 4. Top, Pedigree of family with CETP intron 14 mutation. Proband is indicated by arrow, and serum HDL-C levels are shown below each individual. Bottom, Molecular analysis of CETP intron 14 mutation according to Akita et al.²⁴ Mutant allele is disclosed by appearance of an extra 178-bp band. Electrophoresis lanes match individuals indicated above. n.a. indicates DNA sample not available.

TABLE 3. Summary of Mutation Screening in Subjects With Low (0.2–0.7 mmol/L) or High (1.9–3.6 mmol/L) Serum HDL-C Concentrations

Genotypes and Alleles	Low HDL-C (n=156)		High HDL-C (n=160)	
	n	%	n	%
LCAT _{Fin} (Gly ²³⁰ Arg)	8	5.0	0	0
LCAT Arg ³⁹⁹ Cys	1	0.6	ND	...
LCAT exon 1 insertion of C	0	0	ND	...
CETP exon 15 (D ⁴⁴² G)	ND	...	0	0
CETP intron 14 (G→A)	ND	...	1	0.6
LPL Asn ²⁹¹ Ser variation				
Genotypes				
AsnAsn	142	91.0	155	96.9
AsnSer	13	8.3	5	3.1
SerSer	1	0.6	0	0
Alleles				
Asn	297	95.2	315	98.4
Ser	15	4.8*	5	1.6

ND indicates not determined.

* $P < .05$, low vs high HDL-C.

within the same geographical area in which screening of the two LCAT mutations among smoking men took place.

The frequency of the LPL Asn²⁹¹→Ser allele was higher in the low-HDL-C group than in the high-HDL-C group (4.8% versus 1.6%, $P < .05$). Altogether, 13 heterozygous carriers of the Ser allele and 1 subject homozygous for this allele were identified in the low-HDL-C group, whereas only 5 heterozygous carriers were found in the high-HDL-C group (Table 3). In the high-HDL-C group, 1 subject heterozygous for the CETP intron 14 mutation, previously detected only in the Japanese population, was identified. This proband, his siblings, and his children volunteered for further studies. The proband's parents and grandparents were all of Finnish origin. The proband was a 65-year-old man, who had been a heavy smoker for 40 years and who suffered from elevated blood pressure but was otherwise healthy. There was no history of CAD in this family. The sister and two daughters of the proband were identified as heterozygous carriers of the CETP intron 14 mutation. All the mutation carriers had elevated serum HDL-C levels (Fig 4).

Discussion

Our study confirms earlier findings showing that mutations of LCAT may result in widely different clinical phenotypes (for review, see Reference 8) and specifically describes two mutant LCAT varieties that are associated with unique lipoprotein abnormalities. In addition, we report for the first time the occurrence of a similar mutant LCAT gene in a substantial percentage of subjects with hypoalphalipoproteinemia.

Both the Gly²³⁰Arg (LCAT_{Fin}) and the Arg³⁹⁹Cys point mutations affect exon 6, encoding almost half of the LCAT enzyme.¹⁸ The active site of LCAT, based on homology studies with other serine-dependent esterases²⁸ and site-

directed mutagenesis analysis of LCAT,²⁹ is postulated to be located around Ser¹⁸¹. No mutations affecting the proposed functional area itself have been reported thus far. The LCAT_{Fin} mutation, involving a substitution of a positively charged amino acid for a nonpolar amino acid, is localized to an area of the LCAT protein whose function is poorly understood. The striking effects of the Gly²³⁰Arg substitution on LCAT activity in vitro and on lipoprotein metabolism in vivo suggest that a correct architecture of this region of the LCAT protein is essential for the enzyme activity. Indeed, another nearby mutation, Asn²²⁸Lys, resulting in a charge alteration similar to that present in the product of the LCAT_{Fin} allele, was shown to result in complete LCAT deficiency³⁰ and complete lack of enzyme activity in vitro.³¹

Kuivenhoven and coworkers have sorted LCAT mutations into four categories according to the biochemical phenotype and the enzyme substrate specificity displayed by the individual mutations.⁸ The principal clinical phenotypes, ie, FED characterized by corneal opacities with absence of other major clinical findings and complete LCAT deficiency with corneal opacities, anemia, or renal manifestations, have been maintained unchanged in this classification. If this renovated classification is used as a basis, it appears that the LCAT_{Fin} mutation is associated within the principal phenotype of complete LCAT deficiency, with certain peculiar features related to triglyceride metabolism, whereas homozygosity for the Arg³⁹⁹Cys mutation appears to lead to a unique intermediate phenotype that mixes up not only biochemical but also clinical findings typical of FED and of familial LCAT deficiency. Our study provides one example of the usefulness of molecular genetic data to complement phenotypic classification of inherited diseases.

The proband homozygous for the LCAT_{Fin} mutation had many features compatible with complete LCAT deficiency, including corneal opacities, target cells in peripheral blood, mild proteinuria, and low esterification percentage in all lipoprotein classes. Large amounts of serum cholesterol and phospholipids were present in the VLDL fraction, suggesting disturbances in metabolism of triglyceride-rich lipoproteins. Indeed, further analysis of the proband's apoproteins revealed a striking reduction of the apo B in the LDL density range down to 10% of that in the control subjects (Table 1). These findings could reflect impaired further processing of VLDL particles to IDL and LDL^{32,33} or increased catabolism of LDL. Previous studies reporting reduction of LDL total proteins³⁴ as well as plasma apo B³⁵ in patients with LCAT deficiency suggest that the low content of apo B in the LDL density fraction observed in the present study is due to the absence of LCAT activity itself and not to another unknown defect in apo B metabolism.

Homozygosity for the Arg³⁹⁹→Cys mutation appears to produce a phenotype that can be classified neither as FED nor as familial LCAT deficiency but rather as a unique intermediate type with characteristics of both disorders. The finding that cholesterol esterification percentages in serum and all lipoprotein classes were only slightly reduced is characteristic for FED and as such excludes the phenotype of familial complete LCAT deficiency. However, corneal opacities, typical of FED, were not present, and cholesterol esterifica-

tion rate, which in FED should be close to normal, was found to be only 20% of the control rate. Moreover, although reduced by $\approx 80\%$, serum LCAT α -activity was higher than in a typical case of LCAT deficiency or FED. One of the most striking differences compared with features of FED was the presence of anemia and an excess of stomatocytes in the proband. Although occasional erythrocyte abnormalities have been described in patients with complete LCAT deficiency,³⁶ there are few reports on the occurrence of stomatocytosis in this disorder.³⁷⁻³⁹ Among our patients with three different types of mutant LCAT genes, including carriers of the exon 1 C insertion,⁹ the Arg³⁹⁹Cys mutation, or the LCAT_{Fin} mutation, only those with the Arg³⁹⁹Cys mutation, whether heterozygous or homozygous, presented with a significant degree of this erythrocyte abnormality. Whether occurrence of stomatocytosis indeed is a mutation-specific phenomenon can only be investigated by more detailed compositional studies of red-cell membranes.

Prevalence studies on LCAT deficiency have been hampered by problems arising from phenotypic and genotypic heterogeneity of the disease. In general, LCAT mutations are considered to be uncommon at the population level, with the exception of a specific geographically isolated area of Norway, where the frequency of heterozygous carriers was estimated to be up to 4%.⁴⁰ Our own study shows that heterozygosity for a mutant LCAT gene should be considered in cases with serum HDL-C levels on the order of 0.5 mmol/L; at least in Finland, $\approx 5\%$ appear to be affected. It should be pointed out that all participants of the present study were smokers; because smoking is known to diminish HDL-C levels as such, nonsmoking heterozygous carriers of the LCAT mutations may have on the average slightly higher HDL-C levels than those examined in this study. We are not aware of any other example of molecularly defined mutation of LCAT or other genes that shows a similar prevalence in the population or a portion of it and exerts such a profound effect on serum HDL-C levels. It will be of interest to clarify whether the LCAT_{Fin} mutation occurs in other populations.

The data from our study revealing an increased frequency of the LPL Ser²⁹¹ allele in subjects with diminished serum HDL-C levels (4.8%) compared with those with high HDL-C concentrations (1.6%) is in accordance with the data of Reymer et al,⁴ who found this commonly occurring mutation to be associated with reduced serum HDL-C levels in subjects with and without CAD. This allele has also been suggested to contribute to the expression of familial combined hyperlipidemia⁴¹ and dyslipidemia associated with other genetic factors, such as the presence of apo E allele $\epsilon 2$ ⁴² or a mutant LDL receptor gene.⁴³ It appears, however, that this common mutation does not significantly predispose its carrier to CAD per se.^{4,44} Studies carried out *in vivo*⁴ and *in vitro*^{4,45,46} have indicated that substitution of serine for asparagine at codon 291 of the LPL molecule results in a 30% to 50% reduction of the LPL catalytic activity. Phenotypic expression of this mutation is nevertheless incomplete, an idea substantiated by our demonstration of its presence in 5 of 160 subjects with elevated serum HDL-C levels (Table 2).

CETP deficiency caused by intron 14 (G \rightarrow A) and exon 15 (D442G) point mutations is relatively common among the

Japanese, with estimated frequencies of these mutant alleles of $\approx 2\%$ and 7%, respectively.^{24,47-49} Somewhat unexpectedly, we identified one Finnish subject as a heterozygous carrier of the intron 14 G \rightarrow A mutation. His parents, already deceased, and all of his grandparents were of Finnish origin. Although preliminary information on the occurrence of mutations of CETP in central Europe has been presented,⁵⁰ our study appears to provide the first description of the intron 14 G \rightarrow A defect of CETP in a non-Japanese population.

In conclusion, we have described a unique lipoprotein phenotype, with characteristics of both FED and familial LCAT deficiency, in a subject homozygous for the LCAT Arg³⁹⁹Cys mutation and identified another novel LCAT (Gly²³⁰Arg, or LCAT_{Fin}) mutation causing familial LCAT deficiency and accounting for 5% of cases with hypoalphalipoproteinemia in a specific study population. Most strikingly, either the LCAT_{Fin} or LPL Asn²⁹¹Ser mutation was present in 14% of Finnish men who smoked and had an HDL-C level < 0.7 mmol/L, a finding bearing major impact on molecular diagnosis of hypoalphalipoproteinemia.

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