

# Circulating Oxidized LDL Is Associated With Subclinical Atherosclerosis Development and Inflammatory Cytokines (AIR Study)

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**Objective**—Circulating oxidized LDL (Ox-LDL) is associated with clinical manifestations of atherosclerosis. However, no previous study has examined the relationship between subclinical atherosclerosis and Ox-LDL. The aims of the present study were to investigate the relationship between clinically silent ultrasound-assessed atherosclerotic changes in the carotid and femoral arteries and Ox-LDL and to explore the relationship between Ox-LDL, C-reactive protein, and the inflammatory cytokines interleukin-6 and tumor necrosis factor- $\alpha$ .

**Methods and Results**—The study group (n=391) consisted of clinically healthy, 58-year-old men recruited from the general population. Ox-LDL was measured by using a specific monoclonal antibody, mAb-4E6. The results showed that Ox-LDL was related to intima-media thickness and plaque occurrence in the carotid and femoral arteries. In addition, Ox-LDL was associated with tumor necrosis factor- $\alpha$  and C-reactive protein. Circulating Ox-LDL was also associated with LDL cholesterol but not with blood pressure or smoking. When adjusting for other risk factors, both LDL cholesterol and Ox-LDL seemed to be independent predictors of plaque occurrence in the carotid and femoral arteries (odds ratios for quintile 5 versus quintile 1 were 2.17,  $P=0.049$  and 2.25,  $P=0.050$ , for LDL cholesterol and Ox-LDL, respectively).

**Conclusions**—Ox-LDL was associated with both subclinical atherosclerosis and inflammatory variables, supporting the concept that oxidatively modified LDL may play a major role in atherosclerosis development, although no causality can be shown in this cross-sectional study. (*Arterioscler Thromb Vasc Biol.* 2002;22:1162-1167.)

**Key Words:** oxidized LDL ■ atherosclerosis ■ intima-media thickness ■ plaque ■ inflammation

Inflammation has been postulated to play an important role in atherosclerosis development.<sup>1</sup> Recently, research in this field has been focused on the role of modified lipoproteins, primarily oxidized LDL (Ox-LDL). Several lines of evidence support the concept that Ox-LDL may be a key antigen in atherosclerosis.<sup>2</sup> Ox-LDL as well as antibodies against epitopes of oxidized LDL have been found in several studies in both human and rabbit plasma and in atherosclerotic lesions.<sup>3-6</sup> Until recently it has not been possible to measure circulating Ox-LDL in human plasma. However, Holvoet et al<sup>7,8</sup> have in a number of studies shown that Ox-LDL is related to coronary artery disease in heart transplant patients, as well as in patients with established coronary artery disease.<sup>9,10</sup> Furthermore, Toshima et al<sup>11</sup> recently showed that Ox-LDL was higher in subjects with established coronary heart disease as compared with controls. These results were, however, obtained in selected patients at high risk. As yet, no population-based study has investigated the relationship between early atherosclerosis development and Ox-LDL.

A further role of Ox-LDL in atherosclerosis could be to initiate and affect inflammatory mediators such as C-reactive

protein (CRP), interleukin (IL)-6, and tumor necrosis factor (TNF)- $\alpha$ . A positive correlation between CRP and Ox-LDL in humans has been suggested.<sup>9</sup> In human mesangial cells, Ox-LDL has been shown to stimulate IL-6 expression.<sup>12</sup> Contrary to these results, an inhibitory effect of Ox-LDL on TNF- $\alpha$  expression in rat aortic smooth muscle cells has also been observed.<sup>13</sup> Hence, the results have not been consistent.

The development of the B-mode ultrasound technique has made it possible to noninvasively study the atherosclerotic process. Intima-media thickness (IMT) of the carotid artery has been used as a noninvasive indicator for the atherosclerotic process in the coronary arteries.<sup>14</sup> IMT of the carotid bulb and plaque occurrence and size in the carotid artery have also previously been shown to be associated with coronary atherosclerosis, as measured by coronary angiography.<sup>15</sup>

The aims of the present study were to investigate the relationship between clinically silent atherosclerosis, as measured by ultrasound in the carotid and femoral arteries and Ox-LDL and to explore the relationship among Ox-LDL, CRP, and the inflammatory cytokines IL-6 and TNF- $\alpha$ . These

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observations were made in clinically healthy, 58-year-old men recruited from the general population.

## Methods

### Study Groups

The inclusion criteria were male sex, age of 58 years, and Swedish ancestry. Exclusion criteria were cardiovascular disease (myocardial infarction, angina pectoris, stroke, intermittent claudication, aortic disease), clinical diabetes mellitus or other established disease, treatment with cardiovascular drugs (ie, anti-diabetic, lipid-lowering, antihypertensive, heart failure drugs, or drugs due to angina pectoris) which might disturb the measurements performed in the study, or unwillingness to participate. No subjects with clinically overt diabetes were included. However, fasting blood glucose  $\geq 6.1$  mmol/L was found in 22 subjects (5.6%).

The subjects were randomly selected among men in the County Council register and were invited to a screening examination. The design was a cross-sectional study based on a stratified sampling of randomly selected and screened men ( $n=818$ ) with the aim to include men with different degrees of obesity and insulin sensitivity ( $n=391$ ) as previously described in detail.<sup>16</sup>

The present report is a substudy in a project in which the primary objective was to examine the relationship between insulin peptides, the metabolic syndrome, and atherosclerosis as assessed by ultrasound.<sup>17</sup> A power calculation indicated that at least 750 men had to be screened in order to recruit 390 men in the study.

The subjects received both written and oral information before they gave their consent to participate. The study was approved by the Ethics Committee at Sahlgrenska University Hospital.

### Ultrasonography

#### IMT

Examination was performed with an ultrasound scanner (Acuson 128) with a 7-Mhz linear transducer aperture of 38 mm. The electrocardiographic signal (lead II) was simultaneously recorded to synchronize the image capture of the top of the R wave to minimize variability during the cardiac cycle. Both the left and right carotid arteries were scanned at the level of the bifurcation, and images for IMT measurements were recorded from the far wall in the common carotid artery and the carotid artery bulb and from the right femoral artery. The software program gives the average thickness of the intima-media complex (IMT). 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.<sup>18</sup> 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. At the position of the thickest part of the wall (visually judged), a frozen longitudinal image was captured and recorded on videotape. A short sequence of real-time images was also recorded on videotape to assist in the interpretation of the frozen images. The images were measured in an automated analyzing system,<sup>19</sup> based on automatic detection of the echo structures in the ultrasound image but with the option to make manual corrections by the operator. Interobserver variability for measurement of IMT in the common carotid artery, the carotid artery bulb, and the common femoral artery has been shown to be 5.3%, 6.0%, and 16.9%, respectively.<sup>20</sup> Measurements of carotid (mean of left and right artery) and femoral artery (right artery only) IMT were available in 379 and 373 patients, respectively. A composite mean of IMT in the common carotid artery, the carotid artery bulb, and the femoral artery was also calculated (IMT composite). Measurements of composite mean IMT was available in 365 subjects.

### Assessment of Plaque Occurrence

The carotid and femoral arteries were scanned both longitudinally and transversely to assess the occurrence of plaques.<sup>18</sup> A plaque was defined as a distinct area with an IMT more than 50% thicker compared with neighboring sites (visually judged). A semiquantitative subjective scale was used to grade the size of plaques as grade

1: one or more small plaques ( $< \approx 10$  mm<sup>2</sup>); grade 2: moderate-to-large plaques (the differentiation between grades 1 and 2 was made subjectively in most cases, and quantitative measurements were made in the computerized system<sup>21</sup> only when the correct classification was not obvious to the observer); Grade 3: plaques giving flow disturbances.<sup>19</sup> In the present study, no plaque of grade 3 was found in the femoral artery, and three subjects had plaques of grade 3 in the carotid artery. Therefore, plaques of grade 2 and 3 were merged into one group of moderate-to-large plaques. This analysis included plaques in the near wall as well as the far wall of the vessel. Analyses of plaques were performed in both the right and left carotid artery. The largest plaque in either artery was used in the present analysis. In a rereading reproducibility study ( $n=45$ ) of plaque size, there were high correlation coefficients for the right and left carotid arteries ( $r_s=0.96$  and  $r_s=0.96$ , respectively) and for the right femoral artery ( $r_s=0.86$ ). Measurements of plaques in the carotid and femoral artery were available in 367 and 389 patients, respectively.

### 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.<sup>22,23</sup> LDL cholesterol was calculated as described by Friedewald et al.<sup>24</sup> IL-6, TNF- $\alpha$ , and CRP were measured on serum, and Ox-LDL on plasma, that had been stored at  $-80^\circ\text{C}$ . High-sensitive ELISA kits was used to measure IL-6 (R&D System Europe Ltd), TNF- $\alpha$  (R&D System Europe Ltd), and CRP (Medix Biochemica).

Ox-LDL was measured by a commercially available sandwich ELISA (Mercodia) with the same specific murine monoclonal antibody mAb-4E6 as in the assay described by Holvoet et al.<sup>9</sup> The between-assay variation (different days) for Ox-LDL was 7% ( $r=0.94$ ,  $n=13$ , with a slight systematic difference in mean values 82.3 vs 74.1 U/L,  $P<0.05$ ). Hence, to avoid systematic differences in the present study, two internal controls were repeatedly included on all plates ( $n=10$ ). Mean values and standard deviations for the two controls were  $5.9 \pm 0.4$  (range, 5.4 to 6.7) and  $12.7 \pm 0.7$  (range, 11.9 to 12.7). All analyses were performed at the Wallenberg Laboratory.

### Statistical Analysis

All statistics were analyzed by using SPSS for Windows 10.0. The measurement error was defined as:  $SD/\sqrt{2}$ , where SD is calculated for mean differences between samples.

Coefficient of variation (CV) was calculated as:  $CV = (s \times 100/\bar{x})\%$ . Trend analysis was performed by using the Mantel test. Skewed variables were logarithm-transformed before statistical testing. Multiple regression was used to explore whether the association between circulating levels of Ox-LDL and IMT was independent of other covariates. Logistic regression was used to explore whether the association between circulating Ox-LDL and plaque occurrence in the carotid and femoral arteries was independent of other covariates. Relative odds ratio was calculated as  $e^{\beta(x_4 - x_2)}$ , where  $x_1$ =quintile 5 and  $x_2$ =quintile 1. Simple Spearman's rank correlation coefficients were used to calculate univariate associations.  $P<0.05$  was regarded as statistically significant. For anatomic reasons, there were missing data for plaque occurrence in the carotid and femoral artery ( $n=24$  and  $n=2$ , respectively). For technical reasons, there were also missing data for Ox-LDL in 5 subjects.

## Results

Study group characteristics are summarized in Table 1.

### IMT and Plaque Occurrence in the Carotid and Femoral Arteries in Relation to Circulating Ox-LDL

The carotid and femoral IMT, as well as the composite mean of common carotid, carotid bulb and femoral IMT increased

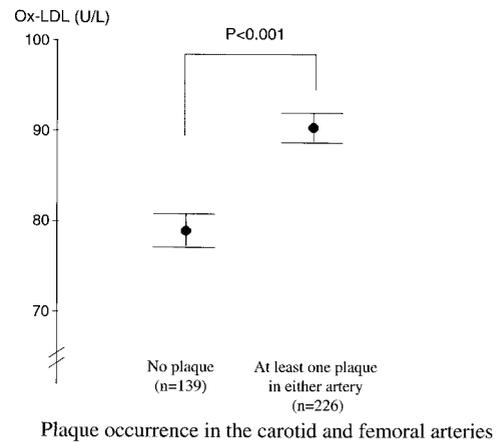
**TABLE 1. Characteristics of the Study Subjects**

Variable	Study Subjects (n=391)
Body mass index, kg/m <sup>2</sup>	26.4±4.4
Waist-hip-ratio	0.94±0.07
Blood pressure, mm Hg	
Systolic	123±17
Diastolic	74±36
Heart rate, bpm	60±8
Serum cholesterol, mmol/L	
Total	6.01±1.11
HDL	1.27±0.37
LDL	4.05±0.97
Serum triglycerides, mmol/L	1.57±1.05
Blood glucose, mmol/L	4.90±1.20
Plasma insulin, mU/L	9.99±6.29
Cigarette-years*	334±413
IMT, mm	
Common carotid artery	0.80±0.13
Carotid bulb	0.99±0.26
Common femoral artery	1.07±0.49

Values are mean±SD.

\*The total number of years of smoking was multiplied by the average number of cigarettes smoked daily.

by tertiles of Ox-LDL (Table 2). Increasing plasma concentrations of Ox-LDL were associated with plaque occurrence in the femoral artery, but not in the carotid artery (Table 2). When combining plaque occurrence in the carotid and femoral arteries, subjects with no plaques had significantly lower levels of Ox-LDL compared with subjects with at least one plaque in either artery (Figure 1).



Scatterplot showing the relationship between Ox-LDL and plaque occurrence in the carotid and femoral arteries.

**CRP and Cytokines by Tertiles of Circulating Ox-LDL**

The serum concentrations of CRP and TNF-α increased by tertiles of Ox-LDL (Table 3). Thirteen subjects had CRP levels above 10 mg/L. No significant association was seen between IL-6 and Ox-LDL (Table 3).

**Correlation Analyses and Multiple Regression**

Circulating Ox-LDL was significantly associated with IMT in the common carotid artery ( $r=0.11, P=0.03$ ), the carotid artery bulb ( $r=0.22, P<0.001$ ), the femoral artery ( $r=0.23, P<0.001$ ), and the composite measure of IMT ( $r=0.27, P<0.001$ ). Ox-LDL was significantly associated with CRP and TNF-α ( $r=0.13, P=0.011$  and  $r=0.25, P<0.001$ , respectively). In addition, Ox-LDL was significantly related to LDL cholesterol ( $r=0.65, P<0.001$ ), but not with systolic blood pressure or cigarette smoking (in years) ( $r=0.07$  and  $r=0.07$ , respectively).

**TABLE 2. IMT and Plaque Occurrence in the Study Group When Divided in Tertiles on Basis of Ox-LDL**

	Tertiles of Ox-LDL			P for Trend
	Lowest (n=129)	Middle (n=128)	Highest (n=129)	
Ox-LDL, U/L	61.5±8.9	83.6±5.8	113.3±17.3	NT
IMT, mm				
Common carotid artery	0.788±0.120	0.794±0.128	0.822±0.137	0.031
Carotid artery bulb	0.95±0.26	0.98±0.23	1.04±0.28	0.003
Femoral artery	0.98±0.23	1.05±0.46	1.15±0.44	0.006
Composite	0.91±0.22	0.94±0.20	1.00±0.19	<0.001
Plaque occurrence in the carotid artery, n (%)				0.103
None	79 (64)	66 (56)	62 (52)	
Small	19 (15)	24 (20)	25 (21)	
Moderate-to-large	26 (21)	29 (24)	32 (27)	
Plaque occurrence in the femoral artery, n (%)				0.002
None	89 (70)	84 (66)	65 (51)	
Small	16 (12)	8 (6)	22 (17)	
Moderate-to-large	23 (18)	36 (28)	41 (32)	

NT indicates not tested for statistical significance because of selection criteria.

TABLE 3. CRP and Cytokines in the Study Group When Divided in Tertiles on Basis of Ox-LDL

	Tertiles of Ox-LDL			P
	Lowest (n=129)	Middle (n=128)	Highest (n=129)	
CRP, mg/L	0.94 (0.03–9.69)	1.37 (0.10–21.46)	1.45 (0.15–31.46)	0.003
IL-6, ng/mL	2.10 (0.23–62.68)	2.39 (0.45–36.88)	2.32 (0.46–11.11)	>0.20
TNF- $\alpha$	1.85 (0.80–5.60)	2.03 (0.83–5.39)	2.30 (1.12–7.52)	<0.001

Values are geometric mean and range.

The composite measure of IMT in the common carotid, the carotid bulb, and the femoral artery was in addition to Ox-LDL also univariately associated with LDL cholesterol ( $r=0.22$ ,  $P<0.001$ ), HDL cholesterol ( $r=-0.11$ ,  $P=0.037$ ), triglycerides ( $r=0.33$ ,  $P<0.001$ ), and CRP ( $r=0.17$ ,  $P<0.001$ ) but not with TNF- $\alpha$  ( $r=0.08$ ,  $P=0.13$ ). In a multiple regression model with the variables univariately associated with the composite measure of IMT as covariates, only triglyceride levels showed an independent association with IMT. However, in a bivariate analysis, Ox-LDL was significantly associated with the composite measure of IMT after adjustment for LDL cholesterol (partial correlation coefficient  $r=0.14$ ,  $P=0.007$ ).

No significant differences in LDL cholesterol, HDL cholesterol, triglycerides, CRP, or TNF- $\alpha$  were observed between subjects with or without plaque occurrence in the carotid artery. Subjects with plaque occurrence in the femoral artery had significantly higher mean values of Ox-LDL, CRP, triglycerides, and LDL cholesterol compared with subjects without plaques. No significant differences were seen for HDL cholesterol or TNF- $\alpha$ .

In a logistic regression model with plaque occurrence in the femoral artery (any plaque) as dependent variable and the above mentioned variables associated with plaque as covariates, no variable showed an independent association with plaque occurrence. However, when combining plaque occurrence in the carotid and femoral arteries (any plaque), subjects with plaque occurrence had significantly higher mean values of LDL cholesterol, Ox-LDL, and triglycerides. In a logistic regression model, both LDL cholesterol and Ox-LDL were independently of each other related to plaque occurrence in the carotid and femoral arteries. The relative risk for having a plaque in the highest quintile versus the lowest quintile was 2.17 ( $P=0.049$ ) and 2.25 ( $P=0.050$ ) for LDL cholesterol and Ox-LDL, respectively.

## Discussion

The contribution of the present study is that it extends the observation of a relationship between circulating Ox-LDL and atherosclerosis to early subclinical lesions in the carotid and femoral arteries in a population-based cohort. In this study, the age factor was kept constant as only 58-year-old men were examined. Age has been established as a confounding factor that covariates with Ox-LDL levels.<sup>9</sup>

The results of the present study showed that Ox-LDL was related to IMT and plaque occurrence in the carotid and femoral arteries. In addition, Ox-LDL was associated with the proinflammatory cytokine TNF- $\alpha$  and CRP. Circulating Ox-LDL was also associated with LDL cholesterol but not with blood pressure or smoking. After adjustment for other risk

factors, the odds ratio for having a plaque in the carotid or femoral artery was 2.27 (95% confidence interval, 1.00 to 5.09) for subjects in the highest versus the lowest quintile of Ox-LDL levels.

These results fit into the concept that oxidatively modified LDL may play a major role in atherosclerosis development. Thus, Ox-LDL has a wide range of atherogenic properties in vivo. Among others, these processes include increased expression of adhesion molecules on endothelial cells, monocyte chemotaxis, upregulation of inflammatory genes, and destabilization of plaques.<sup>25–27</sup> The occurrence of LDL oxidation in vivo is also supported by the fact that Ox-LDL can be detected in both rabbit and human atherosclerotic lesions.<sup>3,5</sup> Furthermore, antibodies against epitopes of Ox-LDL have been found in human and rabbit plasma and in atherosclerotic lesions.<sup>4–6</sup> A significant association between circulating Ox-LDL and sequelae of atherosclerosis has recently been reported in other studies.<sup>7–11</sup> Only one of these studies was prospective, showing that subjects developing angiographically detectable cardiac transplant vasculopathy during a 2-year follow-up ( $n=21$ ) had significantly higher baseline levels of Ox-LDL as compared with subjects not developing vasculopathy ( $n=78$ ).<sup>8</sup> Furthermore, circulating Ox-LDL has also been proposed to give additive information to that provided by Global Risk Assessment Scoring (GRAS).<sup>10</sup>

With the assumption that circulating Ox-LDL mirrors the atherosclerotic disease in different parts of the vascular tree, we chose in the present study to measure subclinical atherosclerosis not only in the common carotid artery, but also in the carotid bulb and the femoral artery. Furthermore, to estimate the overall atherosclerosis burden, we defined subjects without plaque occurrence (both the carotid and the femoral artery) and subjects with plaque occurrence in either or both arteries. Subjects with at least one plaque in the carotid or the femoral arteries had significantly higher Ox-LDL levels as compared with subjects with no plaques. These results were obtained independently of plaque size. However, subjects with plaque occurrence in both arteries also had a high frequency of moderate-to-large plaques in the carotid (59%) as well as in the femoral artery (78%).

With the presently used method (mAb4E6), it is possible to measure very small amounts Ox-LDL containing a conformational epitope in the apolipoprotein B-100 moiety of LDL that is generated as a consequence of substitution of lysine residues of apolipoprotein B-100 with aldehydes.<sup>7</sup> To some extent, the mAb4E6 also detects circulating malondialdehyde LDL. Our study was not designed to examine the occurrence and importance of other epitopes of Ox-LDL. However, Ox-LDL is a very complicated particle, and the measurement

**TABLE 4. Logistic Regression Model With Plaque Occurrence as Dependent Variable and Ox-LDL, LDL, and Triglycerides as Independent Variables**

Covariates*	Plaque Occurrence in the Carotid and Femoral Arteries			Odds Ratio (95% CI), Highest vs Lowest Tertile
	B	SE	P	
Ox-LDL, U/L	0.385	0.180	0.050	2.25 (1.00–5.09)
LDL cholesterol, mmol/L	0.321	0.174	0.049	2.17 (1.01–4.69)
Triglycerides, mmol/L	0.148	0.087	0.087	

\*Divided in quintiles.

of its nature at a single epitope has the inherent problem of not recognizing this heterogeneity. Hence, it might well be that other epitopes on the LDL particle, such as oxidized phospholipids, carry important information with regard to subclinical atherosclerosis development. Another unresolved issue is the origin of circulating Ox-LDL. Previous studies of patients with acute coronary syndromes have suggested that elevated serum levels of Ox-LDL could be explained by ruptured atherosclerotic plaques, ischemic injury, or even remote inflammatory sources.<sup>27</sup> Our data from clinically healthy individuals with mainly normal CRP levels still showed an association between early atherosclerosis and Ox-LDL, refuting the suggested mechanisms as sole explanations. It seems likely that elevated circulating levels of Ox-LDL reflect the turnover of Ox-LDL in newly formed or progressing lesions in the arterial tree. Alternatively, circulating Ox-LDL concentrations are secondary to cholesterol levels in the blood and other risk factors and not directly involved in the atherosclerotic process. However, the causal relationship between Ox-LDL and progression of subclinical atherosclerosis has to be elucidated in future prospective studies. A previous study of transplant-associated coronary artery disease with the same monoclonal antibody as in the present study showed that Ox-LDL predicted future atherosclerotic disease independent of conventional cardiovascular risk factors.<sup>7</sup>

CRP is a sensitive marker of inflammation and infection. The production of CRP is regulated by cytokines, including IL-1, IL-6, and TNF- $\alpha$ .<sup>28</sup> TNF- $\alpha$  induces cellular responses after binding to specific cell surface receptors, TNFR1 and TNFR2.<sup>29</sup> It is unknown whether CRP exerts a direct effect on the atherosclerotic process or if serum CRP elevation is a phenomenon secondary to the impact of other factors. However, as mentioned above, Ox-LDL has been suggested to be involved in the upregulation of inflammatory genes.<sup>26</sup> Ox-LDL has for example been shown to stimulate the release of IL-1 $\beta$ <sup>30</sup> and IL-6.<sup>12</sup> On the other hand, TNF- $\alpha$  expression has been shown to be inhibited by Ox-LDL<sup>13,31</sup> in in vitro experiments.

The results of the present study showed a positive association between Ox-LDL and CRP as well as between Ox-LDL and TNF- $\alpha$ . These results suggest that high levels of circulating Ox-LDL, possibly by back-diffusion from atherosclerotic plaques, may interact with the inflammatory response. The observed discrepancies to previous in vitro studies regarding TNF- $\alpha$  have to be further elucidated. However, the relationship between CRP, cytokines, Ox-LDL, and endothe-

lial cells in vivo are possibly much more complicated than the relationships observed in vitro experiments. In this context, it is interesting to note that it is not known if circulating levels of Ox-LDL interact with endothelial cells thereby increasing the expression of cell-adhesion molecules and inflammatory mediators.

To summarize, the present study showed associations among Ox-LDL concentrations, plaque occurrence, and IMT in the carotid and femoral arteries. The relationship between Ox-LDL and plaque occurrence was independent of other risk factors. However, no independent association was seen between Ox-LDL and IMT. Circulating Ox-LDL was also consistently associated with the pro-inflammatory cytokines TNF- $\alpha$  and CRP. Because this was a cross-sectional study, no conclusions can be drawn on causality.

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