Association Between Carotid Intima-Media Thickness and Low-Density Lipoprotein Size and Susceptibility of Low-Density Lipoprotein to Oxidation in Asymptomatic Members of Familial Combined Hyperlipidemia Families

Ming-Lin Liu, MD; Kati Ylitalo, MD; Ilpo Nuotio, MD, PhD; Riitta Salonen, MD, PhD; Jukka T. Salonen, MD, PhD, MScPH; Marja-Riitta Taskinen, MD, PhD

Background and Purpose—In addition to low-density lipoprotein (LDL) cholesterol, small, dense LDL particles and oxidative modification of LDL have been linked to the pathogenesis of atherosclerosis. The present study was aimed at investigating the association between carotid artery intima-media thickness (IMT) and LDL particle size and susceptibility of LDL to oxidation in vitro in asymptomatic members of familial combined hyperlipidemia (FCHL) families.

Methods—LDL particle size, susceptibility of LDL to oxidation in vitro, and carotid IMT were measured in 148 asymptomatic FCHL family members.

Results—LDL particle size and lag time for LDL oxidation were reduced in hyperlipidemic compared with normolipidemic family members. LDL particle size, serum total cholesterol, and α-tocopherol in LDL were independently associated with lag time for LDL oxidation in multivariate analysis. LDL particle size was associated with carotid mean IMT independently of clinical, lipid, and antioxidant variables in multivariate analysis. Although the susceptibility of LDL to oxidation in vitro was correlated with mean IMT, it did not have a significant independent contribution to variation in mean IMT in the multivariate model.

Conclusions—We conclude that LDL particle size but not susceptibility of LDL to oxidation in vitro is independently associated with carotid IMT in asymptomatic FCHL family members. These results imply that small, dense LDL as an inherent feature of FCHL is an important diagnostic indicator for coronary artery disease risk in FCHL. (Stroke. 2002; 33:1255-1260.)

Key Words: carotid arteries ■ hyperlipoproteinemia, familial combined ■ lipoproteins, LDL cholesterol ■ oxygen radical ■ ultrasonography

Familial combined hyperlipidemia (FCHL), the most common familial lipid disorder, associates with early atherosclerosis and is responsible for >10% of premature coronary artery disease (CAD). The lipid profile typical of FCHL shares common features with the atherogenic lipoprotein profile, namely small, dense low-density lipoprotein (LDL) species, hypertriglyceridemia, and low concentrations of high-density lipoprotein (HDL).

In recent years, small, dense LDL and oxidative modification of LDL have been linked to the development of arteriosclerosis. Small, dense LDL particles penetrate the arterial wall easily and have a high affinity for intimal proteoglycans. LDL-proteoglycan complexes show increased susceptibility to oxidation. Oxidative modification of LDL greatly increases its atherogenicity and is considered an initial step in the development of atherosclerosis. Several clinical studies have provided evidence that small, dense LDL is indeed associated with an increased risk of CAD. The susceptibility of LDL to oxidation in vitro, although reported to be associated with CAD in a previous small-scale study, was only marginally associated with the extent of coronary atherosclerosis in a more recent larger-scale study.

Carotid artery intima-media thickness (IMT) can be measured noninvasively by high-resolution B-mode ultrasonography. This method provides an index to describe the atherosclerotic extent of vascular disease at a population level. Several studies have shown that carotid IMT is associated with the risk of CAD, stroke, and myocardial infarction and predicts the progression of CAD. Until now, reports on associations between carotid IMT and LDL particle size.
or susceptibility of LDL to oxidation in vitro have given conflicting results. In the present study, we investigated the associations between carotid IMT and (1) LDL particle size and (2) susceptibility of LDL to oxidation in vitro in asymptomatic FCHL family members.

Subjects and Methods

Subjects
All study subjects were recruited in Helsinki and Turku University Central Hospitals in Finland as a part of the European Multicenter Study on Familial Dyslipidemias (EUFAM). The study protocol has been reported previously. The FCHL probands were required to be 30 to 60 years of age, have verified coronary heart disease, and have serum total cholesterol (TC) and/or triglycerides (TG) that were greater than or equal to that of age- and sex-specific 90th Finnish population percentiles. Families with ≥2 affected family members presenting different lipid phenotypes were included. Family members who had diabetes or a history of CAD or stroke and those with lipid medication were excluded. Altogether, 148 FCHL (75 affected and 73 nonaffected defined according to the above-mentioned lipid criteria) family members from 38 well-defined Finnish FCHL families participated in the present study. Venous blood samples for the biochemical measurements were drawn from subjects after an overnight fast. EDTA plasma was separated by centrifugation and stored at −80°C until analyzed. Pulse pressure was calculated as the difference between the systolic and diastolic blood pressures (SBP and DBP). Pack-years of cigarette smoking were calculated by multiplying the duration of smoking by the number of cigarettes smoked per day and dividing by 20.

Ultrasound Examinations
Ultrasound scannings were performed with a Hewlett Packard Image Point M2410A ultrasound system equipped with a 10-MHz linear-array transducer and videotaped with a Panasonic AG-MD830E PAL super-VHS video cassette recorder. One physician (K.Y.) carried out all ultrasound examinations. Longitudinal images from 3 projections (anterolateral, lateral, and posterolateral) were displayed for the common carotid artery, carotid bulb, and internal carotid artery. Measurements were carried out at a total of 28 sites at both the far wall and the near wall of 6 arterial segments: right and left distal 1 cm of the common carotid artery, the carotid bulb, and proximal 1 cm of the internal carotid artery. All 3 projections in the common carotid artery and carotid bulb and a single angle in the internal carotid artery with the best visibility were used. IMT measurements were done by a single observer at Oy Jurilab Ltd (www.jurilab.com) with a computerized reading system using Prosound software (Caltech) and a video frame grabber. IMT measurements from videotapes were made at a total of 28 sites corresponding to the 28 sites where the scanning was focused. The mean, maximum, and minimum IMT values were derived from each measurement. The average of all mean IMT measurements over 28 (or fewer) sites was chosen as the outcome variable.

Quantification of LDL Particle Size
LDL particle size was determined through gradient gel electrophoresis on a linear 1-mm-thick gel with 2% to 10% polyacrylamide. The vertical slab gels were run in the Bio-Rad Mini-Protein II Electrophoresis Cell in a cooled room to 4°C. Pre-electrophoresis (20 minutes at 30 V) and electrophoresis (18 hours at 125 V) were performed by use of Tris-glycine buffer, pH 8.3 (0.024 mol/L Tris and 0.192 mol/L glycine). Serum samples were diluted with sample buffer containing 0.6 mol/L Tris (pH 8.3), 8% sucrose, and 0.035% bromophenol blue. Then, 10 μL diluted sample was applied to each well. Gels were stained with newly filtered sudan black B lipid stain (0.3% sudan black B and 1% zinc acetate in 30% methanol, 30% 2-propanol) for 1 hour and destained with 30% 2-propanol for 24 hours. Gels were kept in 5% acetic acid for 4 to 6 hours and dried with the Bio-Rad GelAir Drying System for 4 hours. Gels were photographed with a Kodak Digital Science DC120 camera and analyzed with the Kodak Digital Science Electrophoresis Documentation and Analysis System 120. Two isolated LDL samples used as size references and 1 control sample were applied on each gel. The particle sizes of the 2 reference LDL samples were measured by electron microscopy. The median of the LDL particle diameters was calculated by measuring diameters of at least 100 LDL particles from the electron microscopic photograph. The calculated median diameters of the LDL standards were 27.9 and 23.9 nm. The mean particle diameter of the major LDL peak was determined by comparing the mobility of the sample to the mobility of the calibrated LDL preparation run on each gel. The coefficient of variation for the intergel precision of the used control sample was 1.4%.

Determination of the Susceptibility of LDL to Oxidation In Vitro
LDL was isolated by a short-run ultracentrifugation. EDTA was removed from the LDL through size exclusion chromatography (PD-25 column) just before LDL oxidation in vitro. The pure LDL (100 μg LDL protein/mL) was incubated with 5 μmol/L CuSO4 in a total volume of 2 mL at 27°C. The kinetics of conjugated diene formation were determined by monitoring the change in absorbance at 234 nm in a temperature-controlled spectrophotometer. The change in absorbance over time could be divided into 3 consecutive phases: lag phase, propagation phase, and decomposition phase. The lag time was used as a measure of susceptibility of LDL to oxidation in vitro.

Measurement of Lipid-Soluble Antioxidants in Plasma and LDL
α-Tocopherol, β-carotene, and retinol in plasma and LDL, as well as the total LDL protein content, were measured as previously described. The α-tocopherol, β-carotene, and retinol concentrations of the LDL samples were expressed in relation to the total LDL protein content estimated concomitantly in each sample, ie, nanomoles of antioxidant per milligram of LDL protein.

Other Measurements
All lipid and lipoprotein measurements were done in the Research Laboratory of the Helsinki University Central Hospital as described previously. Briefly, serum TC and TG concentrations were determined enzymatically, serum HDL cholesterol by precipitation procedures, and serum apolipoprotein B (apoB) concentration by an immunoturbidimetric assay. LDL was separated by sequential flotation as described.

Statistical Analyses
Statistical analyses were done with the SPSS 9.0.1 statistical package. Variables with nonnormal distribution were log10 transformed. Values are median (range) for study population (Table 1). Differences in means between affected and nonaffected family members were tested by 2-way analysis of variance in which family number (which indicates that the subject belongs to a certain family) was used as a random factor to at least partly correct for the dependence of the study subjects. The frequency distribution of the categorical variables between the 2 groups was compared by use of the χ2 test. Correlations were tested by multivariate analysis with family number as an independent variable. The predictors for the subsequent multivariate analyses were selected on the basis of the correlation analyses (P<0.20 when adjusted only for family number). Backward multivariate analyses were performed to assess the predictors of lag time for LDL oxidation and carotid mean IMT. Family number, age, and sex were always entered into the models.

Results

Clinical and Other Study Variables of FCHL Family Members
Clinical and other study variables of the subjects are presented in Table 1. When the subjects were subdivided according
to the lipid inclusion criteria (see Subjects and Methods), 75 subjects were affected and 73 were nonaffected. Age, sex distributions, and the number of current/ever smokers were similar in the affected and nonaffected family members. SBP and pulse pressure but not DBP were significantly higher in affected than in nonaffected family members. Carotid mean IMT was comparable in affected and nonaffected family members (0.75 ± 0.02 versus 0.73 ± 0.02 mm, \( P = \text{NS} \); Figure 1A). LDL particle size was significantly smaller (26.0 ± 0.2 versus 27.3 ± 0.1 nm, \( P < 0.001 \); Figure 1B) and lag time for LDL oxidation was significantly shorter (106.3 ± 1.4 versus 112.4 ± 1.2 minutes, \( P < 0.01 \); Figure 1C) in affected than in nonaffected family members. Plasma \( \alpha \)-tocopherol was increased in affected compared with nonaffected (35.3 ± 1.1 versus 27.7 ± 0.7 \( \mu \text{mol/L} \), \( P < 0.01 \)) family members. No significant differences, however, were observed in plasma \( \beta \)-carotene and retinol and in lipid-soluble antioxidants (\( \alpha \)-tocopherol, \( \beta \)-carotene, and retinol) in LDL between affected and nonaffected family members (data not shown).

**Associations Between Susceptibility of LDL to Oxidation In Vitro and Related Variables**

In the correlation analyses, lag time for LDL oxidation was significantly correlated with pulse pressure, smoking pack-years, TC, log TG, apoB, LDL particle size, \( \beta \)-carotene in LDL, and retinol in LDL. In the multivariate analysis, LDL particle size, TC, and \( \alpha \)-tocopherol in LDL were significant predictors of a variation in lag time for LDL oxidation independently of log TG, apoB, and clinical variables (age, sex, pulse pressure, and smoking pack-years; Table 2).

**Associations Between Mean IMT and Selected Variables**

Of the clinical, lipid, and antioxidant variables, mean IMT correlated significantly with age, body mass index (BMI), SBP, DBP, pulse pressure, smoking pack-years, log TG, TC,
TABLE 2. Correlations Between Lag Time for LDL Oxidation and Related Variables: Multivariate Analysis of Predictors Affecting Lag Time for LDL Oxidation

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th>( r )</th>
<th>( \beta )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>148</td>
<td>-0.10</td>
<td>-0.01</td>
</tr>
<tr>
<td>Sex (male = 1, female = 0)</td>
<td></td>
<td>-0.12</td>
<td>0.06</td>
</tr>
<tr>
<td>BMI</td>
<td>148</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Pulse pressure</td>
<td>142</td>
<td>-0.23†</td>
<td>-0.17</td>
</tr>
<tr>
<td>Smoking pack-years</td>
<td>142</td>
<td>-0.23†</td>
<td>NE</td>
</tr>
<tr>
<td>TC</td>
<td>142</td>
<td>-0.32†</td>
<td>-0.23*</td>
</tr>
<tr>
<td>Log TG</td>
<td>148</td>
<td>-0.38‡</td>
<td>NE</td>
</tr>
<tr>
<td>ApoB</td>
<td>148</td>
<td>-0.33‡</td>
<td>NE</td>
</tr>
<tr>
<td>LDL size</td>
<td>148</td>
<td>0.41‡</td>
<td>0.22*</td>
</tr>
<tr>
<td>( \alpha )-Tocopherol in LDL</td>
<td>148</td>
<td>0.15</td>
<td>0.32†</td>
</tr>
<tr>
<td>( \beta )-Carotene in LDL</td>
<td>148</td>
<td>0.20*</td>
<td>NE</td>
</tr>
<tr>
<td>Retinol in LDL</td>
<td>148</td>
<td>-0.23*</td>
<td>-0.14</td>
</tr>
<tr>
<td>Adjusted ( R^2 )</td>
<td></td>
<td></td>
<td>0.242</td>
</tr>
</tbody>
</table>

NE indicates did not enter final model. Correlation coefficient, \( r \), was adjusted for family number. Family number, age, and sex were forced into the multivariate model.

\* \( P<0.05 \), † \( P<0.01 \), ‡ \( P<0.001 \).

LDL cholesterol, ratio of TC to HDL cholesterol, apoB, LDL particle size, lag time for LDL oxidation, \( \alpha \)-tocopherol, and \( \alpha \)-tocopherol in LDL (Table 3). Correlations with SBP, pulse pressure, log TG, TC, apoB, and \( \alpha \)-tocopherol in LDL were weaker after adjustment for family number, age, BMI, and sex. However, the negative correlations with both LDL particle size \( (r=-0.22, P=0.008) \) and lag time for LDL oxidation \( (r=-0.20, P=0.017) \) were less affected by the adjustments. Notably, carotid mean IMT correlated significantly with lag time for LDL oxidation in affected \( (r=-0.310, P=0.007) \) but not in nonaffected \( (r=-0.152, P=NS) \) family members. Likewise, carotid mean IMT correlated significantly with lag time for LDL oxidation in affected \( (r=-0.268, P=0.024) \) but not in nonaffected \( (r=-0.102, P=NS) \) family members.

Figure 2. Relationship between carotid mean IMT and LDL size (A) and lag time for LDL oxidation (B). Significant inverse correlation existed between carotid mean IMT and LDL size in affected \( P<0.05 \) family members. Likewise, carotid mean IMT correlated significantly with lag time for LDL oxidation in affected \( P<0.01 \) family members. Notably, carotid mean IMT correlated significantly with lag time for LDL oxidation in affected \( P<0.001 \) family members but not in nonaffected family members (LDL size: \( r=-0.152, P=0.205 \); lag time for LDL oxidation: \( r=-0.102, P=0.400 \)).

Multivariate analysis was used to identify independent determinants of mean IMT (Table 4). In model 1, which included clinical variables, antioxidants in LDL, and lag time for LDL oxidation, mean IMT was independently associated with age, sex, and \( \alpha \)-tocopherol in LDL but not with lag time for LDL oxidation and \( \beta \)-carotene in LDL. In model 2, which included clinical and lipid variables and LDL particle size, mean IMT was independently associated with age, sex, pulse pressure, and LDL particle size. In model 3, which included...
TABLE 4. Multivariate Analyses of Predictors Affecting Mean IMT

<table>
<thead>
<tr>
<th>Independent Variables</th>
<th>Model 1</th>
<th></th>
<th>Model 2</th>
<th></th>
<th>Model 3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \beta )</td>
<td>( P )</td>
<td>( \beta )</td>
<td>( P )</td>
<td>( \beta )</td>
<td>( P )</td>
</tr>
<tr>
<td>Age</td>
<td>0.805</td>
<td>&lt;0.001</td>
<td>0.697</td>
<td>&lt;0.001</td>
<td>0.721</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sex (male=1, female=0)</td>
<td>0.171</td>
<td>0.002</td>
<td>0.101</td>
<td>0.035</td>
<td>0.150</td>
<td>0.006</td>
</tr>
<tr>
<td>BMI</td>
<td>NE</td>
<td>NS</td>
<td>NE</td>
<td>NS</td>
<td>−0.076</td>
<td>NS</td>
</tr>
<tr>
<td>Smoking pack-years</td>
<td>NE</td>
<td>NS</td>
<td>0.075</td>
<td>NS</td>
<td>0.058</td>
<td>NS</td>
</tr>
<tr>
<td>Pulse pressure</td>
<td>0.263</td>
<td>&lt;0.001</td>
<td>0.199</td>
<td>&lt;0.001</td>
<td>0.199</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TC/HDL ratio</td>
<td>−0.115</td>
<td>NS</td>
<td>−0.190</td>
<td>NS</td>
<td>−0.190</td>
<td>NS</td>
</tr>
<tr>
<td>ApoB</td>
<td>0.087</td>
<td>NS</td>
<td>0.171</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL size</td>
<td>−0.108</td>
<td>0.045</td>
<td>−0.199</td>
<td>0.003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL lag time</td>
<td>−0.090</td>
<td>NS</td>
<td>0.022</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \alpha )-Tocopherol in LDL</td>
<td>0.182</td>
<td>0.002</td>
<td>NE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \beta )-Carotene in LDL</td>
<td>0.100</td>
<td>NS</td>
<td>0.055</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adjusted ( R^2 )</td>
<td>0.722</td>
<td>&lt;0.001</td>
<td>0.752</td>
<td>&lt;0.001</td>
<td>0.790</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

NE indicates did not enter the final model. Family number was forced into each model. TC and log TG were tested in models 2 and 3 but did not enter the final models.

Discussion

To the best of our knowledge, this is the first study to investigate the association between carotid artery IMT and both LDL particle size and the susceptibility of LDL to oxidation in vitro in asymptomatic FCHL family members. Our findings demonstrate that LDL particle size but not the susceptibility of LDL to oxidation in vitro is independently associated with mean IMT in asymptomatic affected FCHL family members. The data suggest that LDL particle size is superior to conventional lipid values in describing cardiovascular risk in FCHL family members.

The predominance of small, dense LDL particles is an inherent feature of FCHL. Whether small, dense LDL in FCHL is a genetically determined trait or a consequence of lipid abnormalities is still debated. In the present study, the affected FCHL family members had consistently smaller LDL particle size compared with nonaffected family members. Numerous studies have shown the association between LDL particle size and the risk of CAD. Recently, LDL particle size has been reported to be associated with carotid IMT in middle-aged healthy subjects in 2 studies. In our asymptomatic affected FCHL family members, LDL particle size correlated significantly with mean IMT even after adjustment for family number, age, BMI, and sex. Importantly, our results from multivariate analysis demonstrate that LDL particle size is independently associated with mean IMT in FCHL.

Small, dense LDL particles are more prone to oxidation than large buoyant LDL particles. In this study, we showed that increased susceptibility of LDL to oxidation in vitro is related to the presence of small, dense LDL. In the multivariate analysis, lag time for LDL oxidation was associated with LDL particle size, TC, and \( \alpha \)-tocopherol in LDL independently of log TG, apoB, and clinical variables. AFFECTED FCHL family members had higher absolute plasma \( \alpha \)-tocopherol levels as a result of higher lipid-soluble antioxidant-carrying capacity caused by an elevation in LDL cholesterol. Nevertheless, the ratio of plasma \( \alpha \)-tocopherol to TC (data not shown) and \( \alpha \)-tocopherol in LDL did not differ between affected and nonaffected family members. Therefore, LDL particle size seems to play more important role in the increased susceptibility of LDL to oxidation in vitro than the LDL-associated antioxidants in FCHL.

Oxidative modification of LDL plays a key role in the development of atherosclerosis. Available studies on the relationship between atherosclerotic disease and autoantibodies against oxidized LDL have given inconsistent results. So far, few studies have investigated the association between LDL oxidizability and the extent of arterial atherosclerosis. Oxidized LDL has been reported to be associated with CAD in a small-scale study and with progression of carotid atherosclerosis in a 3-year follow-up study. In contrast, LDL oxidizability was not associated with carotid atherosclerosis or was only marginally associated with the extent of coronary or peripheral atherosclerosis. The differences in the stages of atherosclerosis and study designs may account for the differences in study results. Also, the assessment of LDL oxidation in vitro is problematic, and the method may affect the findings. Unfortunately, in these studies, LDL particle size was not measured.

Small, dense LDL particles penetrate into the extracellular space more easily and have high binding affinity with proteoglycans. This process promotes LDL modification in.
the arterial wall. Small, dense LDL also has a low binding affinity to the LDL receptor and reduced hepatic clearance, resulting in prolongation of its residence time in the subendothelial space, a prooxidative environment. Modified LDL is able to initiate several processes that further enhance the development of atherosclerosis. Thus, the size of LDL particles may play a crucial role in LDL oxidation and the consequent atherosclerotic process in FCHL. Therefore, it is not surprising that the susceptibility of LDL to oxidation in vitro is not an independent risk factor of atherosclerosis in FCHL but is overshadowed by LDL particle size. However, both measurements may reflect to some extent the same characteristic of LDL.

In conclusion, our results suggest that LDL particle size is independently associated with carotid IMT in asymptomatic FCHL family members. On the other hand, the susceptibility of LDL to oxidation in vitro is closely related to LDL particle size but has no independent association with carotid IMT. Therefore, we conclude that LDL particle size is an important diagnostic indicator for CAD risk in FCHL.

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References

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