Lipoprotein Subfractions Highly Associated With Renal Damage in Familial Lecithin:Cholesterol Acyltransferase Deficiency

Masayuki Kuroda, Adriaan G. Holleboom, Erik S.G. Stroes, Sakiyo Asada, Yasuyuki Aoyagi, Kouju Kamata, Shizuya Yamashita, Shun Ishibashi, Yasushi Saito, Hideaki Bujo

Objective—In familial lecithin:cholesterol acyltransferase (LCAT) deficiency (FLD), deposition of abnormal lipoproteins in the renal stroma ultimately leads to renal failure. However, fish-eye disease (FED) does not lead to renal damage although the causative mutations for both FLD and FED lie within the same LCAT gene. This study was performed to identify the lipoproteins important for the development of renal failure in genetically diagnosed FLD in comparison with FED, using high-performance liquid chromatography with a gel filtration column.

Approach and Results—Lipoprotein profiles of 9 patients with LCAT deficiency were examined. Four lipoprotein fractions specific to both FLD and FED were identified: (1) large lipoproteins (>80 nm), (2) lipoproteins corresponding to low-density lipoprotein (LDL), (3) lipoproteins corresponding to small LDL to large high-density lipoprotein, and (4) to small high-density lipoprotein. Contents of cholesteryl ester and triglyceride of the large LDL in FLD (below detection limit and 45.8±3.8%) and FED (20.7±4.4% and 28.0±6.5%) were significantly different, respectively. On in vitro incubation with recombinant LCAT, content of cholesteryl ester in the large LDL in FLD, but not in FED, was significantly increased (to 4.2±1.4%), whereas dysfunctional high-density lipoprotein was diminished in both FLD and FED.

Conclusions—Our novel analytic approach using high-performance liquid chromatography with a gel filtration column identified large LDL and high-density lipoprotein with a composition specific to FLD, but not to FED. The abnormal lipoproteins were sensitive to treatment with recombinant LCAT and thus may play a causal role in the renal pathology of FLD. (Arterioscler Thromb Vasc Biol. 2014;34:1756-1762.)

Key Words: chromatography, gel □ LDL □ lecithin acyltransferase deficiency □ renal insufficiency

LECithin:cholesterol acyltransferase (LCAT)–deficiency syndromes are rare autosomal recessive diseases, characterized by hypo-α-lipoproteinemia and corneal opacity.1,2 They are caused by mutations in the LCAT gene, of which 88 have been reported to date.3 Severe mutations lead to familial LCAT deficiency (FLD), mild mutations lead to fish-eye disease (FED). In FLD, the mutant LCAT enzyme is either absent in plasma (not secreted from the hepatocyte or rapidly degraded on secretion) or exhibits no catalytic activity on any lipoprotein; in FED, LCAT cannot esterify cholesterol on high-density lipoprotein (HDL; loss of α-activity) but retains its activity on lipoproteins containing apolipoprotein B (β-activity).1,2 Likely, the molecular difference is causal to the major clinical difference between FLD and FED: patients with FLD develop renal failure, whereas patients with FED do not.4

To prevent renal failure in patients with FLD, replacement therapy with recombinant enzyme is currently being developed.5-8 Alternatively, we are developing a long-lasting gene therapy by transplantation of human LCAT gene-transduced autologous adipocytes.7,9 Recombinant LCAT (rLCAT) secreted by the LCAT gene-transduced adipocytes corrected abnormal HDL subpopulations in sera of FED patients in vitro.10 LCAT catalyzes the esterification of cholesterol with acyl groups hydrolyzed from phospholipids, predominantly on HDL particles. This leads to mature lipoproteins with cores filled with cholesterol ester. LCAT dysfunction leads to decreased maturation of the HDL particle and to increased levels of both its substrates: unesterified cholesterol and phosphatidylcholine. In the absence of LCAT activity, abnormal lipid particles have been observed throughout lipoprotein fractions.11-14 The HDL fraction contains disk-shaped particles in rouleaux and small spherical particles. Density-gradient ultracentrifugation followed by electron microscopy...
revealed that the low-density lipoprotein (LDL) fraction contains 3 abnormal particles with different sizes, lipid composition, and associated apolipoproteins,\textsuperscript{11,12} which were proposed to be important in the pathogenesis of renal manifestation in patients with FLD.\textsuperscript{15-18} Of these, lipoprotein-X (LpX)\textsuperscript{19,20} have been postulated to accumulate in glomeruli, associated apolipoproteins,\textsuperscript{11,12} which were potentially causing the renal damage observed in patients with FLD.\textsuperscript{16-18} In 1 patient with FLD, lipid-lowering therapy led to a reduction of LpX and a concomitant reduction in proteinuria.\textsuperscript{21} LpX is phospholipid (PL)-rich and free cholesterol (FC)–rich but triglyceride (TG)-poor particle without apolipoproteins, ranging in size between very low density lipoprotein and large LDL.\textsuperscript{22}

To characterize the abnormal lipoproteins associated with the renal pathology of FLD, we characterized lipoprotein fractions by analyzing patients with different mutations and manifestations in comparison with another LCAT-deficiency syndrome, FED. We applied high-performance liquid chromatography with a gel filtration column (HPLC-GFC) for the first time to characterize the above abnormal lipoproteins and in fact identified lipoprotein subfractions specific to FLD. The lipid contents and particle size were biochemically determined, and the responsiveness of the lipoproteins against incubation with rLCAT was investigated in vitro.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

## Results

### Lipoprotein Subfractions Specific to LCAT-Deficiency Syndromes

Five patients with FLD (1–5) and 4 patients with FED (6–9) were compared with 4 nonaffected normolipidemic controls. Clinical and molecular characteristics and lipid profiles of the patients are given in Tables 1 and 2, respectively. Ultracentrifugation fractionation followed by determination of lipid contents was performed in patients 1, 2, and 5 (Table I in the online-only Data Supplement). LCAT α-activities in the patients’ sera were all <2% of reference. As expected in LCAT deficiency, mature HDL particles found at fraction (Fr.) 16 and 17 of unaffected controls were absent in the 9 patients (Figure 1). Although the lipid profiles of patients were heterogeneous, HPLC-GFC showed 4 lipoprotein fractions in sera of patients with FLD and FED that were not present in sera of unaffected controls: large lipoproteins (>80 nm) in Fr. 1 (Lp1), lipoproteins corresponding to large LDL in Fr. 8 (or Fr. 7–10; Lp8), lipoproteins corresponding to very small LDL and large HDL in Fr. 12 to 16 (Lp12–16), and lipoproteins corresponding to small HDL in Fr. 18 to 20 (Lp18–20). The levels of cholesterol, TG, and PL in these specific fractions varied among the 9 patients (Figure 1). Serum apolipoprotein analyses of Fr. 7 to 10, Fr. 13 to 15, and Fr. 18 to 20 in 3 patients (1, 2, and 5) showed that Fr. 13 to 15 and Fr. 18 to 20 were rich in apolipoprotein A as normolipidemic control although varied among patients (Figure I in the online-only Data Supplement). Apolipoprotein Cs were also rich in Fr. 18 to 20 but not in Fr. 13 to 15. Apolipoprotein B was mostly distributed in Fr. 8 to 10 among the 3 fraction categories. Apolipoprotein E was abundant in all 3 fraction categories when compared with that in the control.

### Abnormal Lipoproteins Are Present in FLD Regardless of Degree of Proteinuria

To study the relationship between lipoproteins and the degree of proteinuria in patients with FLD, lipoproteins between 2 sibling patients with FLD homozygous for the C337Y mutation in LCAT were compared (Figure 1, patients 1 and 3). Patient 1 had proteinuria in the nephrotic range (6 g/24 h), whereas patient 3 had only mild proteinuria (0.45 g/L).\textsuperscript{23} All 4 abnormal lipoproteins were present in both patients (Figure 2A), although 3 lipoproteins (Lp1, Lp8, and Lp18–20) were lower in the younger patient.

### Table 1. Clinical and Molecular Characteristics of Patients With Lecithin:Cholesterol Acyltransferase Deficiency

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age, Y</th>
<th>Race</th>
<th>Renal Failure/Proteinuria</th>
<th>Corneal Opacity</th>
<th>Anemia</th>
<th>CAD</th>
<th>Phenotype</th>
<th>AA Substitution</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>17</td>
<td>White (Morocco)</td>
<td>6 g/24 h</td>
<td>+</td>
<td>11.4 g/dL</td>
<td>–</td>
<td>FLD</td>
<td>C337Y</td>
<td>23</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>61</td>
<td>Japanese</td>
<td>2 g/24 h</td>
<td>+</td>
<td>9.5 g/dL</td>
<td>–</td>
<td>FLD</td>
<td>C98Y</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>12</td>
<td>White (Morocco)</td>
<td>0.45 g/L</td>
<td>+</td>
<td>9.2 g/dL</td>
<td>–</td>
<td>FLD</td>
<td>C337Y</td>
<td>23</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>63</td>
<td>Japanese</td>
<td>0.23 g/24 h</td>
<td>+</td>
<td>10.3 g/dL</td>
<td>–</td>
<td>FLD</td>
<td>G203R</td>
<td>25</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>68</td>
<td>Japanese</td>
<td>0.5 g/L</td>
<td>+</td>
<td>6.6 g/dL</td>
<td>–</td>
<td>FLD</td>
<td>G54S</td>
<td>26</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>38</td>
<td>Japanese</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>FED</td>
<td>T147I</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>58</td>
<td>White (Dutch)</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>FED</td>
<td>T147I</td>
<td>None</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>36</td>
<td>White (Dutch)</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>FED</td>
<td>W99S/T147I</td>
<td>27</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>30</td>
<td>White (Dutch)</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>FED</td>
<td>T147I/V333M</td>
<td>28</td>
</tr>
</tbody>
</table>

Patients 8 and 9 are compound heterozygotes; others are homozygotes for the indicated mutations. AA indicates amino acid; CAD, coronary artery disease; F, female; FED, fish-eye disease; FLD, familial lecithin:cholesterol acyltransferase deficiency; and M, male.
Next, lipoprotein profiles of a patient with FLD with homozygous for the C98Y mutation before and after a fat-restricted diet, which led to a reduction of proteinuria from 2.0 g/gCr to 0.6 g/gCr, were compared (Figure 1, patient 2). All 4 lipoproteins remained present after the diet although Lp1 and Lp8 were decreased to some extent (Figure 2B).

Lp8 and Lp12 to 16 Are Specific to FLD and Not to FED
Next, composition of the 4 Lps was analyzed (Figure II in the online-only Data Supplement). In all lipoproteins, cholesteryl ester (CE) was absent in FLD and low in Lp1, Lp12 to 16, and Lp18 to 20 in FED (panel A). PL in Lp8 was significantly lower in FLD when compared with that in FED (panel D). PL and FC were increased in Lp12 to 16 in FLD when compared with that in FED (panels B and D). FC, TG, and PL in both Lp1 and Lp18 to 20 did not differ between FLD and FED.

Lp8 Is a Large LDL, Rich in FC, PL, and TG, and Different From LpX
In comparison with unaffected controls and to patients with FED, CE in the LDL fractions of FLD sera was significantly decreased, whereas TG was increased (Figure 3A). In patients with both FLD and FED, FC, TG, and PL in Fr. 8 were significantly higher than in Fr. 9, whereas in controls, FC, TG, and PL in Fr. 8 were significantly lower than in Fr. 9 (Figure 3B). As a result, average sizes of Lp8 (Fr. 7–10) in FLD were significantly increased when compared with normal, whereas averaged particle size in FLD was lower than those in FED because of the severe deficiency of CE (Figure 3C). The composition of Lp8 in our patients with FLD is consistent with the previously reported FLD-LDL, and not consistent with the lipid characteristics of LpX.

Abnormal Lipid Compositions of FLD-Specific Lps Are Ameliorated by In Vitro Incubation With rLCAT
In vitro rLCAT incubation was performed followed by HPLC-GFC analyses (Figure III in the online-only Data Supplement). Incubation of patients’ sera with rLCAT increased CE, TG, and PL in Fr. 16 to 18 in both FLD and FED (Figure IV in

<table>
<thead>
<tr>
<th>Patients</th>
<th>TC</th>
<th>TG</th>
<th>HDL-C</th>
<th>LDL-C</th>
<th>CE/TC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>109</td>
<td>179</td>
<td>5.8</td>
<td>67</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>123</td>
<td>307</td>
<td>9.3</td>
<td>52</td>
<td>0.13</td>
</tr>
<tr>
<td>3</td>
<td>47</td>
<td>56</td>
<td>10.1</td>
<td>26</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>47</td>
<td>89</td>
<td>6.3</td>
<td>23</td>
<td>0.13</td>
</tr>
<tr>
<td>5</td>
<td>56</td>
<td>59</td>
<td>2.0</td>
<td>42</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>85</td>
<td>120</td>
<td>4.0</td>
<td>57</td>
<td>0.57</td>
</tr>
<tr>
<td>7</td>
<td>133</td>
<td>120</td>
<td>4.7</td>
<td>104</td>
<td>0.54</td>
</tr>
<tr>
<td>8</td>
<td>144</td>
<td>205</td>
<td>3.9</td>
<td>99</td>
<td>0.57</td>
</tr>
<tr>
<td>9</td>
<td>98</td>
<td>118</td>
<td>4.9</td>
<td>70</td>
<td>0.39</td>
</tr>
</tbody>
</table>

Values for LDL-C were calculated according to Friedewald et al. CE/TC indicates cholesteryl ester/total cholesterol ratio; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; TC, total cholesterol; and TG, triglyceride.
indicating LCAT-mediated maturation of HDL. CE and PL contents of Lp8 were significantly increased and decreased, respectively, in FLD after incubation with rLCAT, whereas TG content was not significantly altered (Figure 4A and 4B). In FED, composition of Lp8 was not significantly altered by the treatment (Figure 4A and 4B). On incubation with rLCAT, Lp8 increased in size in FLD and it decreased in size in FED (Figure 4C). However, FC and PL in Lp12 to 16 decreased on incubation (Figure 4D).

**Discussion**

In this study, 4 lipoprotein fractions specific to LCAT-deficiency syndromes were identified by the HPLC-GFC analysis of samples from genetically diagnosed patients with different mutations and manifestations. Two of these had lipid compositions that were specific to FLD and thus may be involved in causing the renal damage that characterizes FLD. In vitro incubation with rLCAT corrected the abnormal fractions.

Lp1, one of the abnormal lipoproteins characteristic to LCAT-deficiency syndrome, was rich in TG and PL, and associated with the degree of proteinuria in 2 siblings with FLD, and was decreased on fat restriction in another patient with FLD (Figure 2). Indeed, abnormal lipoproteins with size of ≈100 nm corresponding to Lp1 have been identified in patients with LCAT deficiency with renal failure. The lipid composition of Lp1 did not change on incubation with rLCAT (data not shown). Together, this suggests that Lp1 is most likely secondary to renal failure rather than directly caused by LCAT deficiency.

As opposed to controls, Fr. 8 was richer in total cholesterol, TG, FC, and PL than Fr. 9 in the patients with LCAT

![Figure 2](http://atvb.ahajournals.org/)

**Figure 2.** Differences in lipoproteins in patients with familial lecithin:cholesterol acyltransferase deficiency (FLD) with or without renal insufficiency. A, Lipoprotein profiles were compared between a patient with FLD with nephrotic range proteinuria (patient 1, ○) and patient 3 with mild proteinuria (●). B, Lipoprotein profiles were compared between before (○) and after (●) fat-restricted diet.
deficiency (Figures 1 and 4B). Lp8 also differed in composition between FLD and FED: in FLD, it contained increased TG and decreased CE in comparison with FED (Figure 3A).

Importantly, although the levels varied with the severity of renal damage as did those in Lp1, the buoyance of the peak at Fr. 8 did not vary with severity of renal damage (Figure 2), strongly suggesting that Lp8 directly results from a lack of LCAT and not from metabolic disturbances that occur during proteinuria and progressive renal failure.

In addition to the above-mentioned characteristics for Lp8 in LCAT-deficiency syndrome, HPLC-GFC analyses clarified novel unique lipid properties of Lp8 in FLD in comparison with that in FED; the averaged sizes of Lp8 are smaller in FLD than those in FED (Figure 3C). The lipid compositions of Lp8 in FLD were, in part, ameliorated by rLCAT incubation (Figure 4A). The averaged sizes of the Lp8 increased in FLD, whereas those in FED decreased (Figure 4C). rLCAT increased the CE formation in both LDL and HDL fractions in FLD sera. Thus, these findings indicated that the abnormal compositions were most likely caused primarily by the dysfunction of LCAT in the patients, and that the abnormal characteristics of Lp8 were not because of metabolic disturbances that occur during proteinuria and progressive loss of kidney function.

Previous extensive analyses using electron microscopy have identified 3 abnormal lipoproteins in the LDL fraction of FLD: TG-rich and CE-poor particles of sizes similar to normal LDL (FLD-LDL); FC- and PL-containing particles of sizes distributing from 40 to 60 nm (LpX-like particle) 2; particles with a diameter of 100 nm (designated as LM-LDL)17,30 that were later reported to be identical to LpX. 15 LpX is FC- and PL-rich but TG-poor lipid particles (30%, 60%, and 2%, respectively)22 without apolipoproteins, which range from very low density lipoprotein to large LDL fractions in fast performance liquid chromatography analysis.31 The abnormal particles have been shown to be decreased by lipid-lowering therapy in a patient with FLD.21 Lipoproteins in Lp8 were different from LpX in the lipid contents; the fractions were rich in FC and PL and also rich in TG (13.2±1.3%, 41.4±3.3%, and 45.8±3.8%, respectively). The composition analyses suggested that Lp8 corresponds to FLD-LDL, but the calculated sizes of Lp8 were larger than normal LDL using the data obtained by size fractionation with HPLC-GPC in the present study. Thus, the identified Lp8 in LCAT-deficiency syndrome was most likely not identical to LpX in the characteristics.

There is a limitation for the interpretation of the quantitative measurement of LpX in the frozen samples collected in our study because the abnormal lipoproteins were known to be labile to freezing-and-thawing treatment. In this context, fresh sera were collected from patients 2 and 4 and analyzed by agarose gel electrophoresis. The lipid staining of lipoproteins electrophoresed in agarose gel detected the abnormally slowly migrating TG-poor lipoproteins, LpX, at the expectedly migrating position, as well as TG-rich abnormal β-lipoproteins (LDL) in the once-frozen sample, as well as the fresh sample...
in patient 4, although the staining intensity tended to decrease in comparison with the fresh counterpart. However, LpX was not detected in either sample with or without freeze-and-thaw treatment from patient 2. Thus, LpX was indeed labile to freeze/thawing, and the frozen samples were not adequate for the quantitative measurement. However, the presence was still able to be evaluated after once-freezing treatment. On the basis of background data, HPLC-GFC analysis showed that lipid contents in Lp8 were not largely affected by once-freezing treatment in both patients 2 and 4; in contrast, the contents of TG and PL were slightly decreased in lipoproteins with peak of Fr. 5 (data not shown). Additional studies using fresh samples of patients with distinct mutations and manifestations are needed to interpret the significance of novel lipoproteins in comparison with LpX for the development of renal insufficiency in LCAT0deficiency syndrome quantitatively.

In FLD but not in FED, Lp12 to 16 were heterogeneous in size and rich in PL. LCAT decreased PL in these fractions specifically (Figure 5D; Figure II in the online-only Data Supplement). This may suggest that the heterogeneous-sized PL-rich particles in Fr. 12 to 16 converge to normal-sized HDL (Fr. 16–18) on incubation with LCAT, with concomitant esterification of FC.

In conclusion, 4 lipoprotein fractions specific to LCAT-deficiency syndromes were identified by the HPLC-GFC analysis of samples from genetically diagnosed patients with different mutations and manifestations. The composition of 2 of these was unique to only FLD; these were not likely compatible with the previously reported LpX. These abnormal lipoproteins may be causal to the renal pathology in FLD, the main cause of increased morbidity and mortality in this condition. The regular evaluation of these specific lipid fractions during LCAT enzyme replacement therapy in patients with LCAT deficiency may provide guidance for success of the intervention. The value of these lipid fractions for risk of future renal disease needs to be addressed in prospective follow-up studies in patients with FLD with various mutations in the LCAT gene before the onset of proteinuria.

Sources of Funding
This study was supported, in part, by Health and Labour Sciences Research grants for translational research and for research on rare and intractable diseases, Japan (Hideaki Bujo). A.G. Holleboom is supported by a Veni grant (project number 91613031) from the Netherlands Organization for NWO.

Disclosures
None.

References


**Significance**

Lecithin: cholesterol acyltransferase-deficiency syndromes are classified into 2 forms: familial lecithin: cholesterol acyltransferase deficiency and fish-eye disease. Patients with familial lecithin: cholesterol acyltransferase deficiency develop renal failure, whereas fish-eye disease patients do not. This study was performed to identify abnormal lipoproteins associated with the renal damage of patients with different mutations and manifestations. Size fractionation with gel filtration of patients’ sera and in vitro incubation experiments with recombinant lecithin: cholesterol acyltransferase showed abnormal lipoproteins associated with the renal damage. Thus, our novel analytic approach identified large low-density lipoprotein and high-density lipoprotein with a composition specific to familial lecithin: cholesterol acyltransferase deficiency but not to fish-eye disease. The identification of abnormal lipoproteins may shed light on the clarification of renal pathology and the development of treatment for the patients with familial lecithin: cholesterol acyltransferase deficiency.
Lipoprotein Subfractions Highly Associated With Renal Damage in Familial Lecithin:Cholesterol Acyltransferase Deficiency
Masayuki Kuroda, Adriaan G. Holleboom, Erik S.G. Stroes, Sakiyo Asada, Yasuyuki Aoyagi, Kouju Kamata, Shizuya Yamashita, Shun Ishibashi, Yasushi Saito and Hideaki Bujo

Arterioscler Thromb Vasc Biol. 2014;34:1756-1762; originally published online May 29, 2014; doi: 10.1161/ATVBAHA.114.303420
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/34/8/1756

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2014/05/29/ATVBAHA.114.303420.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Materials and methods
Identification of FLD and FED patients

Patients were referred because of a clinical suspicion of LCAT deficiency due to the presence of corneal opacification and HDL deficiency, with or without proteinuria and/or renal insufficiency (Table 1). Lipid profiles of the patients are also given in Table 2. In our clinics, the definitive molecular diagnoses were established for all patients. The renal biopsy analyses were performed to make a diagnosis for renal damage in some patients. Patient(s) 1 and 3 are Moroccan sister. Other patients are unrelated. Pt.2 was treated with a fat-restricted diet (1570 kcal; fat 10 g and protein 45 g) during admission, with the prescription of losartan 50 mg/day for 8 months. Younger sister of Pt. 2 also shows corneal opacification. Informed consent from her was not obtained for genetic analysis and current study. Proteinuria for FLD patients were 6g/24h (Pt. 1), 2 g/24h (Pt. 2), 0.45 g/l (Pt. 3), 0.23g/24h (Pt. 4), and 0.5 g/l (Pt. 5).

Analysis of patient samples

This study was approved by the Ethics Committees of Chiba University School of Medicine and Academic Medical Center, University of Amsterdam, and informed consent was obtained from the participants including unaffected normolipidemic controls. Blood samples were obtained from participants, and serum was prepared and stored at -80 °C until use. LCAT activity (α-activity) was measured using artificial proteoliposomes as substrate. Serum lipoproteins were fractionated by high-performance liquid chromatography with gel filtration column (HPLC-GFC) and analyzed simultaneously by online enzymatic method to quantify total cholesterol (TC), free cholesterol (FC), triglyceride (TG), and phospholipid (PL) (Skylight Biotech, Akita, Japan). The resulting raw chromatograms (elution time versus lipid concentration) were further processed by computer program with the modified Gaussian curve fitting for resolving the overlapping peaks by mathematical treatment. Finally, the system subdivided the lipoprotein particles of normal subjects by size into the following 20 subclasses: chylomicron (CM, >80 nm, fractions 1-2), very low density lipoprotein (VLDL, 30-80 nm, fractions 3-7), low density lipoprotein (LDL, 16-30 nm, fractions 8-13), and high density lipoprotein (HDL, 8-16 nm, fractions 14-20). In this fractionation analysis, standard particle diameters have been reported to be >90, 75, 64, 53.6, 44.5, 36.8, 31.3, 28.6, 25.5, 23.0, 20.7, 18.6, 16.7, 15.0, 13.5, 12.1, 10.9, 9.8, 8.8, 7.6 nm for fraction 1 through 20, respectively. Average sizes of lipoprotein
classes were calculated based on the particle diameter as follows: (sum of particle diameter x lipid concentration in each fraction)/(sum of lipid concentration of the fractions of interest). Ultracentrifugation fractionation followed by determination of lipid contents was performed in Pt. 1, 2 and 5 (Table I in data supplement). For some FLD sera, representative fractions were collected, concentrated by Vivaspin (MWCO=3,000, Sartorius, Weender Landstr., Germany) and subjected to determine apolipoprotein concentrations by Milliplex MAP Human Apolipoprotein Panel kit (Millipore, Billerica, MA) using BioPlex apparatus (BioRad, Hercules, CA).

**Preparation of recombinant LCAT and in vitro incubation with patient’s serum**

Human LCAT gene was transduced into human preadipocytes by retroviral vector as described previously\(^1\). The resulting cells were seeded into T225 flask and grown to confluency in MesenPRO medium (Life Technologies, Carlsbad, CA). The medium was changed to 30 ml of OPTI MEM I (Life Technologies) and the cells were further incubated for seven days to collect culture supernatant. The culture supernatant was concentrated to one-fiftieth of the original volume by Amicon Ultra (MWCO=50 kDa, Millipore)\(^4\). LCAT concentration was titrated by immunoblotting using commercially available human rLCAT (Roar Biomedical, Inc., Calverton, NY) as standard. After mixing with rLCAT-containing culture supernatant at the ratio of 29:71 (v/v), each patient’s serum was incubated at 37 °C for 24 hr (final concentration of rLCAT was 6 \(\mu g/ml\)), and subjected to lipoprotein analysis. Culture supernatant of human preadipocytes without gene transduction was used as control.

**Statistical analysis**

Data are presented as means ± S.D. Comparisons were assessed for significant differences by paired Student’s t-test, or by ANOVA followed by Tukey test, where appropriate. SPSS software was used for statistical analyses. In all cases, P values of less than 0.05 were considered significant.

**REFERENCES:**
proliferative adipocytes are a possible delivery vehicle for enzyme replacement therapy in lecithin: cholesterol acyltransferase deficiency. The Open Gene Ther J 2011;4:1-10


**Supplement Table I. Ultracentrifugation analysis of lipoprotein in FLD patients.**

Patients' sera were subjected to ultracentrifugation fractionation, followed by determination of lipid concentration.

<table>
<thead>
<tr>
<th></th>
<th>Pt. 1</th>
<th>Pt. 2</th>
<th>Pt. 5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HDL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>18.6</td>
<td>12.0</td>
<td>10.8</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>3.6</td>
<td>3.0</td>
<td>4.2</td>
</tr>
<tr>
<td>Phospholipid (mg/dl)</td>
<td>65.4</td>
<td>44.4</td>
<td>42.0</td>
</tr>
<tr>
<td><strong>LDL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>62.4</td>
<td>99.0</td>
<td>39.6</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>77.4</td>
<td>151.2</td>
<td>61.8</td>
</tr>
<tr>
<td>Phospholipid (mg/dl)</td>
<td>106.2</td>
<td>157.8</td>
<td>73.2</td>
</tr>
<tr>
<td><strong>VLDL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>62.0</td>
<td>51.0</td>
<td>9.6</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>190.0</td>
<td>157.8</td>
<td>36.0</td>
</tr>
<tr>
<td>Phospholipid (mg/dl)</td>
<td>103.4</td>
<td>81.8</td>
<td>21.8</td>
</tr>
</tbody>
</table>
Supplement Figure I. Apolipoprotein contents in lipoproteins in FLD patients.

Lipoprotein subfractions were collected and concentrated (MWCO=3,000). Apolipoprotein concentrations in the concentrated samples were determined by ELISA. N; normolipidemic control, 1; Pt. 1, 2; Pt. 2, 5; Pt. 5.
Supplement Figure II. Comparison of lipid concentrations of lipoproteins between FLD and FED.
According to the distribution of lipoproteins shown in Figure 1, CE (panel A), FC (panel B), TG (panel C), and PL (panel D) concentrations in Fr. 1, Fr. 7-10, Fr. 12-16 and Fr. 18-20 were compared between FLD patients (open column, n=5) and FED patients (closed column, n=4). nd, not detected, *p<0.05.
Supplement Figure III. Response to rLCAT *in vitro*

After incubation with culture supernatant of human *LCAT* gene-transduced preadipocytes (closed circle) and un-transduced control (open circle), GFC analyses with simultaneous determination of TC, TG, FC, and PL concentrations were performed. Representative results of FLD (Pt. 1) and FED (Pt. 7) sera were shown.
Supplement Figure IV. *In vitro* rLCAT incubation ameliorated HDL composition in FLD and FED.

After *in vitro* rLCAT incubation and subsequent GFC analyses of lipoproteins shown in Fig. S2, changes of core HDL fractions (Fr. 16-18) were analyzed in FLD (n=4) and FED (n=4) sera (A-C). Lipid concentrations (A), CE/TC ratio (B), and lipid composition (C) were compared between rLCAT containing culture media (closed bar) and the culture media without rLCAT (open bar). *p<0.05.