Circulating Oxidized Low-Density Lipoprotein and Its Association With Carotid Intima-Media Thickness in Asymptomatic Members of Familial Combined Hyperlipidemia Families

Ming-Lin Liu, Kati Ylitalo, Riitta Salonen, Jukka T. Salonen, Marja-Riitta Taskinen

Objective—Oxidized low-density lipoprotein (Ox-LDL) is implicated in the pathogenesis of atherosclerosis. Circulating oxidation-specific epitopes on plasma Ox-LDL has been linked with coronary artery disease, but its determinants and its association with early development of atherosclerosis in familial combined hyperlipidemia (FCHL) has not been very well studied. This study aimed to investigate the determinants of the circulating Ox-LDL and the association between Ox-LDL and carotid intima-media thickness (IMT) in asymptomatic members of FCHL families.

Methods and Results—Ox-LDL, susceptibility of LDL to oxidation in vitro, plasma 8-isoprostane and antioxidants, lipids and lipoproteins, LDL particle size, and carotid IMT were measured in 150 asymptomatic FCHL family members. Affected FCHL family members had reduced LDL particle size and lag time for LDL oxidation, increased plasma levels of Ox-LDL, increased plasma urate and α-tocopherol, and a trend for the increase of 8-isoprostane as compared with nonaffected FCHL. Ox-LDL was independently associated with serum LDL cholesterol, apoB, and 8-isoprostane in multivariate analysis but only univariately correlated with LDL particle size and lag time for LDL oxidation. In addition, Ox-LDL was significantly associated with carotid mean IMT independently of other clinical and biochemical variables in a multivariate model.

Conclusion—Serum LDL cholesterol, apoB levels, and 8-isoprostane were the most important determinants of Ox-LDL. Ox-LDL is independently associated with carotid IMT in asymptomatic FCHL family members and can be used as a marker of early atherosclerosis in FCHL. (Arterioscler Thromb Vasc Biol. 2004;24:1-7.)

Key Words: carotid arteries □ hyperlipoproteinemia □ familial combined □ lipoproteins □ low-density lipoprotein □ oxygen radical □ ultrasonography
als in leukocytes from hypercholesterolemic and hypertriglyceridemic patients. Previous studies also reported the increased formation of isoprostanes, markers of in vivo oxidative stress, in hyperlipidemic patients. Interestingly, our data have shown an adaptive increase of circulating antioxidants with increased oxidative stress in asymptomatic FCHL patients. In addition, LDL from FCHL patients is characterized by a predominance of small dense LDL and an increased susceptibility to oxidative modification. All these factors may influence the metabolism of circulating Ox-LDL in FCHL.

Carotid artery intima-media thickness (IMT), measured noninvasively by high-resolution B-mode ultrasonography, has been associated with the risk of CAD, stroke, and myocardial infarction, and it predicts the progression of CAD. The present study was conducted to investigate the determinants of plasma Ox-LDL and the association between Ox-LDL and carotid IMT in asymptomatic FCHL family members.

**Methods**

**Study Subjects**

The study subjects were recruited according to the study protocol as reported previously. All subjects gave their informed consent to the study protocol, which was approved by the ethical committees. Briefly, the FCHL probands were required to be 30 to 60 years of age, have verified CAD, and have serum total cholesterol (TC) and/or triglycerides (TG) age- and sex-specific levels in >90th Finnish population percentiles. The TC and TG percentiles used in the present study were derived from the results of the surveys based on the Finnish population. Families with >2 affected family members presenting different lipid phenotypes were classified as FCHL. Family members who had diabetes or history of CAD or stroke and those with lipid medication were excluded. As described previously, altogether 150 FCHL (75 affected and 75 nonaffected family members as reported in our previous study) other plasma determinants were comparable between the 2 groups (data not shown). As described, we did not see significant difference in the frequency distribution of the categorical variables between 2 groups was compared by the χ² test. Univariate association was tested by Pearson correlation analysis. The predictors for the subsequent multivariate analysis were selected on the basis of the correlation analyses (P<0.20). Backward multivariate analyses were performed to assess the predictors of Ox-LDL and carotid mean IMT. Both in the ANOVA and the backward multivariate analyses, family number (which indicates belonging to a certain family) was used to correct for the dependence of the study subjects.

**Measurement of Plasma Ox-LDL and LDL Oxidation In Vitro**

Plasma levels of Ox-LDL were measured by a competitive enzyme-linked immunosorbent assay using a specific marine monoclonal antibody 4E6 (mAb-4E6) (Mercodia, Uppsala, Sweden). The coefficient of variation for the assay was 7.4% to 8.3%. The mAb-4E6 is directed against a conformational epitope in the apolipoprotein B-100 (apoB-100) moiety of LDL that is generated as a consequence of substitution of at least 60 lysine residues of apoB-100 with aldehydes. This number of substituted lysines corresponds to the minimal number of substituted lysines required for scavenger-mediated uptake of oxLDL. Substituting aldehydes can be produced by peroxidation of lipids of LDL. LDL for the in vitro oxidation measurement was isolated by a short-run ultracentrifugation. EDTA was removed from LDL using size exclusion chromatography (PD-25 column) just before LDL oxidation in vitro. Altogether 100 μg LDL protein/mL was incubated with 5 μmol/L CuSO₄ in a total volume of 2 mL at 27°C in a temperature-controlled spectrophotometer.

**Other Biochemical Measurements**

Plasma 8-isoprostane levels were measured using an EIA kit (516351; Cayman, Ann Arbor, Mich). Plasma vitamin C, protein-bound thiol groups, urate, α-tocopherol, β-carotene, and retinol were measured as described. LDL particle size was determined using gradient gel electrophoresis. All lipid and lipoprotein measurements were performed as described previously. Briefly, serum TC and TG concentrations were determined enzymatically, serum high-density lipoprotein (HDL) cholesterol by precipitation procedures, and serum apolipoprotein B (apoB) concentration by an immunoturbidimetric assay. LDL was separated by sequential flotation as described.

**Ultrasound Examinations**

Carotid IMT was determined as described previously. Briefly, longitudinal images from 3 projections (anterolateral, lateral, and posteroarteral) were measured by Hewlett-Packard Image Point M2410A ultrasound system for the common carotid artery, carotid bulb, and internal carotid artery. Measurements were performed at a total of 28 sites, both the far wall and the near wall of the arterial segments, right and left distal 1 cm of common carotid artery, carotid bulb, and proximal 1 cm of internal carotid artery. All 3 projections in common carotid artery and carotid bulb, and a single angle in and internal carotid artery with the best visibility were used. The mean, maximum, and minimum IMT were derived from each measurement. The average of all mean IMT measurements over 28 (or fewer) sites was chosen as the outcome variable. The coefficient of variation for mean IMT measurements was 2.4%. Carotid IMT examination was performed at the same visit as blood sampling or within a difference of few weeks.

**Statistical Analysis**

Values are given as means±SE. Variables with nonnormal distribution were log₁₀-transformed. Differences in means between affected and nonaffected family members or among the different Ox-LDL tertile groups were tested by 2-way ANOVA (Figure A). The frequency distribution of the categorical variables between 2 groups was compared by the χ² test. Univariate association was tested by Pearson correlation analysis. The predictors for the subsequent multivariate analysis were selected on the basis of the correlation analyses (P<0.20). Backward multivariate analyses were performed to assess the predictors of Ox-LDL and carotid mean IMT. Both in the ANOVA and the backward multivariate analyses, family number (which indicates belonging to a certain family) was used to correct for the dependence of the study subjects.

**Results**

**Ox-LDL and Other Study Variables of the FCHL Family Members**

Table 1 summarizes clinical and other study variables of the subjects. By definition, TC, TG, LDL cholesterol, and apoB were significantly higher in affected than in nonaffected family members. Affected family members had significantly smaller LDL size, shorter lag time for LDL oxidation, and higher plasma Ox-LDL as compared with those in nonaffected members. Plasma 8-isoprostane levels tended to be higher in affected than in nonaffected family members, but the difference did not reach statistical significance. Plasma α-tocopherol and urate were significantly increased in affected FCHL compared with those in nonaffected family members as reported in our previous study. Other plasma antioxidants (vitamin C, thiol groups, β-carotene, retinol) were comparable between the 2 groups (data not shown). As reported previously, we did not see significant difference in carotid mean IMT between affected and nonaffected family members.
mean IMT in highest Ox-LDL tertile group was significantly thicker than that in lowest Ox-LDL tertile (0.78 ± 0.02 versus 0.69 ± 0.02 mm, \( P = 0.003 \)). In addition, there were more affected subjects in the highest Ox-LDL tertile group (40/50) than in the middle (23/50) and in the lowest (12/50) Ox-LDL tertile groups (\( P < 0.001 \), ANOVA; Figure A). Plasma Ox-LDL correlated with carotid mean IMT in affected and nonaffected family members as well as in the combined group including all family members (Figure B).

Carotid mean IMT correlated significantly with age, body mass index, smoking pack-years, pulse pressure, log TG, LDL cholesterol, apoB, LDL size, lag time for LDL oxidation, plasma urate, and \( \alpha \)-tocopherol, as well as Ox-LDL. However, we did not observe any correlation between 8-isoprostane and mean IMT in univariate analysis. In the multivariate analysis, only age (\( \beta = 0.745, P = 0.001 \)), pulse pressure (\( \beta = 0.158, P = 0.004 \)), LDL size (\( \beta = 0.169, P = 0.022 \)), and Ox-LDL (\( \beta = 0.178, P = 0.038 \)) were independent predictors for the variation of carotid mean IMT. The association between Ox-LDL and mean IMT persisted even after adjustment for logTG, LDL cholesterol, and apoB. These results demonstrate that Ox-LDL is associated with mean IMT in FCHL family members independently of clinical and lipid variables.

**Discussion**

The present study shows that LDL cholesterol, apoB, and 8-isoprostane were independent determinants of plasma Ox-LDL. The Ox-LDL was associated with carotid mean IMT independently of other variables in asymptomatic FCHL family members. Therefore, the Ox-LDL is a useful marker of early atherosclerosis in FCHL.

**Circulating Ox-LDL, 8-Isoprostane, and Antioxidants**

There is increased oxidative stress in dyslipidemia.\(^{16–19}\) In this study, the plasma Ox-LDL was significantly higher in affected FCHL family members as compared with nonaffected subjects. Likewise, there was a trend for an increase of the plasma 8-isoprostane in affected FCHL family members. The present data confirmed our previous results\(^{20}\) showing an adaptive increase of plasma antioxidants (\( \alpha \)-tocopherol and urate) in the presence of the increased oxidative stress in FCHL. Recent data suggested that supplementation of \( \alpha \)-tocopherol cannot prevent lipoprotein oxidation in the vessel wall with the increased levels of \( \alpha \)-tocopherol in the circulation and in the arterial wall.\(^{29}\) A population study showed that \( \alpha \)-tocopherol supplementation in healthy individuals increases plasma levels of \( \alpha \)-tocopherol and reduces LDL oxidative susceptibility and circulating oxidized LDL.\(^{30}\) In contrast, several other clinical studies have reported that supplementation with \( \alpha \)-tocopherol has no effect on autoantibodies against Ox-LDL in hyperlipidemic patients,\(^{31}\) in patients with chronic renal failure,\(^{32}\) or in chronic smokers.\(^{33}\) In agreement with the latter results, the plasma levels of Ox-LDL were increased in affected FCHL family members, despite the elevation of plasma \( \alpha \)-tocopherol. In addition, the low-fat, high-vegetable diet, which increased plasma concentrations of vitamin C, \( \beta \)-carotene, and lycopene, failed to...

**Associations Between Ox-LDL, Related Variables, and Carotid Mean IMT**

In the univariate correlation analysis, Ox-LDL was significantly correlated with age, gender, body mass index, smoking pack-years, log TG, LDL cholesterol, HDL cholesterol, apoB, LDL particle size, lag time for LDL oxidation, plasma urate, \( \alpha \)-tocopherol, and retinol, but not with 8-isoprostane, vitamin C, thiol groups, and \( \beta \)-carotene. Interestingly, plasma Ox-LDL and 8-isoprostane were correlated in affected family members (\( r = 0.349, P = 0.002 \)). In the multivariate analysis, LDL-C, apoB, and 8-isoprostane were associated with plasma Ox-LDL independently of other variables (Table 2).

When FCHL family members were divided by tertiles of Ox-LDL (50 subjects in each tertile group), the carotid mean IMT increased over tertiles of Ox-LDL (Figure A). The present data confirmed our previous results\(^{20}\) showing an adaptive increase of plasma antioxidants (\( \alpha \)-tocopherol and urate) in the presence of the increased oxidative stress in FCHL. Recent data suggested that supplementation of \( \alpha \)-tocopherol cannot prevent lipoprotein oxidation in the vessel wall with the increased levels of \( \alpha \)-tocopherol in the circulation and in the arterial wall.\(^{29}\) A population study showed that \( \alpha \)-tocopherol supplementation in healthy individuals increases plasma levels of \( \alpha \)-tocopherol and reduces LDL oxidative susceptibility and circulating oxidized LDL.\(^{30}\) In contrast, several other clinical studies have reported that supplementation with \( \alpha \)-tocopherol has no effect on autoantibodies against Ox-LDL in hyperlipidemic patients,\(^{31}\) in patients with chronic renal failure,\(^{32}\) or in chronic smokers.\(^{33}\) In agreement with the latter results, the plasma levels of Ox-LDL were increased in affected FCHL family members, despite the elevation of plasma \( \alpha \)-tocopherol. In addition, the low-fat, high-vegetable diet, which increased plasma concentrations of vitamin C, \( \beta \)-carotene, and lycopene, failed to...
decrease plasma levels of Ox-LDL measured with mAb-EO6. The positive correlation between Ox-LDL and plasma antioxidants (Table 2) in FCHL family members may reflect the fact that adaptive increase in plasma antioxidants cannot

### TABLE 1. Clinical and Biochemical Characteristics and Other Study Variables in FCHL Family Members

<table>
<thead>
<tr>
<th>Variable</th>
<th>Total (n=150)</th>
<th>Affected (n=75)</th>
<th>Nonaffected (n=75)</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>40.3±0.9</td>
<td>40.4±1.3</td>
<td>40.1±1.2</td>
<td>NS</td>
</tr>
<tr>
<td>Gender, M/F</td>
<td>56/94</td>
<td>29/46</td>
<td>27/48</td>
<td>NS</td>
</tr>
<tr>
<td>Smoking pack-years</td>
<td>6.6±0.7</td>
<td>7.0±1.1</td>
<td>6.2±0.9</td>
<td>NS</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25.6±0.3</td>
<td>26.5±0.5</td>
<td>24.7±0.4</td>
<td>0.025</td>
</tr>
<tr>
<td>Pulse pressure, mm Hg</td>
<td>47.6±1.0</td>
<td>49.1±1.6</td>
<td>46.1±1.1</td>
<td>0.019</td>
</tr>
<tr>
<td>TC, mmol/L</td>
<td>5.80±0.09</td>
<td>6.40±0.13</td>
<td>5.20±0.09</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TG, mmol/L</td>
<td>1.65±0.12</td>
<td>2.18±0.21</td>
<td>1.13±0.05</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL-C, mmol/L</td>
<td>3.57±0.08</td>
<td>3.99±0.12</td>
<td>3.13±0.09</td>
<td>0.002</td>
</tr>
<tr>
<td>HDL-C, mmol/L</td>
<td>1.49±0.04</td>
<td>1.41±0.05</td>
<td>1.56±0.05</td>
<td>NS</td>
</tr>
<tr>
<td>ApoB, mg/dL</td>
<td>102.0±2.6</td>
<td>119.3±3.6</td>
<td>84.5±2.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL particle size, nm</td>
<td>26.6±0.1</td>
<td>26.0±0.2</td>
<td>27.3±0.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL lag time, min</td>
<td>109.4±0.9</td>
<td>106.3±1.4</td>
<td>112.4±1.2</td>
<td>0.010</td>
</tr>
<tr>
<td>Circulating oxidized LDL, U/L</td>
<td>89.5±2.1</td>
<td>101.9±2.8</td>
<td>77.0±2.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>8-Isoprostane, pg/mL</td>
<td>315.9±16.9</td>
<td>336.3±25.0</td>
<td>295.5±8</td>
<td>NS</td>
</tr>
<tr>
<td>Urate, mmol/L</td>
<td>283.6±6.3</td>
<td>307.7±9.6</td>
<td>259.5±7.2</td>
<td>0.019</td>
</tr>
<tr>
<td>α-Tocopherol, μmol/L</td>
<td>32.1±0.8</td>
<td>35.3±1.1</td>
<td>27.9±0.8</td>
<td>0.001</td>
</tr>
<tr>
<td>Carotid mean IMT, mm</td>
<td>0.74±0.01</td>
<td>0.75±0.02</td>
<td>0.73±0.02</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are means±SE.

M/F indicates male/female; BMI, body mass index; U/L, unit/liter; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides; NS, not significant.

Statistical comparisons between affected and nonaffected family members were performed by 2-way ANOVA. The frequency distribution of the categorical variables between the 2 groups was compared by the χ² test.

### TABLE 2. Determinants of Circulating Oxidized LDL in FCHL Family Members

<table>
<thead>
<tr>
<th>N</th>
<th>Variable</th>
<th>Univariate Analysis</th>
<th>Multivariate Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>r</td>
<td>β</td>
</tr>
<tr>
<td></td>
<td>Age</td>
<td>0.242</td>
<td>−0.051</td>
</tr>
<tr>
<td></td>
<td>Gender</td>
<td>0.254</td>
<td>NE</td>
</tr>
<tr>
<td></td>
<td>BMI</td>
<td>0.350</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Pulse pressure</td>
<td>0.189</td>
<td>NE</td>
</tr>
<tr>
<td></td>
<td>Smoking pack-years</td>
<td>0.176</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Log triglycerides</td>
<td>0.580</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>LDL-C</td>
<td>0.680</td>
<td>0.310</td>
</tr>
<tr>
<td></td>
<td>HDL-C</td>
<td>−0.385</td>
<td>0.055</td>
</tr>
<tr>
<td></td>
<td>ApoB</td>
<td>0.788</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>LDL size</td>
<td>−0.405</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>LDL lag time</td>
<td>−0.257</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>8-Isoprostane</td>
<td>0.136</td>
<td>0.128</td>
</tr>
<tr>
<td></td>
<td>Vitamin C</td>
<td>−0.008</td>
<td>0.921</td>
</tr>
<tr>
<td></td>
<td>Thiol groups</td>
<td>−0.043</td>
<td>0.606</td>
</tr>
<tr>
<td></td>
<td>Urate</td>
<td>0.392</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>α-Tocopherol</td>
<td>0.654</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>β-Carotene</td>
<td>0.107</td>
<td>0.279</td>
</tr>
<tr>
<td></td>
<td>Retinol</td>
<td>0.241</td>
<td>0.013</td>
</tr>
<tr>
<td></td>
<td>Adjusted R²</td>
<td>0.695</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

r indicates correlation coefficient; β, standardized coefficient; NE, does not enter the final model.

Family number was forced into the regression analysis model.
proteins and consequently enhance invasion of LDL into the subendothelium, where the increased oxidative stress as measured by 8-isoprostane will promote the oxidative modification of LDL.

**Association Between Ox-LDL and Carotid IMT**

Oxidative modification of LDL is believed to play an important role in the development of atherosclerosis. Susceptibility of LDL to oxidation in vitro and autoantibodies against Ox-LDL have been related with atherosclerotic diseases in some, but not all, clinical studies. Recently, circulating Ox-LDL measured by different antibodies has been associated with cardiovascular diseases. In the present study, the carotid mean IMT increased by tertiles of Ox-LDL in the combined group (Figure A). In addition, the relative numbers of affected subjects were significantly increased in highest tertile group as compared with those in the middle and in the lowest tertile groups. These data suggest that the subjects with an increased level of Ox-LDL have increased carotid IMT, particularly in affected family members. Furthermore, carotid mean IMT was independently associated with age, pulse pressure, LDL size, and Ox-LDL in the univariate and multivariate analyses. The association between Ox-LDL and mean IMT persisted even after adjustment for logTG, LDL cholesterol, and apoB. The data indicate that Ox-LDL is a useful marker of the early stage of atherosclerosis in FCHL family members without clinical CAD. These results are in keeping with previous studies in which Ox-LDL was associated with the extent of CAD. Likewise, Ox-LDL was univariately correlated with IMT in carotid and femoral arteries and independently associated with subclinical plaque occurrence in carotid and femoral arteries in healthy population. However, no association between carotid IMT and plasma 8-isoprostane was observed in the present study, in line with the other studies. Pulse pressure, a pulsatile component of blood pressure determined by stiffness and elastic properties of arterial walls, is reported to be independently associated with carotid IMT in some studies. In agreement, we observed an independent association between carotid IMT and pulse pressure.

**Conclusion**

Serum levels of LDL cholesterol, apoB, and plasma 8-isoprostane are the most important determinants of Ox-LDL. Ox-LDL and LDL size were associated with carotid IMT independently of other clinical and lipid variables in the FCHL family members without clinical CAD. Therefore, our results suggest that the quantity of LDL particles and the oxidative stress in vivo determine the generation of circulating Ox-LDL, which may be a surrogate marker for CAD risk in the early stage of atherosclerosis in FCHL.

**Acknowledgments**

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