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### Circulating oxidized LDL is associated with the occurrence of echolucent plaques in the carotid artery in 61-year-old men

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## ORIGINAL ARTICLE

# Circulating oxidized LDL is associated with the occurrence of echolucent plaques in the carotid artery in 61-year-old men

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**Objective.** The aim of this study was to elucidate the relationship between the echogenicity of carotid artery plaques and the following risk factors: circulating oxLDL, hsCRP, the metabolic syndrome (MetS), and several of the traditional cardiovascular (CV) risk factors. **Material and methods.** A cross-sectional population-based study of 513 sixty-one-year-old men. The levels of circulating oxLDL were determined in plasma samples by sandwich ELISA utilizing a specific murine monoclonal antibody (mAb-4E6). High-sensitivity CRP was measured in plasma by ELISA. Plaque occurrence, size and echogenicity were evaluated from B-mode ultrasound registrations in the carotid arteries. Plaque echogenicity was assessed based on a four-graded classification scale. **Results.** A higher frequency of echolucent carotid plaques was observed with increasing levels of oxLDL and systolic blood pressure ( $p=0.008$  and  $p=0.041$ , respectively). Subjects with the MetS had a significantly higher frequency of echogenic plaques than subjects without the MetS ( $p=0.009$ ). In a multiple logistic regression analysis, oxLDL turned out to be independently associated with echolucent carotid plaques. **Conclusions.** The occurrence of echolucent carotid plaques was associated with oxLDL and systolic blood pressure, and oxLDL was associated with echolucent carotid plaques independently of systolic blood pressure.

**Keywords:** CRP; OxLDL; plaque; ultrasonography

## Introduction

The response to retention hypothesis assumes that LDL cholesterol is retained in the subintimal space and is modified to oxidized LDL (oxLDL), which is identified by scavenger receptors in the macrophages, leading to an inflammatory reaction and the formation of foam cells [1,2]. The observations that circulating levels of oxLDL and C-reactive protein (CRP) are associated with cardiovascular events have been interpreted as supportive of this hypothesis [3,4]. We have previously shown that high concentrations of circulating oxLDL are associated with development of atherosclerotic plaques in the carotid artery [5].

Most atherosclerotic lesions never lead to cardiovascular disease. During recent years the concept of vulnerable atherosclerotic plaques has been developed. Plaques associated with established cardiovascular disease are characterized by moderate size, a large lipid core and a thin cap with a high content of inflammatory cells [6]. Using high resolution B-mode ultrasound it is possible to identify and characterize plaques in the carotid artery [7–9]. Echolucent plaques seem to be lipid-rich, whereas echogenic plaques have a high content of fibrous tissue and calcification [9].

We, and others, have shown that echolucent carotid plaques, as assessed by ultrasound, are associated with cardiovascular events [10,11].

The metabolic syndrome is associated with an increased risk of cardiovascular events [12], a large intima-media thickness in the carotid artery [13] and elevated CRP [12]. Accordingly, the aim of the present study was to evaluate the hypothesis that high sensitivity CRP and circulating oxLDL are associated with the occurrence of non-stenotic echolucent plaques in the carotid artery.

## Material and methods

The study outline has been described in detail previously [14].

## Population sample-screening examination

The subjects were obtained from a cohort of randomly selected 58-year-old men ( $n=1728$ ) who had replied to a letter and participated in a telephone interview ( $n=1188$ ) in an original study aimed at examining whether insulin resistance was associated

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with atherosclerosis [15]. Two groups were identified from this sample. One group of 237 men had known diabetes mellitus, hypertension, hyperlipidemia or cardiovascular disease (CVD). The other group comprised 391 clinically healthy men, randomly selected from the population sample, with varying degrees of obesity and insulin resistance.

### **Re-examination (present study)**

The present study was performed 3 years after the screening examination. In the group of men with known diabetes mellitus, increased cardiovascular risk and CVD, 231 of the 237 at screening were alive. They were invited to participate in the present study and 168 accepted. In the other group of 391 men at screening, 387 men were alive, and of those 345 participated in the present study. A total of 513 men, 61 years old with Swedish ancestry, were therefore examined.

Examinations took place in the morning on two occasions with an interval of one week. The subjects had fasted overnight and had undergone examination with blood test and ultrasound measurement of the carotid arteries.

All the men received both written and oral information before giving their consent to participation in the study. The study was approved by the Ethics Committee at the Sahlgrenska Academy.

### **Measurements**

Information on general health and smoking habits was obtained through a self-administered questionnaire. Venous blood samples were drawn after a fasting period of 10–12 h, kept at room temperature for 30 min before the serum was separated by centrifugation and thereafter immediately frozen in aliquots at  $-70^{\circ}\text{C}$ .

### **Laboratory examinations**

Lipids and blood glucose were measured by standard methods [16]. hsCRP was measured using commercially available ELISA kits (Medix Biomedica, Kauniainen, Finland).

OxLDL was measured on plasma which had been stored at  $-70^{\circ}\text{C}$ , as previously described [17]. OxLDL was measured using a commercially available sandwich ELISA (Mercodia, Uppsala, Sweden) utilizing the same specific murine monoclonal antibody, mAb-4E6, as in the assay described by Holvoet et al. [18]. The between-assay variation (different days) for oxLDL was 7% ( $r=0.94$ ,  $n=13$ ), with a slight systematic difference in mean values ( $82.3 \text{ U L}^{-1}$  versus  $74.1 \text{ U L}^{-1}$ ;  $p<0.05$ ).

Therefore, in order to avoid systematic differences in the present study, two internal controls were included repeatedly on all plates ( $n=10$ ). Mean values and standard deviations for the two controls were  $5.9\pm 0.4$  (range 5.4–6.7) and  $12.7\pm 0.7$  (range 11.9–12.7). All analyses were done at the Wallenberg Laboratory.

### **Ultrasound measurement**

The visit for the ultrasound examination always took place in the morning. The examination was performed using an ultrasound scanner (Acuson 128; Acuson, Siemens, Mountain View, Calif., USA) with a 7-MHz linear transducer, aperture of 38 mm [19]. An electrocardiographic signal (lead II) was simultaneously recorded to synchronize image capture of the top of the R wave to minimize variability during the cardiac cycle. The laboratory technician carrying out the ultrasound examination was blinded to the clinical status of each subject. The carotid artery was scanned longitudinally and transversely to assess the occurrence of plaques [19]. A plaque was defined as a distinct area with an IMT  $> 50\%$  thicker than that of neighbouring sites (visually judged). A semiquantitative subjective scale was used to grade the size of plaques as: Grade 0=no plaque; Grade 1=one or more small plaques ( $\leq 10 \text{ mm}^2$ ); Grade 2=moderate to large plaques (differentiation between grades 1 and 2 was made subjectively in most cases, and quantitative measurements were produced by the computerized system only when the correct classification was not obvious to the observer); Grade 3=plaques giving flow disturbances [19]. In the present study, 9 subjects out of 513 were unwilling to participate in the ultrasound examinations. Only five plaques of grade 3 were found in the carotid artery. Therefore, plaques of grades 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. Assessment of plaque size was possible in 204 of 207 plaques. In a re-reading reproducibility study ( $n=45$ ) of plaque size, correlation coefficients for both the right and left carotid arteries were high ( $r_s=0.96$  and  $r_s=0.96$ , respectively) [16].

### **Assessment of plaque echogenicity**

Assessment of plaque echolucency was based on the version of classification proposed by Gray-Weale et al. [8], in which plaques are graded from 1 to 4, i.e. 1=dominantly echolucent with a thin echogenic cap;

2=substantially echolucent with small areas of echogenicity; 3=dominantly echogenic lesions with small areas of echolucency (<25 %); and 4=uniformly echogenic lesions (equivalent to homogenous).

Two examiners simultaneously classified plaque echogenicity in all subjects. In cases of discrepancy between the two readers, a third reader was consulted. Since there were few subjects classified as group 1 and group 4 in the present study ( $n=31$  and  $n=5$ , respectively), groups 1 and 2 were merged into one group and groups 3 and 4 into another and defined as predominantly echolucent and predominantly echogenic, respectively. The largest plaque in each artery from four carotid arteries (CCA, bulb on left and right sites) was used to determine the final echogenicity of each subject. Plaques graded as predominantly or substantially echolucent were always chosen as worst cases. Assessment of plaque echogenicity was possible in 194 of 204 plaques (Table I).

In order to investigate the reproducibility of assessment of plaque echogenicity, 76 plaques taken from another study were analysed twice by the same examiner. The classification was the same as that used in the present study and plaques were graded from 1 to 4. The results showed a good agreement ( $r=0.95$ ) between the first assessment of plaque echogenicity and the second one made 6 months

later. Ninety-five percent ( $n=72$ ) of the 76 plaques were classified equally both times [10].

#### Definition of the metabolic syndrome

The definition of the MetS as suggested by the National cholesterol education programme's adult treatment panel III (NCEP) was used [20]. The MetS was defined as at least three of the following factors: 1. Fasting blood glucose  $\geq 5.6$  mmol/L (plasma glucose  $\geq 6.1$  mmol/L); 2. blood pressure  $>130/85$  mmHg or antihypertensive medical treatment; 3. plasma triglycerides  $\geq 1.7$  mmol/L; 4. HDL cholesterol  $<1.0$  mmol/L for men and  $<1.3$  mmol/L for women; 5. waist  $>102$  cm for men and  $>88$  cm for women.

#### Statistics

All statistics were analysed using SPSS for Windows 10.0 (SPSS, Inc., Chicago, Ill., USA). The characteristics of the subjects are described as numbers (%), means and standard deviations. Serum triglycerides, hsCRP and oxLDL were skewed. For these variables, geometric means were calculated and log transformation was performed before any statistical analyses were done. The Pearson chi-squared test was used for comparison of nominal and ordinal variables and Mantel-Haenszel for testing trends in Figures 1 and 2. A multiple logistic regression analysis was applied to test the relationship between single dependent variables and explanatory variables. Spearman's correlation coefficient was calculated for correlation analyses. An independent-samples *t*-test was applied to compare means for two groups of cases such as current smokers (y/n) and MetS (y/n). Two-sided  $p < 0.05$  was considered statistically significant.

#### Results

Characteristics for the entire study group ( $n=513$ ) are presented in Table I. Diabetes mellitus was diagnosed in 74 men (14 %).

#### Risk factors associated with echolucent carotid artery plaques

A higher frequency of echolucent carotid plaques was observed with increasing levels of oxLDL and systolic blood pressure ( $p=0.008$  and  $p=0.041$ , respectively; Figure 1), but not with increasing levels of hsCRP (Figure 1) or LDL cholesterol ( $p=0.655$  and  $p=0.187$ , respectively). There was no significant association between echolucent carotid plaques and the MetS (Figure 1) or current smoking ( $p=0.457$  and  $p=0.182$ , respectively; Figure 1).

Table I. Characteristics.

Variable	Study subjects ( $n=513$ )
Age (years)	61.6 $\pm$ 0.51
BMI (kg/m <sup>2</sup> )	27.2 $\pm$ 4.40
Waist (cm)	99.5 $\pm$ 12.4
SBP (mmHg)	131 $\pm$ 19
HDL cholesterol (mmol/L)	1.25 $\pm$ 0.35
LDL cholesterol (mmol/L)	3.74 $\pm$ 0.94
Serum triglycerides (mmol/L)	1.39 (0.42–8.91)
Blood glucose (mmol/L)	5.4 $\pm$ 1.9
Current smoking, $n$ (%)	88 (17)
hsCRP (mg/L)	1.39 (0.08–27.9)
oxLDL (U/L)	98 (23–222)
MetS, $n$ (%)	149 (29)
Plaque occurrence in the carotid artery	
No, $n$ (%)	245 (48)
Small, $n$ (%)	67 (13)
Moderate/large, $n$ (%)	137 (27)
Plaque morphology in the carotid artery	
Echogenic	35 (7)
Echolucent	159 (31)

Data are presented as the mean value  $\pm$  SD, geometric mean (min-max) or number (%) of patients.

SBP=systolic blood pressure, HDL=high-density lipoprotein, LDL=low density lipoprotein, hsCRP=high-sensitive C-reactive protein, oxLDL=oxidized low density lipoprotein, MetS=metabolic syndrome.

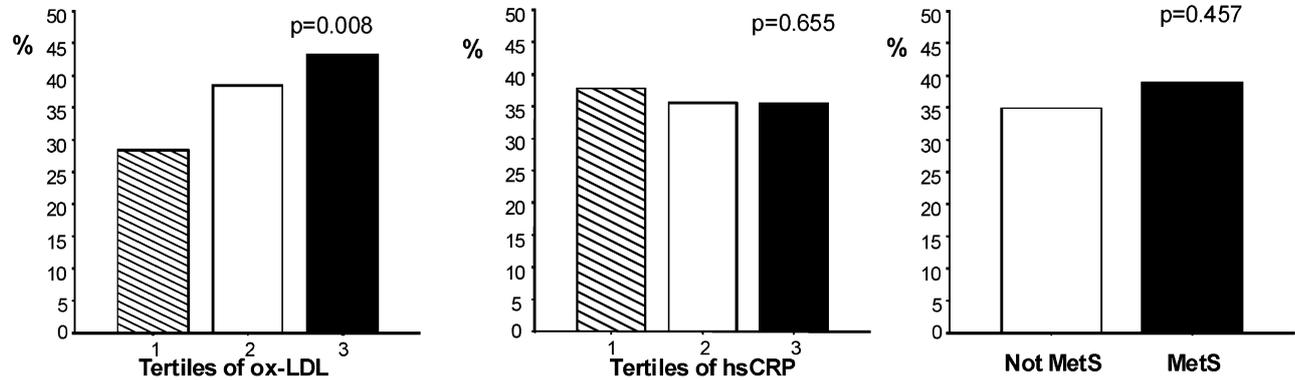


Figure 1. Occurrence of echolucent carotid plaques in relation to levels of oxLDL, hsCRP and to the MetS (y/n).

### Risk factors associated with echogenic carotid plaques

No significant association was observed between circulating levels of oxLDL, hsCRP ( $p=0.809$  and  $p=0.205$ , respectively; Figure 2), LDL cholesterol or systolic blood pressure ( $p=0.959$  and  $p=0.055$ , respectively) in relation to occurrence of echogenic carotid plaques. Subjects with the MetS had a significantly higher frequency of echogenic plaques compared to subjects without the MetS ( $p=0.009$ ; Figure 2). No significant association was observed between echogenic plaques and current smoking ( $p=0.389$ ).

### Multiple logistic regression analysis

In a multiple logistic regression analysis with echolucent carotid plaque as the dependent variable and tertiles of oxLDL and tertiles of systolic blood pressure as covariates, tertiles oxLDL turned out to be independently associated with echolucent carotid plaques (OR=1.34, 95 % CI 1.05–1.71);  $p=0.019$ ).

### Discussion

The main results of the present study are that increased frequency of echolucent carotid plaques is

associated with increasing levels of circulating oxLDL and systolic blood pressure. In a multiple logistic regression analysis, tertiles of oxLDL turned out to be independently associated with echolucent carotid plaques after taking into account systolic blood pressure. Subjects with the MetS had a significantly higher frequency of echogenic plaques compared to subjects without the MetS. Other variables in the study were current smoking, LDL cholesterol and hsCRP, which were not significantly associated with plaque echogenicity.

Association between the MetS and echogenic carotid plaques has not previously been described, as far as we know, and possible mechanisms that might be involved are unclear. Interestingly, there is evidence that echogenic, but not echolucent, carotid plaques are associated with aortic arterial stiffness, but the mechanisms linking arterial stiffness to atherosclerosis are not known at present [21].

The majority of atherosclerotic plaques remain asymptomatic, while others cause clinical syndromes in several ways. Several studies have shown that echolucent carotid plaques predict CV events [10,22,23]. However, the majority of the previous studies investigated the relationship between stenotic

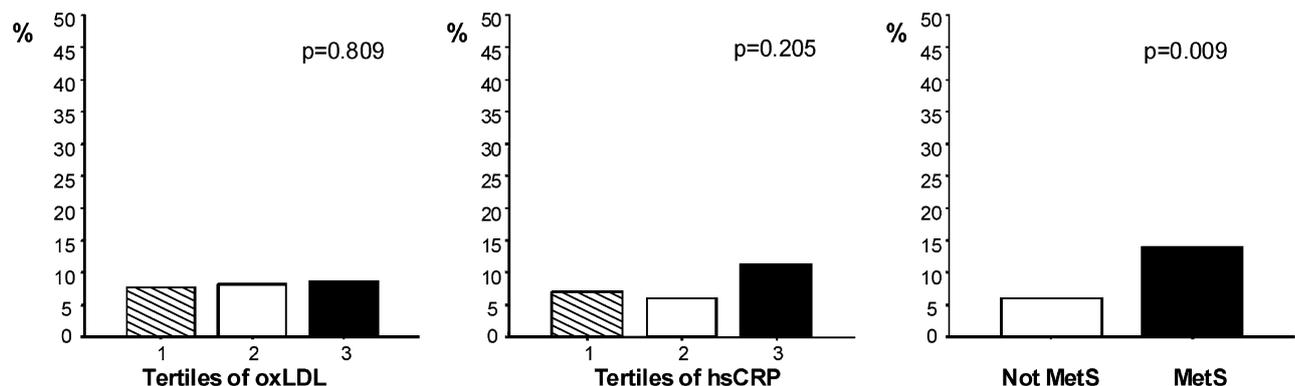


Figure 2. Occurrence of echogenic carotid plaques in relation to levels of oxLDL, hsCRP and to the MetS (y/n).

plaques and events, while the present study was conducted in a population-based sample of middle-aged men with small to moderate plaques without flow disturbances.

Systemic inflammatory markers such as hsCRP have been shown to predict future cardiovascular events, but whether they are associated with early atherosclerosis is uncertain. In a healthy community population, Chapman et al. showed that monocyte count, but not hsCRP or interleukin-6, was an independent risk marker for subclinical carotid atherosclerosis [24]. Muscari et al. found no associations between hsCRP and echolucent carotid plaques [25]. This is in line with our findings showing no significant association between hsCRP and echolucent plaques in the carotid artery. Furthermore, associations between hsCRP and subclinical atherosclerosis seem to be different in the carotid and femoral arteries. As shown in our previous studies, hsCRP was related to intima-media thickness (IMT) and plaque occurrence in the femoral artery, but not in the carotid artery [16].

Circulating oxLDL is a useful biological marker for identifying patients with coronary artery disease [4] and a reliable prognostic marker of subclinical atherosclerosis, as shown in our previous studies where oxLDL level at entry was associated with progression of IMT in the carotid artery also after adjustment for other risk factors [5]. The results of the present study extend previous knowledge showing that oxLDL is also associated with an echolucent plaque phenotype. Circulating oxLDL seems to be a common predictor for the occurrence of echolucent carotid and femoral artery plaques in this study group; results on femoral atherosclerosis has previously been published [26]. Therefore, it might be speculated that it is the same risk factors that drive the development of echolucent plaques in different vascular beds. In line with this hypothesis are recent results showing that not only is the culprit lesion in acute coronary syndromes in a vulnerable state, but also other atherosclerotic plaques in the coronary arterial tree, including small-sized plaques [27,28]. Recent, large, population-based studies showing that stenotic as well as non-stenotic echolucent plaques in peripheral arteries predict CV events is further evidence supporting this hypothesis [10,11,22,23,29].

A limitation of the present study is that it is cross-sectional and included only 61-year-old Caucasian men.

In conclusion, the main results of the present study are that oxLDL was independently associated with echolucent carotid plaques after taking into account systolic blood pressure. This is a cross-sectional study and further prospective studies are needed to elucidate causality.

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