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Circulating Oxidized Low-Density Lipoprotein and Common Carotid Artery Intima-Media Thickness in a Random Sample of Middle-Aged Men

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Key Words
Atherosclerosis · Carotid artery · Intima-media thickness · Low-density lipoprotein · Oxidized

Abstract
Circulating oxidized low-density lipoprotein (oxLDL) has been suggested to play an important role in atherosclerosis development. According to previous observations, oxLDL correlates with clinically manifest coronary and carotid artery disease. We investigated the association between the oxLDL concentration measured directly in plasma and common carotid artery intima-media thickness (IMT) in a population-based, case-control study in middle-aged men from Southern Finland. oxLDL was determined in 214 men by a commercially available sandwich ELISA test (Mercodia). Carotid artery IMT was measured at 12 standardized segments by B-mode ultrasonography (at the near and far wall of the left and right common carotid arteries, bifurcations and internal carotid arteries), and the overall mean maximum IMT (MMaxIMT) was calculated. The MMaxIMT of the carotid arteries was significantly associated with circulating oxLDL ($r_s = 0.16$, $p = 0.018$). In a stepwise multiple regression model with MMaxIMT as dependent variable and systolic blood pressure, smoking, oxLDL, HDL cholesterol and apolipoprotein B as covariates, systolic blood pressure ($\beta = 0.22$, $p < 0.001$), oxLDL ($\beta = 0.15$, $p = 0.022$) and smoking ($\beta = 0.17$, $p = 0.014$) showed an independent association with IMT ($R^2 = 0.10$, $p < 0.001$). Our results show that oxLDL measured directly from plasma is independently associated with subclinical carotid artery atherosclerosis in middle-aged men.

Oxidative processes are postulated to play an important role in the development of atherosclerosis [15]. A high titer of oxidized low-density lipoprotein (oxLDL) antibodies has been found to be an independent predictor of the progression of carotid atherosclerosis [11]. Recently, a new method capable of measuring low concentrations of circulating oxLDL was developed and used to show an association between increased oxLDL levels in plasma and established coronary artery disease (CAD) and carotid atherosclerosis [3, 9, 13].

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Early atherosclerosis of carotid arteries can be assessed noninvasively by high-resolution B-mode scanning in asymptomatic healthy subjects [7, 12]. Even in the absence of discrete plaques or stenosis, the combined thickness of the arterial intima and media, i.e. intima-media thickness (IMT), can be measured with considerable precision by this technique [12]. Age, cigarette smoking, high systolic blood pressure and high cholesterol concentration have been reported to be the most important determinants of IMT [6, 12]. Furthermore, signs of early atherosclerosis captured by B-mode ultrasonography in carotid arteries have been associated with the severity of CAD [14].

Recently, a relationship between subclinical carotid and femoral atherosclerosis and the circulating oxLDL level was reported in a population of clinically healthy 58-year-old men [5]. The aims of the present study were to assess the hypothesis that the concentration of circulating oxLDL in plasma is positively correlated to carotid artery IMT in middle-aged men, and to explore the relationship between the circulating oxLDL level and known risk factors of carotid atherosclerosis.

Patients and Methods

Study Population

The present study is based on the analyses of frozen plasma samples, which were obtained in a cross-sectional population-based study performed 1992–1993. Originally, 300 men selected randomly from a population of 50- to 59-year-old men living in the city of Tampere in Southern Finland (n = 9,058) were invited to participate in the study. There were no exclusion criteria in this study. Of the original study population (n = 300), 33 men refused to participate, 44 men did not answer to the invitation or could not be reached and 9 men were excluded because of missing data. Finally, 214 men were included into the study. The Ethics Committee of the Urho Kaleva Kekkonen Institute approved the study, and all participants gave their written informed consent to the scientific use of the data and samples collected in the study.

Assessments of Clinical Characteristics, Lipids and Lipoproteins

A detailed medical history was obtained including smoking status, cardiovascular and metabolic diseases, and the use of medication. Weight and height were recorded, and body mass index was calculated as weight (kg)/height squared (m^2). Blood pressure was recorded from the dominant arm with a mercury sphygmomanometer after 15 min of supine rest. There were 121 smokers (43 current smokers and 78 former smokers) in the study population. None of the subjects have had a symptomatic cerebrovascular event. Fifty-one subjects had medication for hypertension, 4 for diabetes mellitus, and 10 for cardiovascular disease. Four of the subjects were treated for hyperlipidemia but none of them received statin treatment.

Blood was drawn after an overnight (12-hour) fast. Lipids and lipoprotein fractions were assessed from fresh serum samples. Lipoprotein fractions, including HDL and LDL cholesterol, were determined by ultracentrifugation. Cholesterol and triglycerides were measured with an enzymatic method (CHOD-PAP, Boehringer Mannheim, Mannheim, Germany). Apolipoprotein B was analyzed using an immunonephelometric method (Behring, Behringwerke, Marburg, Germany). Glucose was determined using the glucose dehydrogenase/mutarotase method (Merck Diagnostica, Darmstadt, Germany).

Determination of Circulating oxLDL

EDTA plasma collected during 1992–1993 was separated by centrifugation and stored at −80°C up to 9 years until analyzed. The oxLDL concentrations were determined from frozen plasma using a Mercodia Oxidized LDL ELISA test, which is a solid-phase two-site enzyme immunoassay modified from the original method of Holvoet et al. [3]. It is based on a direct sandwich technique in which two monoclonal antibodies (mAb-4E6 and anti-apolipoprotein B) are directed against separate antigenic determinants on the oxidized LDL-apolipoprotein B. During incubation, oxLDL in the sample reacts with anti-oxidized LDL antibodies bound to microtitration well. After washing, to remove non-reactive plasma components, a peroxidase-conjugated anti-apolipoprotein B antibody recognizes the oxLDL bound to the solid phase. After a second incubation and a simple washing step that removes unbound enzyme-labelled antibody, the bound conjugate is detected by reaction with 3,3′,5,5′-tetramethylbenzidine. Adding acid to give a colorimetric endpoint that is read spectrophotometrically at 450 nm stops the reaction.

Assessment of Carotid IMT

Quantitative carotid artery ultrasonography was performed in 1992–1993 using a standardized protocol adapted to the Finnish population [4, 7]. A high-resolution B-mode ultrasound with a 10-MHz transducer was used (Biosound Phase 2, Biodynamics, Indianapolis, Ind., USA) to examine left and right carotid arteries. The examinations were recorded on videotapes, which were read off-line at the ultrasound reading center, Wake Forest University, Winston-Salem, N.C., USA. The same certified sonographer and reader performed all recordings and measurements.

The right and left carotid arteries were imaged by a circumferential scan including the longitudinal views of lateral, posterior and anterior angles. The regions of interest were 10 mm distal to the common carotid artery, bifurcation and 10 mm proximal to the internal carotid artery. The distance between the media-adventitia interface and the lumen-intima interface represented the IMT. Carotid artery IMT was measured at 12 standardized segments, i.e. at the near and far wall of the left and right common carotid arteries, bifurcations and internal carotid arteries. In analyses, we used mean maximum IMT (MMax IMT), which was calculated as the mean of the 12 maximum IMTs identified in the 12 standard sites. MMaxIMT is an estimate of the extent and severity of carotid atherosclerosis [7, 10].

To assess the intraobserver variability in the recording and measurement of MMaxIMT, repeated scans of a total of 15 randomly selected participants were performed 1 week later. The mean absolute difference between repeated measurements was 0.052 mm in MMaxIMT. The reproducibility of the measurements was good, i.e. the coefficient of variation was 4.1–8.6% for common carotid arteries. These figures are comparable to previously reported data [5, 10].

Statistics

Statistical analysis was performed using SPSS for Windows version 10.1. The Kolmogorov-Smirnov test was used to test the normality of the distribution of study variables. oxLDL, systolic and diastolic blood pressure, apolipoprotein B, body mass index and total, LDL and HDL cholesterol were normally distributed. The variables with skewed distribution, i.e. MMaxIMT, glucose and triglycerides, were analyzed in logarithmically transformed form, but results are expressed in crude form.

Associations between circulating oxLDL, MMaxIMT, and other risk factors for atherosclerosis were calculated with Spearman's rank correlation coefficients. Differences in the mean oxLDL level and MMaxIMT between smokers versus non-smokers were tested by t test for independent samples. A stepwise multivariate linear regression was used to identify the independent variables associated with circulating oxLDL and MMaxIMT. A p value less than 0.05 was considered statistically significant.

Table 1. Clinical characteristics of the study population (n = 214)

	Mean ± SD	Median (range)
Age, years		54 (50–59)
Body mass index, kg/m^2	27.3 ± 3.8	
Systolic blood pressure, mm Hg	132 ± 17	
Diastolic blood pressure, mm Hg	84 ± 10	
Total cholesterol, mmol/l	5.5 ± 0.9	
Serum LDL cholesterol, mmol/l	3.6 ± 0.8	
Serum HDL cholesterol, mmol/l	1.2 ± 0.3	
Serum triglycerides, mmol/l		1.35 (0.34–6.06)
Blood glucose, mmol/l		5.1 (4.3–13.2)
Serum apolipoprotein B, g/l	1.3 ± 0.3	
Serum apolipoprotein A1, g/l	1.5 ± 0.2	
MMaxIMT, mm		1.14 (0.72–2.07)
Plasma oxLDL, mU/l	74 ± 23	

Continuous normally distributed variables (mean ± SD) and skewed distributed variables (medians and ranges) are expressed.

Results

The clinical characteristics of the 214 participants are presented in table 1. There were 121 smokers (57%) and 93 non-smokers (43%) in the study population. In univariate analysis, MMaxIMT correlated statistically significantly with circulating oxLDL ($r^2 = 0.16$, $p = 0.018$), systolic blood pressure ($r^2 = 0.21$, $p = 0.002$), HDL cholesterol ($r^2 = -0.14$, $p = 0.045$) and apolipoprotein B ($r^2 = 0.16$, $p = 0.016$) but not with body mass index, diastolic blood pressure, total and LDL cholesterol, triglycerides, blood glucose and apolipoprotein A1 (table 2). MMaxIMT was statistically significantly higher in smokers than in non-smokers (1.23 vs. 1.14 mm, respectively, $p = 0.008$, t test for independent variables).

A linear regression model was used to explore whether the association between oxLDL and MMaxIMT was independent of other covariates univariately associated with

Table 2. Spearman's correlation coefficients and a stepwise multiple linear regression analysis of factors associated with MMaxIMT

Explanatory variable	Spearman's correlation coefficient		Linear regression analysis $R^2 = 0.10$, $p < 0.001$	
	r_s	p value	β value	p value
Body mass index	0.01	0.913		
Systolic blood pressure	0.21	0.002[a]	0.22	0.001[c]
Diastolic blood pressure	0.13	0.056		
Total cholesterol	0.10	0.144		
Serum LDL cholesterol	0.13	0.051		
Serum HDL cholesterol	−0.14	0.045[a]		0.887
Serum triglycerides	0.10	0.148		
Blood glucose	0.09	0.173		
Serum apolipoprotein B	0.16	0.016[a]		0.610
Serum apolipoprotein A1	−0.06	0.407		
Plasma oxLDL	0.16	0.018[a]	0.15	0.022[c]
Smokers versus non-smokers, t test		0.008[b]	0.17	0.014[c]

[a] Significant univariate association between MMaxIMT and explanatory variable in Spearman's rank correlation test.

[b] Significant difference in MMaxIMT between smokers and non-smokers (t test). These variables are included in the multiple linear regression analysis.

[c] Significant independent association with MMaxIMT in the linear regression model.

Table 3. Spearman's correlation coefficients and a stepwise multiple linear regression analysis of factors associated with oxLDL

Explanatory variable	Spearman's correlation coefficient		Linear regression analysis $R^2 = 0.38$, $p < 0.001$	
	r_s	p value	β value	p value
Body mass index	0.09	0.218		
Systolic blood pressure	−0.02	0.764		
Diastolic blood pressure	−0.04	0.618		
Total cholesterol	0.53	<0.001[a]		0.650
Serum LDL cholesterol	0.53	<0.001[a]		
Serum HDL cholesterol	−0.29	<0.001[a]		0.287
Serum triglycerides	0.32	<0.001[a]	−0.19	0.014[b]
Blood glucose	0.18	0.009[a]		0.900
Serum apolipoprotein B	0.60	<0.001[a]	0.74	<0.001[b]
Serum apolipoprotein A1	−0.08	0.271		
MMaxIMT	0.16	0.016[a]		0.268

[a] Significant univariate association between oxLDL and explanatory variables in Spearman's rank correlation test. These variables are included in the multiple linear regression analysis except for LDL cholesterol, which is part of total cholesterol.
[b] Significant independent association with oxLDL in the stepwise linear regression model.

MMaxIMT. In a stepwise multiple regression model with MMaxIMT as dependent variable and oxLDL, systolic blood pressure, apolipoprotein B, HDL cholesterol and smoking as covariates, systolic blood pressure ($\beta = 0.22$, $p = 0.001$), smoking ($\beta = 0.17$, $p = 0.014$) and oxLDL ($\beta = 0.15$, $p = 0.022$) showed an independent association with MMaxIMT ($R^2 = 0.10$, $p < 0.001$, table 2).

Circulating oxLDL associated statistically significantly with total, LDL and HDL cholesterol, triglycerides, apolipoprotein B, blood glucose and MMaxIMT (Spearman's rank correlation coefficients are shown in table 3). There was no difference in oxLDL levels between smokers and non-smokers ($p = 0.13$, t test for independent samples). In a stepwise multiple linear regression model, we used the variables univariately associated with oxLDL as covariates, except for LDL cholesterol, which is part of total cholesterol. Only the apolipoprotein B level ($\beta = 0.74$, $p < 0.001$) and triglycerides ($\beta = -0.19$, $p < 0.014$) showed an independent association with circulating oxLDL (table 3). The R^2 of the model was 0.39 ($p < 0.001$).

Discussion

The present study confirms another Scandinavian observation by Hulthe and Fagerberg [5] that there is a relationship between circulating oxLDL and early subclinical lesions of carotid atherosclerosis in middle-aged men. Furthermore, our study demonstrated that the association between oxLDL and carotid artery IMT is independent of other covariates associated with IMT. The finding that oxLDL correlates with clinically silent carotid atherosclerosis is an important expansion of the previous observations, which showed that oxLDL correlated with clinically manifest coronary and carotid artery disease [3, 9, 13]. Thus, oxLDL might play an important role in atherosclerosis development.

In the recent study by Hulthe and Fagerberg [5], the carotid IMT was measured from the far wall of the common carotid artery and the carotid bulb. In addition to the far walls of the common carotid arteries and carotid bifurcations, also the near walls of these sites and the near and far walls of the internal carotid arteries were included in the measurement of the MMaxIMT in our study. It is well known that internal carotid artery and carotid bifurcation are predilection sites of carotid atherosclerosis. With the measurement of the MMaxIMT of the near and far walls of both common carotid arteries, bifurcations and internal carotid arteries, we tried to minimize the possibility of underestimating carotid atherosclerosis. Another difference was that there were no exclusion criteria in our study while the patients with cardiovascular disease, diabetes and cardiovascular drugs were excluded in the study by Hulthe and Fagerberg [5]. With a random sample of ten age cohorts, we wanted to guarantee a non-biased study group, which included a whole spectrum of middle-aged men ranging from healthy to diseased. oxLDL was measured in plasma that had been stored at −80 °C both in our

study and in the study of Hulthe and Fagerberg [5]. The analyses were made with the same commercially available sandwich ELISA (Mercodia), and the level of oxLDL was comparable in our study and the study of Hulthe and Fagerberg [5]. In our study the time and methods for storage were the same for all the samples. However, we cannot completely exclude the possibility that the storage had changed the levels of oxLDL. Thus, studies with oxLDL measured on fresh plasma are needed to confirm the results by us and Hulthe and Fagerberg [5].

OxLDL might be one of the key determinants of atherosclerosis. Several recent publications have tested the correlation between the IMT of carotid arteries and LDL oxidation measured by concentrations of circulating oxLDL or autoantibodies against oxLDL. High titer of autoantibodies against oxLDL has been found to be an independent predictor of the progression of carotid atherosclerosis [11]. However, in other studies an inverse correlation has been found between carotid arterial IMT and autoantibodies against oxLDL [1]. Discrepancy among previous reports concerning the association between autoantibodies against oxLDL and carotid atherosclerosis may result from the enormous heterogeneity of oxLDL, which is a complicated particle with many different modifications in the phospholipid and apolipoprotein B components of LDL. Furthermore, the pathophysiological roles of autoantibodies against oxLDL may vary depending on the stages of atherosclerosis, i.e. immune responses may protect from atherogenesis in the early stages of the disease but result in accelerated atherosclerotic progression at the later stages [8]. To our knowledge, there is no discrepancy between the published data concerning the relationship between circulating oxLDL and carotid atherosclerosis, suggesting that circulating oxLDL might be a more specific marker of atherosclerosis than autoantibodies against oxLDL.

The present and previous studies clearly demonstrate that small amounts of LDL containing different oxidation-specific epitopes can be measured in the circulation, although blood is rich in a variety of antioxidants that inhibit the formation of oxLDL [3, 5, 9, 13]. However, the origin of circulating oxLDL is an yet unresolved issue. Previous studies among patients with symptomatic coronary or carotid syndromes have suggested that elevated serum levels of oxLDL could be explained by ruptured atherosclerotic plaques [3, 9, 13]. Our results with clinically asymptomatic men suggest that the increase in circulating oxLDL might be partly due to a backdiffusion of oxLDL from an atherosclerotic arterial wall into the blood in the early phase of atherosclerosis.

The present study confirmed the relationship of hypercholesterolemia and hyperglycemia with oxLDL [3, 5]. oxLDL did not associate with smoking and blood pressure in the present and previous studies [3, 5]. In a multiple regression model, none of these variables were independently associated with the oxLDL level. To our knowledge, there is no evidence that lowering cholesterol or glucose levels affects circulating oxLDL concentration. In the Vitamin E Atherosclerosis Prevention Study, α-tocopherol supplementation reduced circulating oxidized LDL but did not reduce the progression of IMT over a 3-year period [2]. Thus, further studies are needed to confirm the reasons and ways to reduce LDL oxidation.

Our findings suggest that there is an independent association between oxLDL levels and early carotid atherosclerosis. Whether there is causal relationship between oxLDL and carotid atherosclerosis remains to be resolved in future prospective studies.

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