Antibody Titers Against Oxidized LDL Are Not Elevated in Patients With Familial Hypercholesterolemia

Johannes Hulthe, John Wikstrand, Aira Lidell, Inger Wendelhag, Göran K. Hansson, Olov Wiklund

Abstract—Antibodies against oxidized low density lipoproteins (Ox-LDLs) have been proposed to be independent predictors of atherosclerosis development. The main aims of the current study were to (1) compare antibody titers to Ox-LDL in patients with heterozygous familial hypercholesterolemia (n=51) with those in matched controls (n=45) and (2) analyze whether the antibody titers were related to the extent of atherosclerosis, as assessed cross-sectionally and prospectively by ultrasonography in the 2 study groups. Antibody titers were determined with a solid-phase ELISA, and plates were coated with the antigens Ox-LDL or malondialdehyde-treated LDL (MDA-LDL) as well as with the postcoat only (5% dry milk powder). Antibody titers were expressed as absorbance [(value in patient serum minus that in postcoat) divided by (Internal Standard Serum minus postcoat)]. There were no significant differences in antibody titers against Ox-LDL or MDA-LDL between the group of patients with familial hypercholesterolemia and the controls. In cross-sectional comparisons, no significant associations were observed between the intima-media thickness of the carotid or femoral arteries and antibody titers against Ox-LDL or between plaque occurrence and these titers. Patients with a history of myocardial infarction had significantly lower IgM titers against Ox-LDL compared with patients without a history of myocardial infarction and with controls. In conclusion, mean values for antibody titers against Ox-LDL were not increased in the patient group compared with a healthy control group, and no positive, significant relationship was observed between antibody titers and the extent of atherosclerosis, as measured by ultrasound, in the carotid or femoral arteries. Taken together, these findings indicate that the relationship between the autoimmune response to Ox-LDL and the extent of atherosclerosis is more complex than previously anticipated. (Arterioscler Thromb Vasc Biol. 1998;18:1203-1211.)

Key Words: antibody titers ■ familial hypercholesterolemia ■ oxidized LDL

Immune mechanisms have been suggested by several investigators to play a key role in atherosclerosis development. Several antigens have been suggested to be involved in this immune reaction. Recently, research in this field has been partly focused on the role of modified lipoproteins, primarily oxidized (Ox) LDLs. Several lines of evidence support the concept that Ox-LDL may be a key antigen in atherosclerosis. T-cell clones responsive to Ox-LDL have been isolated from human lesions. Immune responses to Ox-LDL have also been observed in apoE knockout mice, an animal model for atherosclerosis development. Furthermore, antibodies against epitopes of Ox-LDL have been found in several studies in both human plasma and in atherosclerotic lesions. A relationship between circulating antibodies against Ox-LDL and atherosclerotic disease has, however, not been unequivocally shown. There are case-control studies suggesting an elevated antibody titer against Ox-LDL in patients with various manifestations of atherosclerotic disease. High titers of antibodies have also been found to be independent predictors of the progression of carotid atherosclerosis. In other recent studies, however, no such relationships have been found between atherosclerotic disease and antibody titers.

The development of the B-mode ultrasound technique has made it possible to study these relations at an early and, in many cases, a preclinical stage of atherosclerosis development. This method also makes it possible to noninvasively follow the development of the disease. An increased intima-media thickness (IMT) is used as a marker of generalized atherosclerosis, including coronary atherosclerosis. We have reported on earlier ultrasound studies in patients with familial hypercholesterolemia (FH) that show an increased IMT, as well as an increased occurrence of plaques, in carotid and femoral arteries.

The main aims of the current study were to (1) compare antibody titers to Ox-LDL in patients with heterozygous FH (n=51) with those in matched controls (n=45) and (2) analyze whether the antibody titers were related to the extent of atherosclerosis, as assessed cross-sectionally and prospectively by ultrasonography in the 2 study groups.
TABLE 1. Anthropometric Data, Blood Pressure (BP), Heart Rate (HR), Serum Lipids and Lipoproteins, and Smoking Habits of Study Participants at the 5-Year Examination

<table>
<thead>
<tr>
<th>Variable</th>
<th>FH (n=51)</th>
<th>Controls (n=45)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>56.9±12.0</td>
<td>56.8±11.8</td>
</tr>
<tr>
<td>Male, n</td>
<td>30</td>
<td>26</td>
</tr>
<tr>
<td>Female, n</td>
<td>21</td>
<td>19</td>
</tr>
<tr>
<td>Body height, cm</td>
<td>173±10</td>
<td>173±8</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>76.1±16.9</td>
<td>73.6±12.3</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25.5±4.7</td>
<td>24.7±3.6</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>129±17</td>
<td>128±20</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>78±10</td>
<td>79±10</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>60±10</td>
<td>60±8</td>
</tr>
<tr>
<td>Serum cholesterol, mmol/L</td>
<td>6.78±1.42</td>
<td>5.15±0.81</td>
</tr>
<tr>
<td>Serum triglycerides, mmol/L</td>
<td>1.81±1.04</td>
<td>1.33±0.53</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.25±0.39</td>
<td>1.24±0.32</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>4.76±1.35</td>
<td>3.33±0.78</td>
</tr>
<tr>
<td>Never smoked, %</td>
<td>31</td>
<td>57</td>
</tr>
<tr>
<td>Past smoker, %</td>
<td>47</td>
<td>28</td>
</tr>
<tr>
<td>Current smoker, %</td>
<td>22</td>
<td>15</td>
</tr>
<tr>
<td>Cigarette-years, mean</td>
<td>250±274</td>
<td>185±327</td>
</tr>
</tbody>
</table>

BMI indicates body mass index; SBP, systolic blood pressure; and DBP, diastolic blood pressure. Values are n, percent, or mean±SD.

Methods

Study Groups: Main Study

FH

Patients with heterozygous FH (Table 1) were recruited from a prospective, observational ultrasound study of carotid and femoral arteries conducted at the lipid clinic of Sahlgrenska University Hospital, Göteborg, Sweden. The diagnosis of FH was based on prespecified criteria. During follow-up, all but 3 patients underwent cholesterol-lowering therapy with cholestyramine (n=12), pravastatin (n=19), pravastatin combined with other lipid-lowering agents (n=14), or simvastatin (n=3). Eighteen patients had a history of myocardial infarction (MI).

Control Group

The subjects in the control group were recruited from the same observational study as referred to above (Table 1). Control subjects had been matched with regard to sex, age, height (±10 cm), and weight (±7 kg). Age matching was within ±5 years except in 2 cases (±6 and ±7 years). All control subjects had serum cholesterol values <6.5 mmol/L when recruited, and they were recruited from a representative population sample in Göteborg. Subjects in this sample with a history of MI (n=3) were excluded, and another 3 declined to participate in follow-up investigations.

Patients and controls were followed up for 5 years. Data relating to group comparisons of cross-sectional data were mainly from the 5-year examination, although data from the 4-year examination were used for 8 patients and 4 controls because of missing 5-year values. In all, 51 patients and 45 control subjects were included in the cross-sectional analysis. Seventy-six subjects had analyzable samples from both the 4- and 5-year examinations, which could be used to study the long-term variability in antibody titers (see below). Prospective data from the ultrasound study were related to differences observed in ultrasound data between the 3- and 5-year follow-up examinations.

Study Group: Prestudy Variability Experiments

Before the main study on antibody titers was commenced, some prestudy variability experiments were performed. Blood samples from healthy controls [n=33; 18 men and 15 women with a mean age of 62.2 years (range, 29 to 72)] were used in these experiments. Samples originated from an ongoing screening examination at the Wallenberg Laboratory.

Lipoprotein Preparation

LDL (1.019 to 1.063 g/mL) was prepared from pooled plasma from 2 healthy human donors by sequential ultracentrifugation in the presence of 0.2% disodium EDTA. The isolated lipoprotein was extensively dialyzed against PBS (0.14 mol/L NaCl and 0.01 mol/L PBS) containing 0.1 mmol/L disodium EDTA, 2.5 μmol/L 4,12-aminooctadecylbenzenesulfonyl fluoride, and 5 mM penicillin-streptomycin (PEST), pH 7.4. LDL was sterile filtered, and the protein content was determined by the method of Lowry et al.

Malondialdehyde-treated (MDA)-LDL was prepared as described by Palinski et al. Ox-LDL was prepared by oxidation of LDL in the presence of 5 μmol/L CuSO₄ for 13 hours at 37°C. As a routine procedure, modifications were checked by controlling the electrophoretic mobility in agarose gel of the modified lipoproteins.

Determination of Antibody Titers Against Modified Lipoproteins

Antibody titers were determined with a solid-phase ELISA, essentially as described by Palinski and coworkers. Disposable 96-well microtitration plates (Sero-Wel) were used. The wells were coated with native (Nat)-LDL, Ox-LDL, or MDA-LDL as the antigens, which were diluted to a protein concentration of 5 μg/mL in phosphate buffer (pH 7.4) containing 2.7 mmol/L EDTA and 20 μmol/L BHT; 50 μL of antigen preparation was added to each well, equivalent to 5 mL per plate. After 24 hours at 4°C, the plates were postcoated with 5% dry milk powder (Semper AB Stockholm) at a concentration of 50 g/L in phosphate buffer for 1 hour, washed 3 times, and then stored frozen until used. Fifty microliters of serum at appropriate dilution (1/2500 and 1/6.25 for IgG and IgM, respectively) was added to the wells and incubated overnight at 4°C. After the first incubation, the plates were aspirated and washed 4 times.

Alkaline phosphatase–conjugated secondary antibodies specific for IgG (DAKO DO 336) and IgM (DAKO DO 337) (50 μL per well, diluted 1/1000 and 1/2000, respectively) were added to the wells and incubated at room temperature for 2 hours. After another 4 washes, 50 μL of phosphatase substrate (Sigma Diagnostic 104) was added to each well (1 mg/mL) and incubated (incubator 500, Organon Teknika) at a constant temperature of 37°C for 30 minutes.

The absorbance was measured at 405 nm in a kinetic automatic microplate reader (MAXline, Molecular Devices). Serum samples were run in quadruplicate. For the determination of background absorbance, plates with only the postcoat were run each time together with plates coated with the antigens Nat-LDL, Ox-LDL, or MDA-LDL. The background value was subtracted from the absorbance of the samples. For each batch of plates, the appropriate serum dilution was determined by running a dilution series of an internal standard serum. The serum dilutions selected were within the linear range of the titration curve.

On each plate an internal standard serum was also run, and the titer was expressed as the ratio between the average absorbance for each sample and the internal standard serum. All blood samples from the main study, including patients with FH and controls, were run on the same batch of plates. Titer was calculated as absorbance of (patient serum minus postcoat) divided by (internal standard serum minus postcoat). For IgG, the postcoated wells gave no absorbance; therefore, this correction was made for IgM only.

Internal Antibody Titer Standard

Because all samples from the study could not be run on the same plate and plates might differ somewhat in coating characteristics, there was a need for an internal standard. Onto each plate, therefore, 2 different internal standard serum samples were added. The absorbances for these 2 samples, named internal control sample (ICS) and internal standard sample (ISS), were used to calculate the ratio of ICS to ISS, which was used as the internal antibody titer standard.
Measures Taken After the Prestudy Variability Experiments

The data from the prestudy variability experiments referred to above indicated that the variability for the measurement of Ox-LDL IgG antibody titers was unsatisfactory. This occurred despite the fact that all patient samples had been normalized against the internal standard serum (PS/ISS). Because outliers had been identified in the analyses of ICS/ISS data in these prestudy experiments, the following decisions were made before samples in the main study were run: if the absorbance of ISS was outside the 90% confidence interval (CI), calculated on the basis of all readings of the internal standard in the experiment, then the entire plate should be reanalyzed. The plate should also be reanalyzed if the ratio of ICS to ISS was outside the 90% CI.

In the main study, SDs for the mean value of the ICS-ISS (ratio ie, internal antibody titer standard) from all plates were 0.19 and 0.09 for Ox-LDL IgG and MDA-LDL IgG titers, respectively, before reanalysis. That is, SDs for the main study were comparable to SDs in the prestudy variability experiments. When prespecified criteria for reanalysis were applied, SDs for the ICS-ISS ratio were 0.05 and 0.06 for Ox-LDL IgG and MDA-LDL IgG titers, respectively. Therefore, for use of these measures in the main study for patients with FH and controls, the data indicated that the between-plate variability was also satisfactory for IgG titers.

Displacement Experiments

To study the specificity of the antibodies bound to LDL or modified LDL, a series of displacement experiments were performed. One milliliter of diluted Nat-LDL, Ox-LDL, or MDA-LDL (in concentrations as indicated in Figures 2 and 3) was added to 1 mL of standard serum (ISS) and incubated for 1 hour at room temperature.

The samples were then added to plates coated with Nat-LDL, Ox-LDL, or MDA-LDL. Addition of Nat-LDL to serum did not displace the antibody binding to the plates coated with Nat-LDL (IgM titer), Ox-LDL, or MDA-LDL (Figures 2 and 3). Some displacement was seen for the IgG titer (Figure 3). Addition of Ox-LDL to serum displaced the antibody binding to plates coated with Ox-LDL and Nat-LDL (Figures 2 and 3). Addition of MDA-LDL to serum also displaced antibody binding to plates coated with Nat-LDL and MDA-LDL (Figures 2 and 3).

These data suggested that there was a modification of Nat-LDL during the coating process, which means that plates coated with Nat-LDL also included oxidized epitopes. We therefore did not use Nat-LDL as a blank but rather used only postcoated plates as the blanks (for further comments, see the Discussion section).

Biochemical Analysis

Blood samples for serum cholesterol, serum triglycerides, and lipoprotein fractions were drawn after a fasting period of 10 to 12 hours. Cholesterol and triglyceride levels were determined by fully enzymatic techniques. HDL was determined after precipitation of apoB-containing lipoproteins with MnCl₂ and heparin. LDL cholesterol was calculated as described by Friedewald et al.

Ultrasongraphy

Examination was performed with an ultrasound scanner (Acuson 128) equipped with a 7-MHz linear transducer and a transducer aperture of 38 mm, as previously described in detail. The examination included ~2 cm of the right common carotid artery, the carotid bulb, and 1 cm of the internal and external carotid arteries. The right femoral artery was scanned ~4 cm proximal and 1 cm distal to the flow divider. If a plaque was present, 3 separate, “frozen” B-mode images of the thickest part of the plaque in the longitudinal view were recorded on videotape. Pulsed Doppler was used to provide information on the velocity of blood flow. Images for IMT measurements were recorded (frozen on top of the R wave on the ECG) from each of the following 3 arterial segments: the carotid bulb, common carotid artery, and common femoral artery.
Measurement of IMT

The ultrasound images were analyzed in a computerized analyzing system, as previously described in detail. IMT was defined as the distance from the leading edge of the lumen-intima interface to the leading edge of the media-adventitia interface of the far wall. The computer program calculated the average thickness along a 10-mm-long section (IMTmean) and also the maximum thickness of the analyzed section (IMTmax). Measurements in the common femoral artery were made in a similar way as for the carotid artery but along a 15-mm-long section proximal to the bifurcation. The mean of 3 separately analyzed images in the common carotid artery, carotid bulb, and common femoral artery were used in the analyses.

Assessment of Plaque Occurrence

A semiquantitative subjective scale was used to grade the size of plaques into no plaques, small plaques, and moderate to large plaques. This analysis included plaques in the near wall as well as the far wall of the vessel. A plaque was defined as a distinct area with an IMT >50% thicker than the neighboring sites (as judged visually).

Statistical Analysis

All statistics were analyzed by using SPSS for Windows 6.1. The measurement variation (s) was defined as SD/√2, where SD was calculated for the mean difference between the 2 samples. Nonparametric Spearman’s rank correlation test was used in the correlation analysis, with the relationships illustrated with Pearson’s correlation coefficient (r). For comparison between groups, the Mann-Whitney U test was used. Furthermore, a t-distributed variable was used to calculate 95% CIs for differences. The difference in distribution for plaque occurrence and sex was tested with a χ² test. Mantel’s test was used to test the correlation between IgM titer against Ox-LDL and a history of previous MI when the influence of sex was eliminated. Because of the large number of correlations performed, the level of significance for these analyses was set at P<0.01.
sided). For all other situations, $P<0.05$ (2 sided) was regarded as statistically significant.

## Results

### Comparison of 4- and 5-Year Antibody Titers in the Merged Group of FH Patients and Controls

Forty patients and 36 controls had antibody titer measurements available from both the 4- and 5-year examinations. The correlation coefficients for the relations between the 4- and 5-year measurements were $r_{5}=0.79$ and $r_{5}=0.83$, respectively, for IgG and IgM titers against MDA-LDL and $r_{5}=0.87$ and $r_{5}=0.77$, respectively, for IgG and IgM titers against Ox-LDL (Figure 4).

### Relationship Between Different Antibody Titers

In this analysis the 2 groups were analyzed together, and the correlation coefficients for the relations between antibody titers against MDA-LDL, Ox-LDL, and Nat-LDL are presented in Table 2. The relatively high correlation between titers against Nat-LDL and Ox-LDL is probably due to the oxidative modification of Nat-LDL on the plates during the coating process.

### Antibody Titers in the 2 Study Groups

No significant differences in means were found between the group of patients with FH and the control group for any of the antibody titers studied (Table 3). When the 2 groups were analyzed together, women had a significantly higher IgM titer against Ox-LDL (1.17, $n=40$) than men (1.03, $n=56$; 95% CI for the difference, 0.04 to 0.24, $P<0.05$). No significant difference in mean values between sexes was observed when the 2 study groups were analyzed separately.

### Antibody Titers Against Ox-LDL in Relation to Serum Cholesterol and Lipoproteins

In the merged group of patients and controls, serum cholesterol, LDL, HDL, and triglycerides were not significantly associated with IgG and IgM titers against Ox-LDL ($P>0.01$).

### IMT and Plaque Occurrence in the 2 Study Groups

There were significant differences in IMT between patients and controls for all arterial segments studied: the common carotid artery, carotid artery bulb, and common femoral artery (Table 4). However, there were no significant differences between patients and controls regarding change in IMT during 2 years of follow-up (Table 4). This may be explained by the fact that patients with FH were treated with cholesterol-lowering drugs. Moderate to large plaques in the carotid artery were observed in 14 patients and 3 controls ($P<0.01$). Moderate to large plaques in the femoral artery were observed in 22 patients and 4 controls ($P<0.001$).

IMT in the common femoral artery was significantly thicker in patients with a history of MI than in patients without a history of MI ($P=0.02$). Otherwise, no significant differences in IMT between the 2 subgroups with FH were observed.

### Antibody Titers in Relation to IMT and Plaque Occurrence

In the merged group of patients and controls or the 2 groups separately, antibody titers (both IgG and IgM) for Nat-LDL, Ox-LDL, and MDA-LDL were tested against IMT$_{mean}$ and IMT$_{max}$ in 3 peripheral arterial regions (the common carotid artery, carotid bulb, and common femoral artery). No significant associations were found. All subjects, patients and controls, with no plaques in the carotid

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**TABLE 2. Correlation Coefficients for the Relations Between IgM and IgG Titers (n=96)**

<table>
<thead>
<tr>
<th></th>
<th>FH+Controls</th>
<th>IgM Titer</th>
<th>IgG Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Correlation Versus</td>
<td>Nat-LDL $r$</td>
<td>Ox-LDL $r$</td>
</tr>
<tr>
<td>IgG titer</td>
<td>Nat-LDL</td>
<td>0.09</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>Ox-LDL</td>
<td>...</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>MDA-LDL</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>IgM titer</td>
<td>Ox-LDL</td>
<td>0.68</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>MDA-LDL</td>
<td>0.64</td>
<td>...</td>
</tr>
</tbody>
</table>

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Figure 4. Scatterplots showing long-term, within-patient variation, illustrated as relationships between IgM titer against Ox-LDL at 4- and 5-year examinations (left) and between IgG titer against Ox-LDL at 4- and 5-year examinations (right). Within-patient variation (s) was calculated as $SD/\sqrt{2}$, where $SD$ was calculated for mean differences between samples.
or femoral arteries were tested against those who had moderate to large plaques in these arteries. No significant differences in antibody titers were found (Figure 5).

Antibody Titers Against Ox-LDL in Relation to Change in IMT During 2 Years of Follow-up

In the merged group of patients and controls or in the 2 groups separately, antibody titers (both IgG and IgM) for Ox-LDL and MDA-LDL were tested against the 2-year change in IMT in the common carotid artery, carotid bulb, and common femoral artery. No significant associations were observed.

Antibody Titer Against Ox-LDL in Relation to a History of MI

Patients with a history of MI (n = 18) had a significantly lower IgM titer against Ox-LDL than did patients as well as controls without a history of MI (P < 0.05, Figure 6). There was still a significant association between IgM titer against Ox-LDL and MI status when the influence of sex was eliminated (P = 0.03).

Discussion

The results of this study showed that mean values of antibody titers against Ox-LDL were no higher in the group of patients with FH than in a group of healthy controls, and no positive, significant relationship was observed between antibody titers and the extent of atherosclerosis, as measured by ultrasound, in the carotid or femoral arteries.

The methodological studies showed a good short-term (weeks) and long-term (1 year) reproducibility with a low within-patient variation for both IgG and IgM titers when measured on the same plate. Therefore, the lack of difference between the group of patients with FH and the control group does not seem to be due to poor reliability of the method.

### TABLE 3. Antibody Titers in the 2 Study Groups

<table>
<thead>
<tr>
<th></th>
<th>FH (n = 51) Mean ± SD</th>
<th>Controls (n = 45) Mean ± SD</th>
<th>Difference Between Groups</th>
<th>95% CI for Differences</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG Nat-LDL</td>
<td>0.81 ± 0.19</td>
<td>0.85 ± 0.18</td>
<td>0.04</td>
<td>-0.03 to 0.12</td>
</tr>
<tr>
<td>Ox-LDL</td>
<td>0.92 ± 0.25</td>
<td>0.93 ± 0.21</td>
<td>0.01</td>
<td>-0.08 to 0.10</td>
</tr>
<tr>
<td>MDA-LDL</td>
<td>0.95 ± 0.15</td>
<td>0.93 ± 0.11</td>
<td>-0.02</td>
<td>-0.07 to 0.03</td>
</tr>
<tr>
<td>Ox-LDL/Nat-LDL (ratio)</td>
<td>1.16 ± 0.26</td>
<td>1.11 ± 0.23</td>
<td>-0.05</td>
<td>-0.15 to 0.05</td>
</tr>
<tr>
<td>MDA-LDL/Nat-LDL (ratio)</td>
<td>1.23 ± 0.30</td>
<td>1.13 ± 0.24</td>
<td>-0.10</td>
<td>-0.20 to 0.02</td>
</tr>
<tr>
<td>IgM Nat-LDL</td>
<td>1.08 ± 0.37</td>
<td>1.05 ± 0.28</td>
<td>-0.03</td>
<td>-0.16 to 0.11</td>
</tr>
<tr>
<td>Ox-LDL</td>
<td>1.09 ± 0.29</td>
<td>1.09 ± 0.23</td>
<td>0.00</td>
<td>-0.10 to 0.11</td>
</tr>
<tr>
<td>MDA-LDL</td>
<td>1.28 ± 0.35</td>
<td>1.27 ± 0.28</td>
<td>-0.01</td>
<td>-0.15 to 0.11</td>
</tr>
<tr>
<td>Ox-LDL/Nat-LDL (ratio)</td>
<td>1.06 ± 0.29</td>
<td>1.09 ± 0.23</td>
<td>0.03</td>
<td>-0.09 to 0.13</td>
</tr>
<tr>
<td>MDA-LDL/Nat-LDL (ratio)</td>
<td>1.24 ± 0.28</td>
<td>1.27 ± 0.35</td>
<td>0.03</td>
<td>-0.11 to 0.15</td>
</tr>
</tbody>
</table>

Antibody titers were calculated as absorbances for serum samples divided by the absorbance for the standard serum measured on each plate. Mean values for the ratio between antibody titers against Ox-LDL and MDA-LDL versus Nat-LDL are also shown.

### TABLE 4. Ultrasound Variables in the 2 Study Groups

<table>
<thead>
<tr>
<th>IMT at Measurement of Antibody Titers</th>
<th>3- to 5-Year Increase ΔIMT, mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls Mean ± SD (n = 45)</td>
</tr>
<tr>
<td>--------------------------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>Common carotid artery</td>
<td></td>
</tr>
<tr>
<td>Mean, mm</td>
<td>0.75 ± 0.16</td>
</tr>
<tr>
<td>Max, mm</td>
<td>0.88 ± 0.18</td>
</tr>
<tr>
<td>Carotid artery bulb†</td>
<td></td>
</tr>
<tr>
<td>Mean, mm</td>
<td>0.86 ± 0.26</td>
</tr>
<tr>
<td>Max, mm</td>
<td>1.16 ± 0.38</td>
</tr>
<tr>
<td>Common femoral artery‡</td>
<td></td>
</tr>
<tr>
<td>Mean, mm</td>
<td>0.93 ± 0.51</td>
</tr>
<tr>
<td>Max, mm</td>
<td>1.19 ± 0.68</td>
</tr>
</tbody>
</table>

*One patient did not attend the ultrasound examination.
†Cross-sectional data missing in 2 patients and 2 controls and prospective data missing in 5 patients and 6 controls (anatomic reasons).
‡Cross-sectional data missing in 4 patients and 1 control and prospective data missing in 6 patients and 1 control (anatomic reasons).
The method for antibody determination used was essentially the same as that used by several other investigators. However, we preferred to express the titers as the ratio to a standard serum, which was included in each analysis. Nonspecific binding was corrected for by subtracting absorbance in postcoated wells only. Others have used the ratio between binding to Ox-LDL and binding to Nat-LDL as a titer of specific antibodies. In our methodological studies, we observed, however, that plates coated with “Nat” LDL probably exposed epitopes common with modified LDL. This suggests that at least in our experience, LDL is to some extent modified during the coating process, despite the presence of both EDTA and BHT. Oxidative modification may explain why antibody titers against Nat-LDL were associated with titers against Ox- and MDA-LDL. Under these circumstances, we found it unreliable to use the ratio to Nat-LDL to express the antibody titers, although using this ratio would not have changed our main results (see Table 2). Unfortunately, other investigators have not presented data on the degree of oxidation of Nat-LDL in their experiments.

Elevated antibody titers against Ox-LDL or MDA-LDL have been suggested to be independent markers for the progression of atherosclerotic disease and specifically for coronary heart disease. However, available studies are inconsistent and to some extent contradictory. Elevated titers in patients with coronary heart disease, peripheral vascular disease, and hypertension have been found in several studies. On the other hand, in a recently published study, Uusitupa and coworkers concluded that antibody titers against Ox-LDL did not seem to be associated with excess cardiovascular mortality, morbidity, or IMT of the carotid artery, as observed during long-term follow-up. In a previous study by Van de Vijer and coworkers, no significant association was found between antibody titer against Ox-LDL and the extent of coronary atherosclerosis. Our results corroborate results from these later studies. In a post hoc analysis we found significantly lower titers of IgM against Ox-LDL in patients with a history of MI. One might speculate that these results would rather suggest that antibodies against Ox-LDL could have a protective effect.

Although immune mechanisms are known to be present in the atherosclerotic lesion, the role of the immune system in atherosclerosis is still unclear. Cell-mediated immune responses by macrophages and T lymphocytes occur in lesions in both humans and experimental animal models. These are to a large extent caused by specific CD4 T cells responding to Ox-LDL. This suggests that Ox-LDL is an important local antigen in atherosclerosis and that such local T-cell responses may induce B-cell activation, with concomitant systemic antibody production.

The recent development of genetic models of atherosclerosis in mice has permitted dissection of the precise role of such responses. Therefore, mice that are both atherosclerosis prone due to targeted gene deletion of apoE and that lack mature macrophages due to the op/op mutation develop significantly less atherosclerosis than do mice that lack apo E but contain macrophages. Similarly, apoE-deficient mice that cannot respond to the T-cell cytokine interferon-γ exhibit significantly reduced lesion formation. Together, these data imply that proinflammatory immune responses are important for the development of atherosclerosis.
However, the role of immunocompetent B and T cells is not as well clarified: a compound-mutant mouse lacking both B and T cells (RAG-1 knockout) and apoE develops significantly less atherosclerosis than do apoE single-knockout mice when fed standard chow.31 Surprisingly, this difference is eliminated when mice are fed a cholesterol-rich diet.32 This may indicate that the immune system plays a modifying role in atherosclerosis under more “normal” conditions. In severely proatherogenic, hypercholesterolemic situations, this role may be overshadowed by the proatherogenic lipoproteins. Such a differential role of the immune system in more normal versus more extreme conditions could explain the discrepancy between our finding, ie, a lack of correlation between antibody titers and the extent of atherosclerosis in FH, and those of others who have reported a correlation between titers and extent of disease in atherosclerotic patients not suffering from this particular metabolic disease.

Furthermore, the importance of anti–Ox-LDL responses could vary, depending on the stage of the disease. We have recently observed that apoE knockout mice develop strong, systemic anti–Ox-LDL responses in the phase of fatty streak formation, with gradual alleviation during the progression to advanced, fibrofatty lesions (X. Zhou et al, unpublished observations). Finally, the majority of patients in our study were treated with cholesterol-lowering drugs; it is not known whether these substances may affect antibody production against Ox-LDL. For ethical reasons, it is not possible to study patients with FH without lipid-lowering treatment.

In summary, our analysis of anti–Ox-LDL titers in patients with FH did not reveal any titer increase when compared with a control group. Furthermore, there was no association between antibody titer and the extent of atherosclerosis in the carotid or femoral artery as evaluated by ultrasonography. However, in a post hoc analysis, we found lower IgM titers in patients with a history of MI. It is evident that the role of antibodies with respect to modified lipoproteins in atherosclerosis is still a controversial issue that needs further investigation.

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Antibody Titers Against Oxidized LDL Are Not Elevated in Patients With Familial Hypercholesterolemia

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