Lipid Oxidation in Carriers of Lecithin:Cholesterol Acyltransferase Gene Mutations

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Objective—Lecithin:cholesterol acyltransferase (LCAT) has been shown to play a role in the depletion of lipid oxidation products, but this has so far not been studied in humans. In this study, we investigated processes and parameters relevant to lipid oxidation in carriers of functional LCAT mutations.

Methods and Results—In 4 carriers of 2 mutant LCAT alleles, 63 heterozygotes, and 63 family controls, we measured activities of LCAT, paraoxonase 1, and platelet-activating factor-acetylhydrolase; levels of lyso phosphatidylcholine molecular species, arachidonic and linoleic acids, and their oxidized derivatives; immunodetectable oxidized phospholipids on apolipoprotein (apo) B–containing and apo(a)-containing lipoproteins; IgM and IgG autoantibodies to malondialdehyde-low-density lipoprotein and IgG and IgM apoB-immune complexes; and the antioxidant capacity of high-density lipoprotein (HDL). In individuals with LCAT mutations, plasma LCAT activity, HDL cholesterol, apoA-I, arachidonic acid, and its oxidized derivatives, oxidized phospholipids on apo(a)-containing lipoproteins, HDL-associated platelet-activating factor-acetylhydrolase activity, and the antioxidative capacity of HDL were gene-dose–dependently decreased. Oxidized phospholipids on apoB-containing lipoproteins was increased in heterozygotes (17%; P<0.001) but not in carriers of 2 defective LCAT alleles.

Conclusion—Carriers of LCAT mutations present with significant reductions in LCAT activity, HDL cholesterol, apoA-I, platelet-activating factor-acetylhydrolase activity, and antioxidative potential of HDL, but this is not associated with parameters of increased lipid peroxidation; we did not observe significant changes in the oxidation products of arachidonic acid and linoleic acid, immunoreactive oxidized phospholipids on apo(a)-containing lipoproteins, and IgM and IgG autoantibodies against malondialdehyde-low-density lipoprotein. These data indicate that plasma LCAT activity, HDL-associated platelet-activating factor-acetylhydrolase activity, and HDL cholesterol may not influence the levels of plasma lipid oxidation products.

Key Words: gene mutations ▪ lecithin cholesterol:acyl transferase ▪ lipoproteins ▪ lipid oxidation

Decreased levels of high-density lipoprotein (HDL) cholesterol (HDL-c) are strongly associated with risk of coronary artery disease. HDL is considered to exhibit antiatherogenic properties mostly through its role in reverse cholesterol transport, but HDL can also provide protection against oxidation through established actions of its associated proteins and enzymes. These include paraoxonase-1 (PON1), platelet-activating factor-acetylhydrolase (PAF-AH), and lecithin:cholesterol acyltransferase (LCAT).

Oxidized phospholipids (OxPL) are key mediators of further lipid oxidation and inflammation, while the detrimental effects of OxPL are attenuated upon removal of the sn-2–oxidized fatty acids. This reaction can be catalyzed by LCAT, an enzyme that is secreted into the circulation where it is primarily associated with HDL. LCAT hydrolyzes the acyl group at the sn-2 position of phosphatidylincholine (or lecithin) and transfers this fatty acid to free cholesterol forming a cholesteryl ester (CE). Through this reaction, it generates most of the CE present in human plasma, thereby maturing HDL. Through this phospholipase-A 2 (PLA 2 ) activity, LCAT can also hydrolyze oxidized acyl chains from phosphatidylincholine-based OxPL, generating the less

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bioactive lysophosphatidylcholine (lysoPC)\(^7\) and oxidized free fatty acids, which may be subsequently used by LCAT to esterify diacylglycerol, thereby generating triglycerides in addition to the formation of CEs.\(^6\) Several in vitro and animal studies have shown that through this PLA\(_2\) activity, LCAT can apparently remodel vascular lipids, resulting in an apparent decrease in oxidative stress by hydrolyzing oxidized sn-2 fatty acids from phosphatidylcholine-based OxPL.\(^7,16-18\)

In humans, mutations in LCAT resulting in a loss of enzymatic LCAT activity cause reductions of HDL-c levels.\(^19\) It is, however, not known what impact the loss of LCAT activity might have on lipid oxidation. To study this, we used plasma of carriers of 1 or 2 defective LCAT alleles and of the mutations is based on guidelines of the Human Genome Mutation Database (hgmd). Controls were first-, second-, or third-degree family members or spouses. Inform consent was obtained for blood sampling, storage, genetic, and biochemical analysis. The study was approved by the Institutional Review Board of the Academic Medical Center in Amsterdam.

### Methods

#### Study Design

Homozygous, compound heterozygous, and heterozygous carriers of deleterious LCAT mutations were enrolled in this study. Nomenclature of the mutations is based on guidelines of the Human Genome Variation Society (for LCAT, this means that 24 aa positions—leader sequence—are added to previous annotations). Controls were first-, second-, or third-degree family members or spouses. Inform consent was obtained for blood sampling, storage, genetic, and biochemical analysis. The study was approved by the Institutional Review Board of the Academic Medical Center in Amsterdam.

#### Plasma Lipids and LCAT Activity

Serum and EDTA plasma blood samples were obtained after an overnight fast and stored at \(-80^\circ\)C until use. Total cholesterol, low-density lipoprotein (LDL) cholesterol, HDL-c, triglycerides, and apoA-I and apoB were measured using a COBAS MIRA analyzer. LCAT activity was measured in whole plasma using a proteoliposome substrate.\(^23\) Lipoprotein a (Lp(a)) was measured using quantitative immunoprecipitation analysis with commercially available antisera (Diasorin, Stillwater, MN).

#### Quantification of LysoPC Molecular Species

Internal standard (1000 ng 19:0 Lyso-PC) was added to human plasma, and lipids were extracted 3× by a modified Dole extraction method.\(^24\) The combined organic extracts were dried under N\(_2\) flow and then the dried lipids resuspended in water/acetonitrile/2-propanol (8/1/1) and injected onto an Agilent C8 column (2×150 mm, 5 \(\mu\)m) for separation using Waters HPLC 2690. LysoPC molecular species were detected using electrospray ionization in positive ion mode with multiple reaction monitoring using the trichloroacetic acid precipitation procedure using \([\text{H}]\)-PAF (100 \(\mu\)mol/L final concentration, specific activity 15 000 cpm/nmol) as a substrate.\(^25\) Fifty microliters of HDL diluted 1:3 (vol/vol) with HEPES buffer (pH 7.4) were mixed with HEPES in a final volume of 90 \(\mu\)L and used as the source of the enzyme. Incubations were performed for 10 minutes, and PAF-AH activity was expressed as nmol PAF degraded per minute per mL of HDL.

#### PAF-AH Activity

1-O-hexadecyl-2-[\(\text{H-acetyl}\]-sn-glycero-3-phosphocholine (Hexadecyl PAF, [acetyl-\(\text{H}\)], 0.1 mCi/mL, specific activity range 10–30 Ci/mmol), was purchased from Perkin Elmer (Waltham, MA). Unlabeled PAF (1-O-hexadecyl-2-acetyl-sn-glycerol-3-phosphocholine) was from Sigma Aldrich. PAF-AH activity in HDL, prepared by the dextran-Mg\(^2+\) method (similar to PAF-AH activity in HDL prepared by ultracentrifugation),\(^25\) was measured by the trichloroacetic acid precipitation procedure using \([\text{H}]\)-PAF (100 \(\mu\)mol/L final concentration, specific activity 15 000 cpm/nmol) as a substrate.\(^25\) Fifty microliters of HDL diluted 1:3 (vol/vol) with HEPES buffer (pH 7.4) were mixed with HEPES in a final volume of 90 \(\mu\)L and used as the source of the enzyme. Incubations were performed for 10 minutes, and PAF-AH activity was expressed as nmol PAF degraded per minute per mL of HDL.

#### OxPL per Apolipoprotein, ApoB-Immune Complexes, and Autoantibodies to Oxidized LDL

The content of OxPL on apoB and apo(a) lipoproteins was measured by ELISA, as previously described.\(^27\) All samples for a given analysis were studied in a single assay. Each sample was assayed in triplicate, and data are expressed as relative light units in 100 ms. The levels of circulating OxPL/apoB and OxPL/apo(a) were measured as described, using a sandwich ELISA with anti-apoB or anti-apo(a) capture antibodies\(^20\) and the murine monoclonal antibody E06, which binds the phosphocholine headgroup of OxPL but not native phospholipids.\(^20,28\) Titers of IgG and IgM autoantibodies binding to malondialdehyde (MDA)-LDL were determined at 1:200 dilutions of plasma as previously described.\(^28,30\) ApoB-100-immune complexes were measured as previously described.\(^28,30\)

#### Tandem Mass Spectrometry Quantification of AA and LA and Their Oxidation Products

High-performance liquid chromatography with on-line electrospray ionization tandem mass spectrometry was used to quantify total levels (ie, free fatty acids, plus esterified fatty acids to cholesterol, phospholipids, and triglycerides) of multiple distinct oxidation products of AA and LA, including individual hydroxyeicosatetraenoic acids (HETEs) and hydroxyoctadecadienoic acids (HODEs), using established methods.\(^21,30\) Briefly, 10 ng each of 2 deuterated internal standards, 15(S)-hydroxy-5,8,11,13-eicosatetraenoic-5,6,8,9,11,12,14,15-d8 acid and prostaglandin F\(_2\alpha\) (Cayman Chemical Company, Ann Arbor, MI), were added to plasma samples, fatty acids were released by base hydrolysis with 1 N NaOH at 60°C for 120 minutes under argon atmosphere, samples acidified to pH 3.0 with 2 mol/L HCl, and then fatty acids were extracted twice with 4 mL hexane. The combined hexane extracts were dried under N\(_2\) flow and resuspended in 200 \(\mu\)L 50% methanol/water (vol/vol). Samples were injected onto a C-18 column (2×150 mm, 5 \(\mu\)m, ODS, Phenomenex, Rancho Palos Verdes, CA) at a flow rate of 0.2 mL/min. Separations were performed using a gradient starting from 85% methanol over 4 minutes, then to 100% methanol over 4 minutes, followed by 100% methanol for 8 minutes. High-performance liquid chromatography column effluent was introduced into a triple quadrupole mass spectrometer (Quattro Ultima, Micromass Inc, Manchester, UK). Analyses were performed using electrospray ionization in negative ion mode with multiple reaction monitoring.
of parent and characteristic daughter ions specific for each isomer monitored. The transitions monitored were mass-to-charge ratio (m/z): m/z 295→171 for 9-HODE; m/z 295→195 for 13-HODE; m/z 279→261 for LA; m/z 319→115 for 5-HETE; m/z 319→155 for 8-HETE; m/z 319→151 for 9-HETE; m/z 319→167 for 11-HETE; m/z 319→179 for 12-HETE; m/z 319→175 for 15-HETE; m/z 303→259 for AA; m/z 327→182 for 15(S)-hydroxy-5,8,11,13-eicosatetraenoic-5,6,8,9,11,12,14,15-d8 acid. Collision-induced dissociation was obtained using argon gas. The internal standard 15(S)-hydroxy-5,8,11,13-eicosatetraenoic-5,8,9,11,12,14,15-d8 acid was used for quantification of HETEs, as well as to calculate extraction efficiencies of HODEs and HETEs (which control studies revealed were >85%). The internal standard prostaglandin F2α was used for quantification of F2-isoprostanes.

**DCF Assay**

HDL was prepared by the dextran-Mg2+ method as described.22 The antioxidant capacity of HDL was tested in the presence or absence of LDL as described,22 with some modifications. LDL was isolated from normal plasma by ultracentrifugation as described.33 After isolation, LDL was exposed to normal atmospheric conditions with gentle mixing at room temperature for 6 hours to generate mildly oxidized LDL (OxLDL). Then LDL was stored at 4°C under nitrogen. 2,7 dichlorofluorescin diacetate (DCFH-DA, Molecular Probes/Invitrogen, Carlsbad, CA) was dissolved in fresh methanol at 2.0 mg/mL and incubated at room temperature in the dark for 20 minutes, resulting in the release of DCFH. Upon interaction with lipid peroxidation products, DCFH is oxidized to fluorescent 2,7-dichlorofluorescein (DCF). HDL from normolipidemic healthy individuals inactivates OxLDL and therefore prevents the oxidation of DCFH and release of DCF. To determine the functional properties of HDL, patient and control HDL was used in this assay in the presence or absence of OxLDL. To compare the HDL antioxidant properties of affected and unaffected subjects by the DCF assay, we normalized for differences in HDL-c concentration. Thus, any differences observed in our study are a result of reduced HDL functionality and not reduced HDL-c levels. HDL (final concentration 50 μg cholesterol/mL) in the presence or absence of OxLDL (final concentration 100 μg cholesterol/mL) was added into a black 96-well plate in a final volume of 100 μL. The plate was incubated at 37°C on a rotator for 1 hour in the dark. At the end of this incubation period, 10 μL of DCFH solution (0.2 mg/mL) was added to each well, mixed, and incubated for an additional 2 hours at 37°C with rotation in the dark. Fluorescence was measured with a plate reader (Fluo-Star Galaxy, bMG) at an excitation wavelength of 465 nm and an emission wavelength of 535 nm.

**Statistical Analyses**

All data presented are means with SDs, unless otherwise indicated. Parameters were compared between 2 groups using Student t test for continuous variables and χ² test for categori-cal variables. ANOVA was applied to study differences over 3 groups in case of stepwise differences. In case of a significant difference in univariate analysis, multivariate analysis by linear regression was carried out to assess independence of observed effects. Statistical analyses were performed using SPSS software (version 12.0.2, SPSS Inc, Chicago, IL). P<0.05 was considered statistically significant.

**Results**

**Study Group Characteristics**

Two patients with familial LCAT deficiency (FLD) and 2 patients with fish-eye disease (FED) participated. One patient with FED was homozygous for the p.T123I mutation34 (which is now annotated p.T147I, including the 24-aa leader sequence, following the guidelines of the Human Genome Variation Society to describe mutations), the other compound heterozygous for p.T147I and p.V333M.35 The patients with FLD were homozygous for either p.C337Y36 or p.T345M.37 In addition, 63 heterozygous carriers of LCAT gene mutations and 63 unaffected family controls participated in the study. Twenty heterozygotes carried a mutation known to cause FLD when present on both alleles; 39 heterozygotes carried a mutation known to cause FED when present on both alleles; finally, 4 heterozygotes carried point mutations of which it is unknown whether they cause FED or FLD when present on both alleles (no homozygous patients have been described to help in this regard). Table I in the online-only Data Supplement gives an overview of the exact molecular LCAT defects in the heterozygotes.

Table 1 summarizes the demographic, lifestyle, and clinical characteristics of cases and controls. Age, body mass index, alcohol use, and the percentage of smokers were similar in heterozygotes and family controls. There were more men among the heterozygotes, but this did not reach statistical significance. Heterozygotes experienced more cardiovascular disease (P<0.01) concordant with a more frequent use of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase inhibitors (statins) compared with controls (P<0.001). HDL-c and apoA-I were gene-dose–dependently decreased in carriers of LCAT mutations, whereas levels of LDL-c, apoB, and triglycerides did not differ between the groups. The gene-dose–dependent decrease in total cholesterol could therefore be attributed to the decrease in HDL-c. Carriers of 2 defective LCAT alleles presented with severe HDL-c deficiency and severely reduced Lp(a).

**LCAT, PON1, and PAF-AH Activities**

To study the role of LCAT in lipid oxidation in humans, we studied all HDL-related enzymes with an antioxidative function comparable with LCAT (ie, hydrolysis of OxPL). LCAT16,17 and PAF-AH16 are well known in this respect; for PON1, this has been strongly suggested.34,35 Table 2 shows a highly significant gene-dose–dependent decrease of plasma LCAT and HDL-associated PAF-AH activities in carriers of LCAT mutations (for both, P for ANOVA <0.001). HDL-associated PON1 activity levels were similar in heterozygotes and controls, whereas carriers of 2 defective LCAT alleles showed a 85% reduction in PON1 activity (P<0.001).

**LysoPC Molecular Species**

LCAT, via its PLA₂ activity, hydrolyzes phospholipids removing sn-2 fatty acyl groups for subsequent esterification (via acyltransferase activity) to cholesterol and releasing lysoPC. As a measure of LCAT PLA₂ activity, we quantified individual molecular species of lysoPC. Table 3 shows slightly lower concentrations of all molecular species of lysoPC in the heterozygotes compared with controls, but this did not reach significance for any of the individual lysoPC species. In the homozygotes/compound heterozygotes however, all molecular species of lysoPC were significantly reduced (average reduction 58%) compared with controls.
Loss of LCAT activity was associated with decreased total levels of essential fatty acids (free plus esterified; Table 4): total plasma levels of AA were gene-dependently decreased in carriers of \( \text{LCAT} \) mutations (\( P \) for ANOVA=0.049). Total plasma levels of LA were also significantly decreased in heterozygotes compared with controls (\( P =0.007; P =0.02 \) after adjustment for age and sex). Carriers of 2 defective alleles, however, showed similar levels of LA as the heterozygotes.

Measuring oxidation products of AA, we found that all HETEs (ie, 5-, 8-, 9-, 11-, 12-, 15-HETEs) were significantly reduced in carriers of 2 defective LCAT alleles compared with controls. The 8-, 9-, 11-, and 12-HETEs levels were also significantly reduced when comparing heterozygotes and controls, and for these parameters we also observed a gene-dose effect (ANOVA: \( P =0.008, P =0.024, P =0.006, P =0.005, \) respectively). Among the multiple LA oxidation products monitored, we found no significant differences in the LA oxidation products 9- and 13-HODEs when comparing the carriers of \( \text{LCAT} \) mutations and controls.

Because absolute fatty acid levels were decreased, we calculated the ratio of oxidized to total fatty acids. Table II in the online-only Data Supplement shows that ratios of HETEs/AA and HODEs/LA are not different in heterozygotes and controls. In carriers of 2 defective LCAT alleles, 9-, 11-, and 12-HETEs/AA ratios were significantly lower compared with controls.

OxPL/ApoB and OxPL/Apo(a), ApoB-Immune Complexes, and Autoantibodies Against MDA-LDL

OxPL/apoB were significantly increased in heterozygotes compared with family controls (\( P =0.01 \); Table 5) and remained

### Table 1. Clinical Characteristics, Lipids, and Lipoproteins

<table>
<thead>
<tr>
<th></th>
<th>Homozygous/Compound Heterozygotes (n=4)</th>
<th>Heterozygotes (n=63)</th>
<th>Family Controls (n=63)</th>
<th>( \text{PHZ/cHT vs Controls} ) (Unadjusted)</th>
<th>( \text{PHeterozygotes vs Controls} ) (Unadjusted)</th>
<th>( \text{PHeterozygotes vs Controls} ) (Adjusted)</th>
<th>( P ) for ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>34.0±19</td>
<td>44.8±1.7</td>
<td>44.2±2.1</td>
<td>0.69</td>
<td>0.82</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Men, n (%)</td>
<td>2 (50)</td>
<td>38 (60)</td>
<td>28 (44)</td>
<td>0.92*</td>
<td>0.07*</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>23.4±4.8</td>
<td>25.6±0.64</td>
<td>24.5±0.55</td>
<td>0.44</td>
<td>0.19</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Alcohol (units per wk)†</td>
<td>3 (6)</td>
<td>4 (5)</td>
<td>3 (6)</td>
<td>0.59</td>
<td>0.64</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Current smokers, n (%)</td>
<td>0 (0)</td>
<td>6 (10)</td>
<td>5 (8)</td>
<td>0.72*</td>
<td>0.76*</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>CVD cases, n (%)</td>
<td>0 (0)</td>
<td>7 (11)</td>
<td>1 (2)</td>
<td>0.79*</td>
<td>&lt;0.01*</td>
<td>0.08*</td>
<td></td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (3)</td>
<td>0.15*</td>
<td>0.15*</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>Statin use, n (%)</td>
<td>2 (50)</td>
<td>15 (24)</td>
<td>2 (3)</td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>115±56</td>
<td>178±45</td>
<td>195±41</td>
<td>&lt;0.001</td>
<td>0.034</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>LDL-cholesterol, mg/dL</td>
<td>87±40</td>
<td>126±35</td>
<td>130±30</td>
<td>&lt;0.01</td>
<td>0.55</td>
<td>0.041</td>
<td></td>
</tr>
<tr>
<td>HDL-cholesterol, mg/dL</td>
<td>6.4±2.5</td>
<td>33±12</td>
<td>55±20</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Triglycerides, mg/dL †</td>
<td>89 (63)</td>
<td>118 (114)</td>
<td>92 (80)</td>
<td>0.49</td>
<td>0.11</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Apo A-I, mg/dL</td>
<td>31±0.2</td>
<td>126±26</td>
<td>159±33</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Apo B, mg/dL</td>
<td>74±8.6</td>
<td>103±26</td>
<td>100±23</td>
<td>0.12</td>
<td>0.56</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Lp(a), mg/dL †</td>
<td>0.35 (0.1)</td>
<td>1.9 (38)</td>
<td>2.0 (25)</td>
<td>&lt;0.001</td>
<td>0.86</td>
<td>0.03</td>
<td></td>
</tr>
</tbody>
</table>

BMI indicates body mass index; cHT, compound heterozygote; CVD, cardiovascular disease; LDL, low-density lipoprotein; HDL, high-density lipoprotein; HZ, homozygote; Apo, apolipoprotein; Lp(a), lipoprotein a; N/A, not applicable.

\*P for \( \chi^2 \) test. Data for alcohol use, triglycerides, and Lp(a) were log-transformed before \( t \) test/ANOVA because of a skewed distribution.

†Median (interquartile range).

‡Diabetes mellitus type 2.

### Table 2. LCAT, PAF-AH, and PON1 Activities

<table>
<thead>
<tr>
<th></th>
<th>Homozygous/Compound Heterozygotes (n=4)</th>
<th>Heterozygotes (n=63)</th>
<th>Family Controls (n=63)</th>
<th>( \text{PHZ/cHT vs Controls} ) (Unadjusted)</th>
<th>( \text{PHeterozygotes vs Controls} ) (Unadjusted)</th>
<th>( \text{PHeterozygotes vs Controls} ) (Adjusted)</th>
<th>( P ) for ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCAT activity, nmol/mL per hour</td>
<td>0.45±0.2</td>
<td>9.6±2</td>
<td>13.6±2</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PAF-AH activity, nmol/min per mL*</td>
<td>1.4 (1.8)</td>
<td>3.2 (2.0)</td>
<td>3.9 (1.6)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PON1 activity, U/L*</td>
<td>7.5 (38)</td>
<td>46 (79)</td>
<td>51 (66)</td>
<td>&lt;0.001</td>
<td>0.39</td>
<td>N/A</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

LCAT indicates lecithin:cholesterol acyltransferase; PAF-AH, platelet-activating factor acetyl hydrolase; PON1, paraoxonase-1; cHT, compound heterozygote; HZ, homozygote.

\( \text{PHZ/cHT vs controls} \): \( P \) for unpaired 2-tailed \( t \) test homozygotes/compound heterozygotes vs controls.

\( \text{P (unadjusted)} \): \( P \) for unpaired 2-tailed \( t \) test.

\( \text{P (adjusted)} \): \( P \) for linear regression model including age and sex as potential confounders.

\( \text{P for ANOVA} \) to assess differences over the 3 groups.

*Median and interquartile range. Data were log-transformed before statistical analysis because of a skewed distribution.
significantly increased ($P=0.017$) after correction for age and sex. This difference also retained significance after exclusion of participants who had experienced atherosclerotic cardiovascular disease (7 heterozygotes and 1 control; $P=0.030$). In a linear regression model with only PAF-AH activity as a potential confounder, the difference also retained significance ($P=0.049$). However, significance was lost in a linear regression model including age, sex, and PAF-AH activity ($P=0.125$). In carriers of 2 defective LCAT alleles, OxPL/apoB were similar to controls.

In heterozygotes, OxPL/apo(a) were similar to controls ($P=0.68$) but were markedly decreased in carriers of 2 defective LCAT alleles ($87\%$ reduction; $P<0.001$).

We also measured IgG and IgM immune complexes on apoB-containing lipoproteins and IgG and IgM titers against MDA-LDL (Table 5). Of these 4 parameters, 1 parameter was significantly different among the groups: IgM immune complexes on apoB-containing lipoproteins were significantly decreased in heterozygotes compared with controls ($P=0.03$).

### Antioxidant Capacity of HDL

The capacity of HDL to inhibit oxidized LDL from forming DCF (cell-free assay) was gene-dose-dependent decreased. The fluorescence intensity reflecting the potential of HDL to inhibit the action of OxLDL in this assay was $1.3\times10^5$ arbitrary units in carriers of 2 mutant alleles, $3.0\times10^4$ arbitrary units in heterozygotes, and $2.3\times10^4$ arbitrary units in controls (Table 6; $P<0.001$ for all comparisons).

### Table 3. Lysophosphatidylcholine Molecular Species

<table>
<thead>
<tr>
<th>Homozygous/Compound Patients (n=4)</th>
<th>Heterozygotes (61)*</th>
<th>Family Controls (n=60)*</th>
<th>$P$ HZ/cHT vs Controls (Unadjusted)</th>
<th>$P$ Heterozygotes vs Controls (Unadjusted)</th>
<th>$P$ for ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>1.3 (1.2)</td>
<td>5.0 (3.1)</td>
<td>5.5 (3.3)</td>
<td>$&lt;0.001$</td>
<td>0.89</td>
</tr>
<tr>
<td>16:0</td>
<td>186 (135)</td>
<td>388 (151)</td>
<td>440 (185)</td>
<td>$&lt;0.001$</td>
<td>0.29</td>
</tr>
<tr>
<td>17:0</td>
<td>4.1 (3.6)</td>
<td>7.3 (3.7)</td>
<td>7.9 (4.2)</td>
<td>$&lt;0.001$</td>
<td>0.95</td>
</tr>
<tr>
<td>18:0</td>
<td>31 (35)</td>
<td>99 (50)</td>
<td>107 (52)</td>
<td>$&lt;0.001$</td>
<td>0.38</td>
</tr>
<tr>
<td>18:1</td>
<td>34 (37)</td>
<td>64 (40)</td>
<td>64 (33)</td>
<td>0.01</td>
<td>0.52</td>
</tr>
<tr>
<td>18:2</td>
<td>51 (91)</td>
<td>78 (41)</td>
<td>73 (50)</td>
<td>0.69</td>
<td>0.59</td>
</tr>
<tr>
<td>20:0</td>
<td>0.13 (0.05)</td>
<td>0.26 (0.17)</td>
<td>0.29 (0.15)</td>
<td>$&lt;0.001$</td>
<td>0.13</td>
</tr>
<tr>
<td>20:4</td>
<td>0.99 (2.2)</td>
<td>9.7 (5.7)</td>
<td>8.5 (5.0)</td>
<td>0.33</td>
<td>0.10</td>
</tr>
<tr>
<td>22:6</td>
<td>0.99 (2.2)</td>
<td>1.7 (0.94)</td>
<td>1.8 (1.1)</td>
<td>0.058</td>
<td>0.96</td>
</tr>
</tbody>
</table>

Data are median plasma concentration in μmol/L, with interquartile ranges in parentheses. Data were log-transformed before statistical analysis because of a skewed distribution. $P$ HZ/cHT vs controls: $P$ for unpaired 2–tailed t test homozygotes/compound heterozygotes vs controls. N/A indicates not applicable; cHT, compound heterozygote; HZ, homozygote.

$P$ (unadjusted): $P$ for unpaired 2–tailed t test. $P$ for ANOVA to assess differences over the 3 groups.

*Because of lack of plasma, lysophosphatidylcholines could not be measured in 3 heterozygotes and 2 family controls.

### Table 4. Arachidonic and Linoleic Acids and Their Oxidized Derivatives

<table>
<thead>
<tr>
<th>Nonoxidized fatty acids</th>
<th>Arachidonic acid, nmol/L</th>
<th>Linoleic acid, nmol/L</th>
<th>P HZ/cHT vs Controls (Unadjusted)</th>
<th>P Heterozygotes vs Controls (Unadjusted)</th>
<th>P for ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>20:4</td>
<td>2.3×10^4±7.2×10^3</td>
<td>10.4×10^4±1.4×10^4</td>
<td>2.7×10^4±7.8×10^3</td>
<td>3.3×10^4±1.8×10^4</td>
<td>0.27</td>
</tr>
<tr>
<td>20:5</td>
<td>47.0±21</td>
<td>101.±10</td>
<td>88.1±39</td>
<td>0.041</td>
<td>0.863</td>
</tr>
<tr>
<td>20:6</td>
<td>20.9±7.9</td>
<td>26.2±11</td>
<td>21.3±7.3</td>
<td>45.4±25</td>
<td>0.054</td>
</tr>
<tr>
<td>20:4</td>
<td>22.1±11</td>
<td>49.7±22</td>
<td>41.5±17</td>
<td>59.1±32</td>
<td>0.047</td>
</tr>
<tr>
<td>20:6</td>
<td>19.3±7.3</td>
<td>41.5±17</td>
<td>50.1±26</td>
<td>0.020</td>
<td>0.033†</td>
</tr>
<tr>
<td>18:2</td>
<td>12.6±9.4</td>
<td>40.2±17</td>
<td>40.2±17</td>
<td>0.014</td>
<td>0.029†</td>
</tr>
<tr>
<td>18:2</td>
<td>50.1±26</td>
<td>74.8±34</td>
<td>81.7±35</td>
<td>0.025</td>
<td>0.28</td>
</tr>
<tr>
<td>18:2</td>
<td>83.4±26.5</td>
<td>111±80</td>
<td>113±62</td>
<td>0.36</td>
<td>0.91</td>
</tr>
<tr>
<td>18:2</td>
<td>133±35</td>
<td>203±106</td>
<td>213±85</td>
<td>0.07</td>
<td>0.57</td>
</tr>
</tbody>
</table>

HETE indicates hydroxyeicosatetraenoic acids; HODE, hydroxyoctadecadienoic acid; CVD, cardiovascular disease; N/A not applicable; cHT, compound heterozygote; HZ, homozygote.

$P$ HZ/cHT vs controls: $P$ for unpaired 2–tailed t test homozygotes/compound heterozygotes vs controls.


*Because of lack of plasma, arachidonic and linoleic acids and their oxidized derivatives could not be measured in 3 heterozygotes and 2 family controls.

†Retained significance after exclusion of 8 participants (7 heterozygotes, 1 control) who had experienced atherosclerotic CVD.
Table 5. (Auto-)Antibodies to Oxidized Phospholipids and Apolipoproteins

<table>
<thead>
<tr>
<th></th>
<th>Homozygous/ Compound Heterozygous Patients (n=4)</th>
<th>Heterozygotes (63)</th>
<th>Family Controls (n=63)</th>
<th>PHZ/chHT vs Controls (Unadjusted)</th>
<th>P Heterozygotes vs Controls (Unadjusted)</th>
<th>P Heterozygotes vs Controls (Adjusted)</th>
<th>P for ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidized phospholipids/ apoB (RLU)</td>
<td>2.2×10^4 (3.1×10^4)</td>
<td>2.8×10^4 (8.6×10^4)</td>
<td>2.4×10^4 (6.6×10^4)</td>
<td>0.27 &lt;0.01*</td>
<td>0.017*,†</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Oxidized phospholipids/ apo(a) (RLU)</td>
<td>726 (1.1×10^4)</td>
<td>4.4×10^4 (3.5×10^4)</td>
<td>5.8×10^3 (2.4×10^4)</td>
<td>&lt;0.001</td>
<td>0.68</td>
<td>N/A &lt;0.01</td>
<td></td>
</tr>
<tr>
<td>IgG immune complexes/ apoB (RLU)</td>
<td>1.9×10^4 (1.1×10^4)</td>
<td>1.8×10^4 (1.1×10^4)</td>
<td>1.9×10^4 (817)</td>
<td>0.74</td>
<td>0.89</td>
<td>N/A N/A</td>
<td></td>
</tr>
<tr>
<td>IgM Immune complexes/ apoB (RLU)</td>
<td>1.9×10^3 (1.3×10^3)</td>
<td>931 (840)</td>
<td>1.2×10^4 (833)</td>
<td>0.11</td>
<td>0.03</td>
<td>0.04 N/A</td>
<td></td>
</tr>
<tr>
<td>IgG MDA-LDL (RLU)</td>
<td>1.9×10^3 (1.6×10^4)</td>
<td>1.8×10^3 (1.2×10^3)</td>
<td>1.8×10^3 (1.6×10^3)</td>
<td>0.87</td>
<td>0.36</td>
<td>N/A 0.65</td>
<td></td>
</tr>
<tr>
<td>IgM MDA-LDL (RLU)</td>
<td>9.3×10^3 (7.0×10^4)</td>
<td>9.5×10^3 (6.4×10^4)</td>
<td>1.1×10^3 (6.4×10^4)</td>
<td>0.49</td>
<td>0.40</td>
<td>N/A 0.59</td>
<td></td>
</tr>
</tbody>
</table>

Data are medians (interquartile range). Data were log-transformed before statistical analysis because of a skewed distribution. Apo indicates apolipoprotein; RLU, relative light unit; MDA-LDL, malondialdehyde-low-density lipoprotein; N/A, not applicable; CVD, cardiovascular disease; PAF-AH, platelet-activating factor acetyl hydrolase; cHT, compound heterozygote; HZ, homozygote.

PHZ/chHT vs controls: P for unpaired 2-tailed t test homozygotes/compound heterozygotes vs controls.
P (unadjusted): P for unpaired 2-tailed t test.
P (adjusted): P for linear regression model including age and sex as potential confounders.

*Retained significance after exclusion of 8 participants (7 heterozygotes, 1 control) who had experienced atherosclerotic CVD.
†Retained significance in linear regression model with PAF-AH activity as only potential confounder; significance lost upon inclusion of PAF-AH activity in the model already including age and sex.
‡Independent of age, sex, activities of PON1 and PAF-AH and of HDL-c, BMI, and statin use.

ANOVA). Multivariate regression analysis shows that this difference retained significance after adjustments for age, sex, body mass index, HDL-c, and statin use (P<0.001). Additional statistical corrections for PON1 and PAF-AH activities in HDL did not change this result either (P<0.001).

After exclusion of participants who had experienced atherosclerotic cardiovascular disease, the antioxidative capacity of HDL also remained significantly lower in heterozygous carriers of LCAT gene mutations compared with controls.

Discussion

First proposed by Klimov et al,18 LCAT can hydrolyze oxidized fatty acids from the sn-2 position of OxPL.7,17 In particular long-chain oxidized fatty acids, whereas PAF-AH has been reported to hydrolyze short-chain oxidized fatty acids.16

LCAT overexpression decreased autoantibodies to OxLDL,40 but the role of LCAT in remodeling lipid pools by selective depletion of lipid oxidation products in plasma in humans is unknown. In this case–control study, we therefore investigated various processes and products related to lipid oxidation in patients with mutations in the LCAT gene. The main and surprising finding is that loss of LCAT activity is not associated with pronounced effect on plasma parameters of lipid peroxidation.

LCAT Activity and Molecular Species of LysoPC

Measuring LCAT activity using exogenous apoAI-containing lipoproteins as substrate, we show that heterozygotes have a diminished capacity to esterify free cholesterol, whereas this is severely reduced in carriers of 2 mutations. However, the activity of LCAT that is specifically responsible for

Table 6. Antioxidative Capacity of HDL

<table>
<thead>
<tr>
<th></th>
<th>Homozygous/ Compound Heterozygous Patients (n=4)</th>
<th>Heterozygotes (63)</th>
<th>Family Controls (n=63)</th>
<th>PHZ/chHT vs Controls (Unadjusted)</th>
<th>P Heterozygotes vs Controls (Unadjusted)</th>
<th>P Heterozygotes vs Controls (Adjusted)</th>
<th>P for ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antioxidative potential of HDL to LDL (arbitrary units)*</td>
<td>1.3×10^3 (7.5×10^4)</td>
<td>3.0×10^4 (1.1×10^4)</td>
<td>2.3×10^4 (5.5×10^4)</td>
<td>&lt;0.001</td>
<td>&lt;0.001†</td>
<td>&lt;0.001; †</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

HDL indicates high-density lipoprotein; CVD, cardiovascular disease; PON1, paraoxonase-1; PAF-AH, platelet-activating factor acetyl hydrolase; HDL-c, HDL cholesterol; BMI, body mass index; cHT, compound heterozygote; HZ, homozygote.

PHZ/chHT vs controls: P for unpaired 2-tailed t test homozygotes/compound heterozygotes vs controls.
P (unadjusted): P for unpaired 2-tailed t test.
P (adjusted): P for linear regression model including age, sex, activities of PON1 and PAF-AH and of HDL-c, BMI, and statin use as potential confounders.

*Median and interquartile range. Data were log-transformed before statistical analysis because of a skewed distribution.
†Retained significance after exclusion of 8 participants (7 heterozygotes, 1 control) who had experienced atherosclerotic CVD.
remodeling of oxidized fatty acids from OxPL species and thus its apparent antioxidant properties relates to the PLA₂ activity of LCAT. Vanloo et al⁴¹ have previously shown that natural LCAT mutations, including several of the mutations investigated in the present study, primarily reduce the PLA₂ activity of LCAT. To verify that this holds true for all mutations studied here, we measured most molecular species of lysoPC, as a proxy for PLA₂ activity of LCAT, and observed nonsignificantly reduced levels of all lysoPC species in heterozygotes compared with controls. On the other hand, the levels of lysoPC in plasma reflect not only rates of formation by LCAT PLA₂ activity but also rates of catabolism (eg, determined by the activities of LysoPC acyltransferase and lysophospholipases). In carriers of 2 defective LCAT alleles, however, all molecular species of lysoPC were significantly reduced (average reduction of 58%) compared with controls. This latter finding is in line with a study by Aoki et al in plasma of 1 FLD patient.⁴² Combined, these data indicate that the mutations studied have an impact on both cholesterol esterification and the generation of hydrolyzed OxPL species.

**Activities of PON1 and PAF-AH**

In vitro studies have demonstrated that PON1 can inhibit the biological activity of minimally OxLDL,¹⁸ and OxPL,³⁹ suggesting that PON1, like LCAT and PAF-AH, can also hydrolyze OxPL.⁴⁸,⁴⁹ Complete HDL deficiency in carriers of 2 defective LCAT alleles in the present study is associated with very low PON1 activity levels. A 40% reduction in HDL-c in heterozygotes for LCAT mutations, however, has no effect on HDL-associated PON1 activity, suggesting that HDL may become rate limiting for PON1 activity only at very low HDL-c levels. These findings also suggest that PON1 is unlikely to contribute to altered lipid oxidation in heterozygotes for LCAT mutations.

There is abundant evidence that HDL-associated PAF-AH has antiatherogenic and anti-inflammatory properties through its ability to abrogate the biological activity of OxLDL through hydrolysis of OxPL.¹⁶,⁴³ The clear gene-dose–dependent reduction in the HDL-associated PAF-AH activity in our carriers of LCAT mutations suggests that PAF-AH activity is more sensitive than PON1 activity to disturbances of HDL homeostasis. LCAT has been reported to hydrolyze PAF but at a 200x slower rate compared with PAF-AH.⁴⁴ It is unlikely that the loss of LCAT activity contributed to the decreased hydrolysis of PAF in the PAF-AH activity assay.

Apart from PAF-AH and LCAT, PON1 on HDL has also been reported to hydrolyze PAF.⁴⁵ However, in our patients heterozygous for an LCAT mutation, the contribution of PON1 to PAF-AH activity is probably of little significance, because PAF-AH activity was significantly decreased in these subjects compared with controls, whereas PON1 activity did not differ between these groups. (In the limited group of homozygotes/compound heterozygotes, both PAF-AH activity and PON1 activity were very strongly decreased.) Notwithstanding, the reduced PAF-AH activity in our patients may contribute to altered degradation of OxPL.

The reductions in activities of PAF-AH and PON1 in HDL of carriers of 2 defective LCAT alleles contrast with the observations in patients with genetic PAF-AH deficiency who have normal HDL-c levels.⁶⁶ In these patients, the loss of plasma PAF-AH activity is not accompanied by a decrease in activities of LCAT or PON1,⁴⁶ indicating that the disturbed HDL maturation resulting from LCAT deficiency in our patients underlies the observed decreased activity of PAF-AH as well as of PON1 in HDL.

**AA and LA and Their Oxidized Derivatives**

To study whether the lower HDL-c resulting from a loss of LCAT activity combined with a loss of HDL-associated PAF-AH activity would be associated with altered lipid oxidation products, we measured plasma levels of multiple specific fatty acid oxidation products.¹¹ Compared with controls, none of the oxidized derivatives of AA or LA was increased in carriers of 1 or 2 defective alleles. On the contrary, we found 4 of the oxidized derivatives of AA to be reduced in a gene-dose–dependent manner.

The lower oxidized fatty acids found in our carriers are most probably secondary to the decrease in plasma levels of total AA and LA, from which they are derived. In turn, the lower total plasma levels of AA and LA are probably the result of a reduction in CE caused by the loss of LCAT activity.⁷⁷,⁴⁸ The total plasma levels of AA and LA represent the sum of these fatty acids, present as free fatty acids but also as acyl groups esterified to cholesterol, triglycerides, and phospholipids. In a recent systematic review, Hodson et al⁴⁹ estimated that 19% of total fatty acid in plasma is esterified to cholesterol. Based on a meta-analysis, they furthermore reported that LA on average accounts for 52% of all fatty acids esterified to cholesterol.⁴⁹ Thus, the reduction in CE can very well explain our observations of modestly lower total LA in our patients. The 30% reduction in AA in both carriers of 1 and 2 defective alleles is more difficult to explain, and Hodson et al⁴⁹ also report that AA accounts for only 5% of all fatty acids esterified to cholesterol. After normalization for total levels of LA or AA (Table II in the online-only Data Supplement), none of the oxidized fatty acids was elevated in LCAT mutation carriers. In conclusion, this analysis does not support the hypothesis of increased lipid oxidation products of AA and LA in these patients.

**(Auto-)Antibodies Against Oxidized Phospholipids and Apolipoproteins**

In the general population, increased levels of OxPL/apoB measured with the E06 antibody were recently found to be associated with increased risk of future coronary artery disease.⁶⁰ In our study, OxPL/apoB (measured with the same antibody) was higher in the heterozygotes for mutations in LCAT, also after statistical correction for age and sex. Thus, PLA₂ activity of LCAT may be relevant for the hydrolysis of OxPL in humans, but the reductions in HDL-associated PAF-AH and 40% decrease of HDL-c could also play a role here, especially because measures of PLA₂ activity were only mildly (LCAT activity) or nonsignificantly (molecular species of lysoPC) reduced in heterozygotes. Linear regression analysis with only PAF-AH activity as potential confounder
showed that the difference on OxPL/apoB between heterozygotes and controls was still statistically significant, but this significance was lost upon inclusion of PAF-AH activity in the previous model already including age and sex. In our 4 patients with 2 defective LCAT alleles, we did not find a further increase in OxPL/apoB. However, Itabe et al.\textsuperscript{31,32} previously showed that 5 patients with FLD had increased plasma levels of LDL-associated OxPL. We have no explanation for this discrepancy other than the low number of patients in both studies.

Interestingly, OxPL on Lp(a) was not increased in carriers of 1 LCAT mutation, whereas in carriers of 2 defective LCAT alleles, this was in fact significantly reduced. The latter observation is likely associated with significant reductions of Lp(a) levels as has been previously reported in patients with LCAT deficiency\textsuperscript{33}; for apo(a) to bind to apoB-containing particles and thus form Lp(a), these particles need to contain sufficient amounts of CE.\textsuperscript{34} In heterozygotes for LCAT mutations, Lp(a) levels were comparable with those in family controls, implying that a modest reduction in LCAT activity does not affect the CE content of apoB-containing lipoproteins to the extent that apo(a) can no longer normally bind to these lipoproteins.

We also measured antibodies against MDA-LDL as a model of oxidation-specific epitopes that occur on OxLDL and other oxidative processes.\textsuperscript{35} Both IgM and IgG autoantibodies against MDA-LDL were not significantly different in subjects with LCAT gene mutations compared with controls, possibly consistent with the data that overall there is no gross disturbance of lipid oxidation. These data need to be interpreted with care however, because we observed large interindividual variations in these parameters.

IgG and IgM autoantibodies can bind to circulating LDL-bearing oxidation epitopes.\textsuperscript{35} In carriers of LCAT mutations, we also observed no differences in IgG-LDL immune complexes, whereas there were significantly decreased IgM-LDL immune complexes in the heterozygotes. In general, there are considerable data that IgM autoantibodies to oxidation-specific epitopes are inversely associated with cardiovascular disease, possibly by binding to oxidized lipids and blocking adverse effects and inhibiting OxLDL uptake by macrophages.\textsuperscript{36} IgM-LDL immune complexes were, however, unaffected in carriers of 2 defective alleles.

### Antioxidant Capacity of HDL

On the basis of the original publication of Navab et al.,\textsuperscript{22} we have set up an assay that assesses the ability of HDL to inactivate OxLDL. We have previously shown that this assay provides similar results when standardized for HDL-c, HDL phospholipids, or apoA-I.\textsuperscript{37} In accordance with most studies,\textsuperscript{22,58–61} here we have standardized for HDL-c. Application of this assay revealed that HDL of patients with LCAT mutations has a strongly decreased ability to inhibit exogenously isolated OxLDL from releasing the fluorescent DCF. In this assay, we normalized for HDL-c. The observed decrease might be directly caused by a loss of LCAT on HDL. Also, these effects may be related to gene-dose–dependent loss of HDL-associated PAF-AH in our study. However, statistical adjustments for PAF-AH but also differences in PON1 and other possible confounders, as well as exclusion of subjects treated with statins did not affect the strong association between reduced LCAT activity and reduced antioxidant capacity of HDL. It has been shown that HDL of LCAT mutation carriers consists of altered HDL subpopulations with altered composition of apolipoproteins compared with controls.\textsuperscript{62} Thus, one can speculate that changes in composition of HDL may affect the antioxidant properties of HDL.

### Mutations in LCAT Associated With FLD or FED

Functional mutations on both LCAT alleles cause clinical phenotypes of which FED and FLD can be regarded as the extremes.\textsuperscript{19} Studying possible heterogeneity between carriers of mutations associated with either phenotype, we did, however, not find clear differences in lipid oxidation parameters between our 2 patients with FED and 2 patients with FLD. The same holds true for heterozygotes for mutations associated with FED (n=39) or FLD (n=20; Tables III and IV in the online-only Data Supplement). Taken together, it seems that loss of LCAT function on either apoAI- or apoB-containing particles is not associated with differences in lipid oxidation parameters.

### Conclusions

This study shows that carriers of LCAT gene mutations display a mild increase of OxPL on apoB-containing lipoproteins but no other indications of enhanced lipid oxidation. These results are in line with the finding that these patients have only mildly increased atherosclerosis.\textsuperscript{63} On the other hand, these findings are surprising when considering that LCAT is expected to play an important role in the selective depletion of lipid oxidation products\textsuperscript{7} but also with the observed marked reductions of HDL-c and apoA-I, which by themselves have been implicated to confer antioxidative properties.\textsuperscript{64} In addition, a significant and dose-dependent decrease of HDL-associated PAF-AH in carriers of LCAT gene mutations would also be expected to translate in a more severe phenotype. The apparent mild oxidative status could be related to unchanged HDL-associated PON1 activity levels in carriers of 1 defective LCAT allele. However, a severe reduction of PON1 in carriers of 2 defective LCAT alleles did not result in markers of enhanced lipid oxidation in these 4 patients. On the other hand, our patients did show a strong decrease in the potential of their HDL to inhibit exogenously isolated OxLDL from releasing fluorescent DCF, but it is difficult to predict what the in vivo implications are.

It is possible that in FLD and FED, OxPL are rapidly cleared via the kidney where poorly lipiddated and pre-β HDL are catabolized.\textsuperscript{65} On the other hand, apoAI fractional synthesis rates are normal in FLD and FED patients,\textsuperscript{67} and combined with a reduced LCAT and PAF-AH activity as shown in the present study, it is not likely that this will be a major pathway for catabolism of OxPL.

Our findings combined suggest that alternative mechanisms, such as clearance of OxLDL by CD36 and other
scavenger receptors, could be important in protection from lipid oxidation.

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Disclosures
None.

References
50. Holleboom et al. Antioxidation by HDL and Lipid Oxidation in LCAT Deficiency 3075


Lipid Oxidation in Carriers of Lecithin:Cholesterol Acyltransferase Gene Mutations
Adriaan G. Holleboom, Georgios Daniil, Xiaoming Fu, Renliang Zhang, G. Kees Hovingh, Alinda W. Schimmel, John J.P. Kastelein, Erik S.G. Stroes, Joseph L. Witztum, Barbara A. Hutten, Sotirios Tsimikas, Stanley L. Hazen, Angeliki Chroni and Jan Albert Kuivenhoven

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Lipid Oxidation in Carriers of LCAT Gene Mutations
Holleboom et al.

DATA SUPPLEMENT

Supplementary Table I. Molecular LCAT defects in heterozygotes

<table>
<thead>
<tr>
<th>Heterozygotes for FLD mutations in LCAT (n=20)</th>
<th>Amino acid substitution</th>
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<td>3</td>
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</tr>
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<td>11</td>
<td>p.R182C&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>p.R268H&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>p.C337Y&lt;sup&gt;4&lt;/sup&gt;</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Heterozygotes FED mutations in LCAT (n=39)</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>6</td>
<td>p.P34Q&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>22</td>
<td>p.T147I&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>N131D&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>V333M&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Heterozygotes for unspecified LCAT mutations (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>5-HETE/AA (mmol/mol)</td>
</tr>
<tr>
<td>8-HETE/AA (mmol/mol)</td>
</tr>
<tr>
<td>9-HETE/AA (mmol/mol)</td>
</tr>
<tr>
<td>11-HETE/AA (mmol/mol)</td>
</tr>
<tr>
<td>12-HETE/AA (mmol/mol)</td>
</tr>
<tr>
<td>15-HETE/AA (mmol/mol)</td>
</tr>
<tr>
<td>9-HODE/LA (mmol/mol)</td>
</tr>
<tr>
<td>13-HODE/LA (mmol/mol)</td>
</tr>
</tbody>
</table>

P (unadj.): P for unpaired two–tailed T test, P (adj.)
$^\dagger$ Due to lack of plasma, arachidonic and linoleic acids and their oxidized derivatives could not be measured in three heterozygotes and two family controls
### Supplementary Table III. Lipid oxidation in homozygous/compound heterozygous FED and FLD patients

<table>
<thead>
<tr>
<th>Pt.</th>
<th>LCAT def. sy.</th>
<th>LCAT mutations</th>
<th>Age (y)</th>
<th>Gender (m/f)</th>
<th>HDL-c (mg/dl)</th>
<th>LDL-c (mg/dl)</th>
<th>LCAT activity (nmol/ml/h)</th>
<th>PAF-AH activity (nmol/min/ml)</th>
<th>PON1 activity (U/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt. 1</td>
<td>FED</td>
<td>T147I&lt;sup&gt;6&lt;/sup&gt;</td>
<td>58</td>
<td>m</td>
<td>0.12</td>
<td>118</td>
<td>0.57</td>
<td>1.3</td>
<td>53.4</td>
</tr>
<tr>
<td>Pt. 2</td>
<td>FED</td>
<td>T147I/V333M&lt;sup&gt;6, 8&lt;/sup&gt;</td>
<td>28</td>
<td>f</td>
<td>0.13</td>
<td>83</td>
<td>0.42</td>
<td>1.6</td>
<td>8.1</td>
</tr>
<tr>
<td>Pt. 3</td>
<td>FLD</td>
<td>C337Y&lt;sup&gt;4&lt;/sup&gt;</td>
<td>13</td>
<td>f</td>
<td>0.26</td>
<td>32</td>
<td>0.46</td>
<td>0.73</td>
<td>3.6</td>
</tr>
<tr>
<td>Pt. 4</td>
<td>FLD</td>
<td>T345M&lt;sup&gt;1&lt;/sup&gt;</td>
<td>37</td>
<td>m</td>
<td>0.15</td>
<td>114</td>
<td>0.33</td>
<td>3.0</td>
<td>6.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pt.</th>
<th>LCAT def. sy.</th>
<th>Non-oxidized fatty acids</th>
<th>Oxidized fatty acids</th>
<th>Anti-oxidative potential of HDL to LDL (arbitrary units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AA&lt;sup&gt;*&lt;/sup&gt; (nmol/l)</td>
<td>LA&lt;sup&gt;*&lt;/sup&gt; (nmol/l)</td>
<td>5-HETE (nmol/l)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(nmol/l)</td>
<td>(nmol/l)</td>
<td>(nmol/l)</td>
</tr>
<tr>
<td>Pt. 1</td>
<td>FED</td>
<td>2.1*10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>9.9*10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>54</td>
</tr>
<tr>
<td>Pt. 2</td>
<td>FED</td>
<td>3.3*10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>11.1*10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>72</td>
</tr>
<tr>
<td>Pt. 3</td>
<td>FLD</td>
<td>1.6*10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>12.1*10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>22</td>
</tr>
<tr>
<td>Pt. 4</td>
<td>FLD</td>
<td>2.2*10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>8.6*10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>41</td>
</tr>
</tbody>
</table>

<sup>*</sup> AA: arachadonic acid, LA: linoleic acid

### LysoPC molecular species

<table>
<thead>
<tr>
<th>LCAT def. sy.</th>
<th>14:0</th>
<th>16:0</th>
<th>17:0</th>
<th>18:0</th>
<th>18:1</th>
<th>18:2</th>
<th>20:0</th>
<th>20:4</th>
<th>22:6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt. 1</td>
<td>FED</td>
<td>0.99</td>
<td>229</td>
<td>4.82</td>
<td>59</td>
<td>39</td>
<td>42</td>
<td>0.15</td>
<td>5.87</td>
</tr>
<tr>
<td>Pt. 2</td>
<td>FED</td>
<td>1.48</td>
<td>161</td>
<td>3.32</td>
<td>25</td>
<td>28</td>
<td>44</td>
<td>0.1</td>
<td>5.27</td>
</tr>
<tr>
<td>Pt. 3</td>
<td>FLD</td>
<td>1.11</td>
<td>66</td>
<td>1.18</td>
<td>17</td>
<td>27</td>
<td>58</td>
<td>0.15</td>
<td>3.71</td>
</tr>
<tr>
<td>Pt. 4</td>
<td>FLD</td>
<td>2.5</td>
<td>210</td>
<td>5.52</td>
<td>37</td>
<td>72</td>
<td>158</td>
<td>0.1</td>
<td>19.8</td>
</tr>
</tbody>
</table>

Data are median plasma concentrations in µM.
Supplementary table IV. Lipid oxidation in heterozygotes for FLD and FED mutations in *LCAT*

<table>
<thead>
<tr>
<th></th>
<th>Heterozygotes for FLD mutation (n=20)</th>
<th>Heterozygotes for FED mutation (n=39)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender (y)</td>
<td>48.7 ± 13</td>
<td>44.8 ± 13</td>
<td>0.41</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>33.5 ± 11</td>
<td>33.2 ± 13</td>
<td>0.95</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>130 ± 34</td>
<td>128 ± 34</td>
<td>0.86</td>
</tr>
</tbody>
</table>

**Enzyme activities**

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>LCAT activity (nmol/mL/h)</td>
<td>9.7 ± 2</td>
<td>9.4 ± 1</td>
<td>0.61</td>
</tr>
<tr>
<td>PAF-AH activity (nmol/min/ml)</td>
<td>4.0 (3.7)</td>
<td>3.1 (1.8)</td>
<td>0.16</td>
</tr>
<tr>
<td>PON1 activity (U/l)</td>
<td>35 (77)</td>
<td>49 (79)</td>
<td>0.17</td>
</tr>
</tbody>
</table>

**LysoPC molecular species**

<table>
<thead>
<tr>
<th>LysoPC molecular species</th>
<th>Heterozygotes for FLD mutation (nmol/mL/h)</th>
<th>Heterozygotes for FED mutation (nmol/mL/h)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>5.7 (4.6)</td>
<td>4.7 (2.9)</td>
<td>0.07</td>
</tr>
<tr>
<td>16:0</td>
<td>284 (330)</td>
<td>388 (155)</td>
<td>0.64</td>
</tr>
<tr>
<td>17:0</td>
<td>7.3 (4.7)</td>
<td>7.1 (3.0)</td>
<td>0.45</td>
</tr>
<tr>
<td>18:0</td>
<td>94 (81)</td>
<td>98 (50)</td>
<td>0.89</td>
</tr>
<tr>
<td>18:1</td>
<td>54 (39)</td>
<td>65 (38)</td>
<td>0.34</td>
</tr>
<tr>
<td>18:2</td>
<td>81 (45)</td>
<td>64 (53)</td>
<td>0.81</td>
</tr>
<tr>
<td>20:0</td>
<td>0.25 (0.17)</td>
<td>0.26 (0.17)</td>
<td>0.97</td>
</tr>
<tr>
<td>20:4</td>
<td>6.3 (9.5)</td>
<td>9.7 (5.3)</td>
<td>0.10</td>
</tr>
<tr>
<td>22:6</td>
<td>1.6 (1.4)</td>
<td>1.7 (0.91)</td>
<td>0.82</td>
</tr>
<tr>
<td>Non-oxidized fatty acids</td>
<td>Heterozygotes for FLD mutation (n=20)</td>
<td>Heterozygotes for FED mutation (n=39)</td>
<td>P</td>
</tr>
<tr>
<td>--------------------------</td>
<td>--------------------------------------</td>
<td>--------------------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>Arachidonic acid (nmol/l)</td>
<td>$2.4\times10^4 \pm 8.3\times10^3$</td>
<td>$2.8\times10^4 \pm 5.6\times10^3$</td>
<td>0.16</td>
</tr>
<tr>
<td>Linoleic acid (nmol/l)</td>
<td>$10.2\times10^4 \pm 2.0\times10^3$</td>
<td>$10.1\times10^4 \pm 1.9\times10^4$</td>
<td>0.96</td>
</tr>
</tbody>
</table>

**Oxidized fatty acids**

<table>
<thead>
<tr>
<th>5-HETE (nmol/l)</th>
<th>65 ± 37</th>
<th>88 ± 42</th>
<th>0.074</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-HETE (nmol/l)</td>
<td>34 ± 20</td>
<td>35 ± 14</td>
<td>0.85</td>
</tr>
<tr>
<td>9-HETE (nmol/l)</td>
<td>41 ± 29</td>
<td>49 ± 21</td>
<td>0.28</td>
</tr>
<tr>
<td>11-HETE (nmol/l)</td>
<td>35 ± 23</td>
<td>41 ± 16</td>
<td>0.31</td>
</tr>
<tr>
<td>12-HETE (nmol/l)</td>
<td>34 ± 20</td>
<td>39 ± 16</td>
<td>0.35</td>
</tr>
<tr>
<td>15-HETE (nmol/l)</td>
<td>62 ± 39</td>
<td>74 ± 33</td>
<td>0.28</td>
</tr>
<tr>
<td>9-HODE (nmol/l)</td>
<td>91 ± 42</td>
<td>110 ± 86</td>
<td>0.45</td>
</tr>
<tr>
<td>13-HODE (nmol/l)</td>
<td>186 ± 78</td>
<td>200 ± 110</td>
<td>0.68</td>
</tr>
</tbody>
</table>

**(Auto-) antibodies to oxidized phospholipids and apolipoproteins**

<table>
<thead>
<tr>
<th>Oxidized phospholipids/apoB (RLU)</th>
<th>2.4<em>10^4 (5.1</em>10^3)</th>
<th>2.8<em>10^4 (9.5</em>10^3)</th>
<th>0.009</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidized phospholipids/apo(a) (RLU)</td>
<td>3.0<em>10^3 (2.2</em>10^4)</td>
<td>4.0<em>10^3 (2.1</em>10^5)</td>
<td>0.93</td>
</tr>
<tr>
<td>IgG immune complexes/apoB (RLU)</td>
<td>1.9<em>10^3 (1.6</em>10^3)</td>
<td>1.8<em>10^3 (1.5</em>10^3)</td>
<td>0.53</td>
</tr>
<tr>
<td>IgM immune complexes/apoB (RLU)</td>
<td>902 (1.1*10^3)</td>
<td>954 (1.0*10^3)</td>
<td>0.88</td>
</tr>
<tr>
<td>IgG MDA-LDL (RLU)</td>
<td>2.3<em>10^3 (2.8</em>10^3)</td>
<td>1.7<em>10^3 (1.1</em>10^3)</td>
<td>0.35</td>
</tr>
<tr>
<td>IgM MDA-LDL (RLU)</td>
<td>9.7<em>10^3 (3.6</em>10^3)</td>
<td>9.3<em>10^3 (6.7</em>10^3)</td>
<td>0.65</td>
</tr>
</tbody>
</table>

**Anti-oxidative potential of HDL to LDL (arbitrary units)**

| 2.9*10^5 (1.4*10^6) | 3.0*10^4 (1.6*10^5) | 0.25  |

*median (interquartile range). Data were log-transformed before statistical analysis because of a skewed distribution. † P for X^2 test. P for unpaired two–tailed T test. FLD mutation: mutation known to cause familial LCAT deficiency (FLD) in patients homozygous for the mutation. FED mutation: mutation known to cause fish eye disease (FED) in patients homozygous for the mutation.


