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### Genetic disorders of HDL metabolism: from model to mechanism

Holleboom, A.G.

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# Chapter 10

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## Compromised LCAT Function Is Associated With Increased Atherosclerosis

G. Kees Hovingh, MD; Barbara A. Hutten, PhD; Adriaan G. Holleboom, MD; Wilma Petersen, BSc; Patrick Rol, BSc; Anton Stalenhoef, MD, PhD; Aeilko H. Zwinderman, PhD; Eric de Groot, MD, PhD; John J.P. Kastelein MD, PhD; Jan Albert Kuivenhoven, PhD

From the Departments of Vascular Medicine (G.K.H., A.G.H., W.P., P.R., E.d.G., J.J.P.K., J.A.K.) and Clinical Epidemiology and Biostatistics (B.A.H., A.H.Z.), Academic Medical Center, Amsterdam, the Netherlands, and Department of Medicine (A.S.), University Medical Centre Nijmegen, Nijmegen, the Netherlands.

## Abstract

**Background.** Prospective epidemiological studies have shown that low plasma levels of HDL cholesterol (HDL-C) are associated with an increased risk for cardiovascular disease (CVD). Despite nearly 40 years of research, however, it is unclear whether this also holds true for individuals with severely reduced levels of HDL-C due to mutations in the lecithin:cholesterol acyltransferase (*LCAT*) gene. Better insight into CVD risk in these individuals may provide clues toward the potential of *LCAT* as a pharmaceutical target to raise HDL-C levels.

**Methods and Results.** Lipids, lipoproteins, high-sensitivity C-reactive protein (CRP), and carotid artery intima-media thickness (IMT) were assessed in 47 heterozygotes for *LCAT* gene mutations and 58 family controls. Compared with controls, heterozygotes presented with a mean 36% decrease in HDL-C levels ( $P<0.0001$ ), a 23% increase in triglyceride levels ( $P<0.0001$ ), and a 2.1-fold increase in CRP levels ( $P<0.0001$ ). Mean carotid IMT was significantly increased in heterozygotes compared with family controls ( $0.623\pm 0.13$  versus  $0.591\pm 0.08$  mm). After adjustment for age, gender, and alcohol use, this difference proved statistically significant ( $P<0.0015$ ).

**Conclusions.** The data show that heterozygosity for *LCAT* gene defects is associated with low HDL-C levels and elevated concentration of triglycerides and CRP in plasma. This phenotype underlies increased IMT in carriers versus controls, which suggests that *LCAT* protects against atherosclerosis. This in turn indicates that targeting *LCAT* to raise HDL-C may reduce CVD risk.

## Introduction

Despite the widespread use of pharmacological agents that can effectively decrease LDL cholesterol (LDL-C), the vast majority of patients at increased risk for atherosclerosis continue to have cardiovascular disease (CVD). This underscores the need for additional therapeutic strategies. In this respect, drugs that increase HDL cholesterol (HDL-C) hold promise. In addition to its pivotal role in reverse cholesterol transport, HDL also exerts antithrombotic, antioxidant, and antiinflammatory properties,<sup>1,2</sup> which illustrates that increasing HDL-C levels may result in various beneficial effects.

Hereditary disorders of human HDL metabolism, such as deficiencies of apolipoprotein (apo) A-I, ATP binding cassette A1 (*ABCA1*), lecithin:cholesterol acyltransferase (*LCAT*), and cholesteryl ester transfer protein (*CETP*) have been crucial to our understanding of the reverse cholesterol transport pathway. Consequently, they have proven important in the identification of therapeutic targets to increase HDL-C. This is exemplified by the recent materialization of inhibitors of *CETP* that exhibit strong HDL-C-raising potential in clinical trials.<sup>3,4</sup> Also, increasing the function of *ABCA1*, a transmembrane protein that promotes the efflux of cholesterol and phospholipids, is thought to reduce CVD risk. This assumption is among others that are based on the finding that mutations in the *ABCA1* gene are associated with increased risk for atherosclerosis in humans.<sup>5</sup> This has led to the search for molecules that selectively enhance *ABCA1* function.<sup>6</sup> HDL deficiency and increased risk of coronary artery disease have also been described in families with apoA-I mutations.<sup>7,8</sup> Supported by convincing epidemiological evidence of the protective role of apoA-I, this has further established this protein as a promising target to reduce coronary artery disease risk. This knowledge has recently prompted the use of infusion of apoA-I-containing phospholipid complexes in patients with acute coronary syndrome, which was shown to induce regression of coronary atheroma.<sup>9</sup>

*LCAT* deficiency represents another rare, recessive genetic disorder that underlies HDL deficiency. *LCAT* is a plasma enzyme that esterifies free cholesterol, primarily at the surface of the HDL particle, after which the cholesteryl ester molecules migrate to the inner core of this lipoprotein. Through this action, *LCAT* plays a key role in the maturation of HDL particles.<sup>10</sup> Norum and Gjone<sup>11</sup> first described that *LCAT* gene mutations underlie familial *LCAT* deficiency. Later, it was recognized that a less severe clinical phenotype, fish-eye disease, results from mutations in this very gene.<sup>12</sup> Familial *LCAT* deficiency and fish-eye disease are both characterized by HDL deficiency (5% to 10% of normal HDL-C levels). (For a review of the effect of *LCAT* mutations on lipid metabolism, see Pritchard and Hill.<sup>13</sup>) Although *LCAT* has thus long been known to play an important role in HDL metabolism, its association with atherosclerosis has remained elusive. This is mainly because of the very limited numbers of carriers of *LCAT* gene mutations. In addition, animal data have provided conflicting results.<sup>14,15</sup> We therefore set out to study the relationships between *LCAT* and atherosclerosis using B-mode ultrasound intima-media

thickness (IMT) measurements of the carotid arteries as a validated marker for CVD risk and atherosclerosis in large cohort of *LCAT* mutation carriers.

## Methods

### *Study Groups*

Over the past 14 years, our laboratory has, in collaboration with the laboratory of Dr. P.H. Pritchard, characterized 5 families of Dutch Caucasian descent with *LCAT* deficiency disorders. Families 1,<sup>16</sup> 2,<sup>17,18</sup> and 3<sup>19</sup> have been described previously. The probands of these 3 families experienced corneal clouding and consulted ophthalmologists before being referred to our Lipid Clinic. More recently, we identified 2 additional probands with *LCAT* deficiency disorders (families 4 and 5, respectively), but these data were not published.

Briefly, the proband of family 4 male, aged 68 years, was referred to our Lipid Clinic for low HDL-C levels. Upon physical examination, corneal opacities were noted, whereas laboratory tests showed an HDL-C level of 0.15 mmol/L. *LCAT* activity was assessed by using a proteoliposome substrate<sup>20</sup> and was severely reduced, which was related to compound heterozygosity for 2 missense mutations (underlying P10Q and V309M, respectively). The proband of family 5, a 20-year-old woman, was referred to our Lipid Clinic for corneal clouding. She had HDL deficiency (HDL-C 0.09 mmol/L), and her plasma *LCAT* activity was severely reduced due to compound heterozygosity for point mutations that caused amino acid substitutions at positions 123 (T123I) and 309 (V309M) of the mature *LCAT* protein.

For the present cross-sectional analysis, the probands and their family members of the aforementioned 5 families were invited to participate in an IMT study irrespective of cardiovascular or genetic status; no inclusion or exclusion criteria were used. The recruitment of study individuals was performed as follows: we contacted the index patients of the families who were previously found to be homozygous or compound heterozygous for *LCAT* gene mutations. Using these individuals as central spokespersons, family members were invited to participate by our physicians and genetic field workers (sometimes through the organization of family reunions).

This was done through a letter and/or by telephone. Family members included both first- and second-degree relatives and those who had married into the family.

All *LCAT* gene defects under study have previously been shown to underlie marked loss of *LCAT* activity (30% to 42% compared with family controls), which has as its direct consequence a 30% to 38% reduction of HDL-C levels.<sup>16,19,21</sup> We therefore only genotyped the participants (using previously described methods<sup>16,18,19,21</sup>) to distinguish between carriers and noncarriers. Heterozygotes for *LCAT* gene mutations do not have clinical signs or complaints despite having these marked reductions of HDL-C levels. Thus, importantly, there existed no clinical recruitment bias for the heterozygotes. From families 1, 2, 3, 4, and 5, we recruited 3, 2, 30, 6, and 6 heterozygotes and 6,

0, 45, 3, and 4 controls, respectively. Nine homozygotes or compound heterozygotes, characterized by near complete HDL-C deficiency, also underwent IMT measurements (average IMT was  $0.73 \pm 0.02$  mm). However, the low number and the completely different age distribution ( $60.9 \pm 15.6$  versus  $41.9 \pm 16.1$  and  $42.2 \pm 17.4$  years in controls and heterozygotes, respectively) unfortunately could not provide the required statistical foundation for solid conclusions with regard to the impact of *LCAT* on atherosclerosis. The data from this group were therefore not used for further analysis. Past medical history, presence of cardiovascular risk factors, and use of medication were assessed by questionnaire. The data on clinical events were verified by studying the clinical records. Informed consent was obtained for plasma sampling, storage, genetic analysis, and IMT measurements. The study was approved by the Ethics Committee of the Academic Medical Center in Amsterdam.

### *Blood Analyses*

Blood was collected in EDTA-coated tubes after overnight fasting. Total cholesterol, triglycerides, and HDL-C were measured by established methods, and LDL-C was calculated by the equation of Friedewald.<sup>22</sup> Plasma apoA-I and apoB were measured with the Nephelometric BNII System (Dade Behring).<sup>23,24</sup> High-sensitivity C-reactive protein (hs-CRP) levels were measured by the Dade Behring method. *LCAT* activity measurements were performed with an exogenous apoA-I proteoliposome substrate as described previously.<sup>20</sup>

### *Carotid Artery IMT and CVD*

IMT measurements were performed in a standardized fashion for both the carriers of *LCAT* gene mutations and the controls, as described previously.<sup>25</sup> Briefly, an Acuson 128XP/10v (Acuson Corporation) equipped with a 7.0-MHz linear-array transducer was used to obtain B-mode ultrasound images. The following wall segments were bilaterally scanned over a length of 10 mm: the common carotid artery, the carotid bulb, and the internal carotid artery. Images were saved as JPEG image files, and a reader, blinded for the genetic status of the patient, measured the IMT of the far wall of the respective segments. The mean combined outcome of the 6 segments was used for analysis. A vascular event was defined by the presence of at least 1 of the following: acute myocardial infarction, percutaneous coronary intervention, coronary artery bypass grafting, and angina pectoris, as well as by the presence of peripheral or cerebrovascular disease. Cardiovascular events were ascertained by use of questionnaires and verified by studying the clinical records.

### *Statistical Analysis*

Results are expressed as means (SD), except for triglycerides, lipoprotein(a), and hs-CRP levels, which are expressed as medians (interquartile range) because of a skewed distribution. These variables were log-transformed before statistical analysis. Differences

in terms of demographic and lifestyle characteristics between heterozygotes for *LCAT* gene mutations and family controls were evaluated with linear or logistic regression analyses with generalized estimating equations in the SAS procedure GENMOD to account for correlations within families. For differences in blood pressure, smoking, and alcohol use, we made adjustments in a multivariate model for age and gender. To evaluate differences between the 2 groups in biochemical characteristics and mean carotid IMT, we used the same SAS procedure, allowing for clustering within families (due to clustering of genetic and/or environmental factors). For the biochemical characteristics, we adjusted for age, gender and smoking using multivariate models. For mean carotid IMT, the main outcome of this study, a more elaborate procedure was used. We first explored univariately the relation between mean carotid IMT and baseline variables.

Hereafter, using multivariate models, we identified independent predictors after stepwise backward selection. For all generalized estimating equation models, the exchangeable correlation structure was used. The difference between controls and *LCAT* heterozygotes was tested by assessing their interaction (age and group). Probability values < 0.05 were considered significant. For statistical analyses the SAS package (release 8.02; SAS Institute Inc) was used.

## Results

### *Genetic and Demographic Characteristics of the Study Groups*

A total of 47 heterozygotes for *LCAT* gene mutations and 58 controls (ascertained by genotyping) were recruited from 5 families of Dutch descent originating from different parts of The Netherlands. Mean average age was nearly identical among the heterozygotes and controls (42.2 versus 41.9 years of age, respectively; Table 1). Males were slightly more prevalent among the heterozygotes (62%) than among controls (47%), but this did not reach statistical significance ( $P = 0.062$ ). Systolic and diastolic blood pressure and

**Table 1.** Demographic and Lifestyle Characteristics and LCAT Activity Levels of Heterozygotes for LCAT Gene Mutations and Family Controls

	Controls (n=58)	Heterozygotes (n=47)	P(unadjusted)	P(Adjusted)*
Age, y	41.9±16.1	42.2±17.4	0.848	...
Male gender, n (%)	27 (47)	29 (62)	0.062	...
Diastolic blood pressure, mmHg	81.8±21.9	88.8±25.9	0.183	0.303
Systolic blood pressure, mmHg	131.6±24.0	126.5±34.5	0.951	0.909
Smoking, n (%)	8(14)	10(21)	0.007	0.015
Alcohol users, n (%)	41 (71)	33 (72)	0.805	0.580
LCAT activity, nmol*ml <sup>-1</sup> *h <sup>-1</sup>	>25	14-18	...	...

\*P values for the variables blood pressure (diastolic and systolic), smoking, and alcohol use were adjusted for age and sex. †LCAT levels as measured against an apoA-I– containing liposome substrate. Data were derived from previous publications.16,18,19,21

alcohol use did not differ among the groups. The percentage of smokers, however, was significantly higher in the heterozygotes than in the controls (21% versus 14%;  $P = 0.015$ ). One heterozygous carrier and 2 subjects in the control group had been prescribed a statin. No patient had diabetes mellitus, defined as use of oral antidiabetic drugs or insulin. The data on *LCAT* activity levels represent data from previous publications.<sup>16,18,19,21</sup> In these reports, the *LCAT* mutations in the respective families have been shown to result in 30% to 42% reductions in *LCAT* activity, with concomitant 30% to 38% reductions in HDL-C levels in the heterozygous carriers compared with family controls.

### *Lipids, (Apo)lipoproteins, and C-Reactive Protein Levels*

Table 2 gives the raw data accompanied by probability values adjusted for age, gender, smoking, and family. Compared with family controls, heterozygotes exhibited a mean 36% decrease in HDL-C levels and a mean 22% decrease in apoA-I levels ( $P < 0.0001$  for both). Mean triglyceride levels were, in contrast, increased by 23% ( $P = 0.026$ ). In the presence of unchanged total cholesterol levels, the heterozygotes also displayed a

**Table 2.** Biochemical Characteristics of Heterozygotes for *LCAT* Gene Mutations and Family Controls

	Controls (n=58)	Heterozygotes (n=47)	P*
HDL-c, mg/dl	49.8 ± 15.1	31.7 ± 9.3	<0.0001
ApoA-I, g/l	1.56 ± 0.28	1.21 ± 0.16	<0.0001
Triglycerides, mg/dl	88.5 (58.4-121.2)	108.8 (77-175.2)	0.026
Total cholesterol, mg/dl	177.2 ± 38.2	176.4 ± 44.4	0.453
LDL-c, mg/dl	107.7 ± 33.6	115.4 ± 32.8	<0.0001
ApoB, g/l	0.93 ± 0.25	1.00 ± 0.33	0.187
Lipoprotein(a), mg/l	104 (62-176)	93 (52-248)	0.819
hs-CRP, mg/l	2.00 (1.20-4.20)	4.40 (1.90-7.20)	<0.0001

Values are means (SD) or median (interquartile range). \*Adjusted for age, gender, and smoking.

significant 7.1% increase in LDL-C levels ( $P < 0.0001$ ), with a concomitant nonsignificant 7.5% increase in apoB levels. Finally, heterozygotes presented with a marked 2.2-fold increase in C-reactive protein (CRP) plasma levels ( $P < 0.0001$ ).

### *IMT and Cardiovascular Events*

Mean IMT values were 0.591\_0.08 mm for controls and 0.623\_0.13 mm for heterozygotes (Table 3). After adjustment for age, gender, use of alcohol, and family, this difference in average IMT proved to be highly statistically significant ( $P = 0.0015$ ). This probability value was obtained with multivariate backward stepwise regression analysis, whereby age, gender, and use of alcohol remained in the model but smoking and blood pressure did not. Although the family controls did not experience cardiovascular events, 1 male



**Table 3.** Carotid IMT and CVD Events for Heterozygotes for LCAT Gene Mutations and Family Controls

	Controls (n=58)	Heterozygotes (n=47)	P(Adjusted)*
Mean carotid IMT, mm	0.591±0.008	0.623±0.13	0.0015
Cardiovascular events, n (%)	0 (0)	2 (4)	...

\*Adjusted for all demographic and lifestyle characteristics that stayed in the model with backward stepwise regression analysis (age, gender, and alcohol use).

heterozygote had angina pectoris at age 50 years and a myocardial infarction at age 54 years, and a second heterozygote had a myocardial infarction at age 58 years.

## Discussion

It has long been difficult to assess the risk of atherosclerosis in individuals with genetically determined low HDL-C levels. This knowledge, however, can provide important insight when it comes to targeting HDL metabolism to reduce CVD risk. After almost 40 years of LCAT research, the present study provides evidence that heterozygotes for *LCAT* gene defects, who present with an average 36% decrease in HDL-C levels, exhibit an increased risk for atherosclerosis as assessed by IMT measurements. These data suggest that intact LCAT function is important in the protection against atherosclerotic vascular disease.

### *LCAT, Lipids, and Lipoproteins*

The present analysis confirms the strong impact of *LCAT* gene mutations on HDL-C levels. All *LCAT* gene defects under study have previously been shown to underlie marked loss of LCAT activity (30% to 42% compared with family controls), and as a direct consequence, to result in 30% to 38% reductions in HDL-C levels<sup>16,19,21</sup> (for a review, see Pritchard and Hill<sup>13</sup>). LCAT is primarily active at the phospholipid monolayer of nascent HDL particles, where it converts cholesterol into cholesteryl ester on activation by its cofactor, apoA-I. Even a single change in the amino acid sequence of LCAT has been shown to result in a decreased interaction with apoA-I, with a consequential decrease in LCAT activity.<sup>26</sup> This will, in turn, cause defective maturation of small HDL into larger, spherical, cholesteryl ester–enriched HDL and hence lower HDL-C levels. The data show that this effect is not counteracted by upregulation of the unaffected *LCAT* allele in heterozygotes or by other key players in HDL metabolism. Additionally, the present study shows the effect of *LCAT* mutations on plasma triglycerides levels, as has been reported previously.<sup>20,27</sup> Although heterozygotes presented with a moderate 22% increase in triglyceride levels, homozygotes presented with a marked 337% increase in triglyceride levels (data not shown). Mild hypertriglyceridemia is also observed in other genetic HDL deficiencies.<sup>28,29</sup> For LCAT, hepatic triglyceride overproduction and reduced lipoprotein lipase activity, as found in LCAT-deficient mice, may explain this phenotype.<sup>30</sup> Finally, we identified a small but significant increase in LDL-C levels compared with controls, for which we have no explanation.

### *LCAT and Atherosclerosis*

Despite the fact that *LCAT* has long been recognized as a key regulator of HDL metabolism, the role of this enzyme in human atherogenesis has remained controversial. Prospective studies that have assessed *LCAT* activity or *LCAT* concentration at baseline are nonexistent, whereas cross-sectional observational studies have reported increased and decreased *LCAT* activity in subjects with CVD.<sup>31,32</sup> Initially, the paradoxical finding of complete HDL deficiency and the reported absence of CVD in *LCAT*-deficient patients has been used to reject the hypothesis that HDL is important in the protection against atherosclerosis. Potential mechanisms to explain these findings were subsequently postulated, such as preferential clearance of HDL fractions that have less atherogenic potential.<sup>33</sup> In addition, decreased LDL-C and apoB levels have also been put forward as an explanation for the lack of marked CVD. In the large group of *LCAT* mutation carriers in the present study, however, LDL-C was clearly not decreased, which refutes this argument. Studies focusing on the role of *LCAT* in human atherosclerosis have thus far been hampered by the paucity of clinical events in small numbers of individuals with *LCAT* mutations. The advent of validated surrogate markers for atherosclerosis has proved very useful in this context. IMT measurements have been used to show that carriers of *ABCA1* and apoA-I defects are at increased risk for coronary artery disease,<sup>8,34,35</sup> which illustrates the power of this tool to study small, interesting groups of patients. The present study now shows that heterozygotes for *LCAT* mutations also have increased atherosclerosis compared with family controls. This finding was not based on a few heterozygous outliers with a very thick carotid intima-media complex but on solid IMT data. This is illustrated by the fact that the 30 heterozygotes of the largest family (family 3) presented with a thicker IMT than their 45 family control members (0.614 versus 0.589 mm). This effect, however, did not reach statistical significance, which illustrates the need to recruit and study more families with the same rare genetic disorder to answer our primary research question. During the preparation of this report, Ayyobi et al<sup>36</sup> also reported vascular abnormalities in a small cohort of 9 heterozygotes for 1 specific *LCAT* mutation. The investigators could not, however, study family controls, and because of the small number of study subjects, these investigators could not draw conclusions regarding the risk for atherosclerosis.<sup>36</sup> A potential but dangerous drawback of studying atherosclerosis in relatively small cohorts, such as investigated here, is the referral bias of the index patients. In this respect, we would like to emphasize that the probands of the currently studied families came to our attention because they experienced corneal opacifications, or they were referred because of previously identified low HDL-C levels. This excludes a bias for selecting those families with *LCAT* mutations with an increased or established risk for CVD. Exactly how reduced *LCAT* function affects IMT, however, cannot be deduced from the present study. *LCAT* mutations may directly compromise reverse cholesterol transport by reducing the flux of cholesterol from cholesterol-loaded peripheral macrophages to the HDL fraction. The markedly reduced HDL-C levels in carriers also may affect endothelial function directly.<sup>37</sup> Alternatively, loss of *LCAT* activity may cause enhanced oxidation of LDL.<sup>38</sup>

### *LCAT and Inflammation*

Elevated levels of CRP are an established predictor for coronary artery disease,<sup>39</sup> but there is much debate about whether this plasma factor plays an active role in atherosclerosis or whether it merely represents an innocent bystander. We were surprised to find that hs-CRP levels were 2.2-fold increased in the heterozygotes. From the present study, however, it can not be appreciated whether increased levels of CRP are a direct consequence of reduced LCAT activity or of the consequentially reduced HDL-C levels. Pirro and colleagues<sup>40</sup> also reported significantly higher CRP levels in other subjects with hypoalphalipoproteinemia, which suggests a proinflammatory status in subjects with low HDL-C levels per se.

### *Concluding Remarks*

Upregulation of LCAT function has been proposed as an HDL-C increasing therapy, but its atheroprotective effects have been questioned.<sup>41</sup> The present study suggests that increasing LCAT activity may reduce atherosclerosis progression, at least in subjects with subnormal levels of LCAT. Efforts to develop LCAT protein therapy have failed, but *LCAT* gene therapy protocols are more promising, as recently illustrated by decreased atherosclerosis in dyslipidemic obese mice<sup>42</sup> after adenovirus-mediated transfer of the *LCAT* gene.

Also, Zhang et al<sup>43</sup> recently showed that a similar strategy increased reverse cholesterol transport in hamsters but, interestingly, not in mice. The present report indicates that increasing LCAT activity in plasma might be speculated to result not only in increased HDL-C levels but also in decreased concentrations of triglycerides and hs-CRP. Moreover, increased LCAT activity might also result in an augmented capacity of HDL to exhibit antioxidative, antiinflammatory,<sup>44</sup> and antithrombotic effects.

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