



Circulating oxidized low-density lipoprotein is associated with echolucent plaques in the femoral artery independently of hsCRP in 61-year-old men

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Abstract

Objectives: The aim of the study was to test the hypothesis that circulating markers of inflammation (high-sensitive C-reactive protein, hsCRP) and oxidative modification of lipids (oxidized low-density lipoprotein, oxLDL) were associated with the occurrence of echolucent rather than echogenic femoral artery plaques in a cross-sectional population based cohort of 513, 61-year-old men.

Background: The relationships between circulating oxLDL, hsCRP and the occurrence of echolucent plaques in the femoral artery have not previously been investigated.

Methods: The levels of circulating oxLDL and hsCRP were determined in plasma by ELISA. Plaque occurrence, size and echogenicity were measured by B-mode ultrasound in the right femoral artery. Assessment of plaque echogenicity was based on the classification (grades 1–4) proposed by Gray-Weale et al.

Results: A higher frequency of echolucent femoral plaques was observed in subjects with the metabolic syndrome and current smokers ($p=0.01$ and $p<0.001$, respectively) as well as with increasing levels of oxLDL and hsCRP ($p=0.002$ and $p=0.005$, respectively). In a multiple logistic regression analysis oxLDL and current smokers turned out to be independent associated with the presence of echolucent femoral artery plaques.

Conclusions: The results of the present study support our hypothesis that circulating oxLDL is a marker of an unstable echolucent plaque phenotype in the femoral artery in man.

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1. Introduction

The composition of an atherosclerotic plaque in the coronary arteries is considered more important than the degree of stenosis for defining cardiovascular risk [1]. However, there

are conflicting data regarding the carotid arteries, both stenosis and composition have been suggested as important factors [2,3]. Much less is known about the femoral arteries in relation to cardiovascular diseases (CVD). However, it has been speculated that atherosclerosis starts earlier in the femoral artery compared to the carotid artery [4].

Ultrasound plaque appearance or echogenicity can in principle be classified into plaques with low-level echoes with a thin often incomplete shell on the luminal surface (echolucent plaque) and plaque with medium and high level echoes (echogenic plaques) [5,6]. Interestingly, we have previously

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shown that echolucent plaques in the femoral artery predict CVD, to a large extent coronary heart disease, during 6.6 years of follow up in initially clinically healthy men [7].

We have previously shown that there is a stronger relation between inflammation (high-sensitive C-reactive protein, hsCRP) and a quantitative measure of atherosclerosis, such as plaque size, in the femoral artery compared to the carotid artery [8]. However, no data are available regarding inflammation (hsCRP) and echogenicity reflecting the qualitative measure of plaques in the femoral artery.

Recently, it has been shown that microinflammation, as assessed by hsCRP, is associated with both progression of atherosclerosis [9] as well as the metabolic syndrome (MetS) [10,11]. However, it is at present not known if it is the grade of atherosclerosis per se or the MetS that contribute most to the “microinflammatory” state seen in these subjects; or which association hsCRP and the MetS have with plaque echogenicity.

Ex vivo studies has shown that echolucent plaques have increased levels of lipids and oxidized low-density lipoprotein (oxLDL) [12–14]. Assays to measure different epitopes on oxLDL in the circulation have been developed and have been shown to be associated with coronary heart diseases [15,16]. So far, however, the association between circulating oxLDL and plaque echogenicity has not been studied.

Since unstable plaques ex vivo previously have been shown to be rich in inflammatory cells and oxidized LDL we hypothesized that circulating markers of inflammation (hsCRP) and oxidative modification of lipids (oxLDL) were associated with the occurrence of echolucent rather than echogenic plaques. In the present study results from femoral artery are reported; results on carotid atherosclerosis are as yet unpublished observations.

2. Materials

This cross-sectional study outline has previously been described in detail [17].

2.1. 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 that was aimed to examine whether insulin resistance is associated with atherosclerosis [18]. From this sample of 1188 men, two groups were identified. One group of 237 men had known diabetes mellitus, hypertension, hyperlipidemia or CVD. The other group consisted of 391 clinically healthy men, randomly selected from the population sample, and with varying degrees of obesity and insulin resistance.

2.2. Re-examination (present study)

Three years after the screening examination the present study was performed. In the group of men with known diabetes mellitus, increased cardiovascular risk and CVD, 231 of the 237 men at screening were alive. They were invited to the present study, and 168 participated. In the other group of 391 men at screening, 387 men were alive, and of those 345 participated in the present study.

Hence, a total number of 513 men, 61-year-old with Swedish ancestry were examined.

The examinations were performed in the morning at two occasions with an interval of 1 week. The subjects had fasted overnight and underwent examination with blood test and ultrasound measurement of the right femoral artery.

All men received both written and oral information before consenting to participate in the study. The study was approved by the ethics committee at Sahlgrenska University Hospital.

3. Methods

Information on general health and smoking habits were obtained by a self-administered questionnaire.

Venous blood samples were drawn after a fasting period of 10–12 h, kept at room temperature in 30 min before the serum was separated by centrifugation and thereafter immediately frozen in aliquots at -70°C .

3.1. Laboratory examinations

Lipids and blood glucose were measured by standard methods [8]. hsCRP was measured by commercially available ELISA kits (Medix Biomedica, Kauniainen, Finland).

oxLDL was measured on plasma which had been stored at -70°C as previously described [19]. oxLDL was measured by a commercially available sandwich ELISA (Merckodia, Uppsala, Sweden) utilizing the same specific murine monoclonal antibody, mAb-4E6, as in the assay described by Holvoet et al. [20]. 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 U/L versus 74.1 U/L, $p<0.05$). Hence, in order to avoid systematic difference 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 ± 0.4 (range 5.4–6.7) and 12.7 ± 0.7 (range 11.9–12.7). All analyses were performed at the Wallenberg Laboratory.

3.2. Ultrasound measurement

The visit for the ultrasound examination always took place in the morning. The examination was performed with an ultrasound scanner (Acuson 128; Acuson, Siemens, Mountain view, CA, USA) with a 7 MHz linear transducer with the aperture of 38 mm [21]. An 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. The laboratory technician performing the ultrasound examination was blinded as to the clinical status of each subject. The right femoral artery were scanned distal to the inguinal ligament along a section approximately 4 cm proximal and 1 cm distal to the flow divider (the site where the artery divides into the superficial and profound femoral arteries) to assess the occurrence of plaques [21]. A plaque was defined as a distinct area with an IMT >50% thicker than that of neighbouring sites (visually judged). A semi-quantitative subjective scale was used to grade the size of plaques as grade 0, no plaque; grade 1, one or more small plaques (less than approximately 20 mm² in the femoral artery); grade 2, moderate to large plaques (the differentiation between grades 1 and 2 was made subjectively in most cases, and quantitative measurements were made by the computerized system only when the correct classification was not obvious to the observer) and grade 3, plaques giving flow disturbances [21]. In the present study, 9 subjects of 513 had missing ultrasound examinations because of unwillingness to participate. One plaque of grade 3 was found in the femoral artery. Therefore, plaques of grades 2 and 3 were merged into 1 group of moderate to large plaques. This analysis included plaques in the near wall as well as the far wall of the vessel. Assessment of plaque size was possible in 187 plaques. In a re-reading reproducibility study of femoral plaque size, there were a high correlation coefficient ($r_s=0.86$; $n=45$) [8].

3.3. Assessment of plaque echogenicity

The assessment of plaque echogenicity was based on the version of classification proposed by Gray-Weale et al. [6] where plaques are graded from 1 to 4:

- (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%).
- (4) Uniformly echogenic lesions (equivalent to homogeneous).

Two examiners simultaneously did the classification of plaque echogenicity performed in all subjects. In cases of discrepancy between the two readers, a third reader was consulted. Very few subjects were classified as groups 1 and 4 in the present study ($n=8$ and $n=1$, respectively). Hence, groups 1 and 2, and 3 and 4 were merged into two groups defined as predominantly echolucent and predominantly echogenic. Assessment of plaque echogenicity was possible in 178 of 187 plaques.

In order to investigate the reproducibility of assessment of plaque echogenicity in the femoral artery, 50 plaques were analysed twice by the same examiner. The results showed a good agreement between the first assessment of plaque echogenicity and the second one made 6 months later ($r=0.90$) [7].

3.4. Definition of the metabolic syndrome

The definition of the MetS suggested by the working group National cholesterol education program's adult treatment panel III (NCEP), was used [22]. The MetS is defined as at least three of the following factors: (1) fasting blood glucose ≥ 5.6 mmol/L (plasma glucose ≥ 6.1 mmol/L), (2) blood pressure $>130/85$ mmHg or antihypertensive medical treatment, (3) plasma triglycerides ≥ 1.7 mmol/L, (4) HDL cholesterol <1.0 mmol/L for men and <1.3 mmol/L for women and (5) waist >102 cm for men and >88 cm for women.

3.5. Statistics

All statistics were analysed by using SPSS for Windows 10.0 (SPSS Inc., Chicago, IL, USA). The characteristics of the subjects are described as numbers, percentage, 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.

Pearson Chi-square test was used for comparison of nominal and ordinal variables and Mantel–Haenszel for test of trends in Figs. 1 and 2. A multiple logistic regression analysis was applied to test the relationship between single dependent variables and explanatory variables. A bivariate Spearman correlations analyses was applied to measure how variables

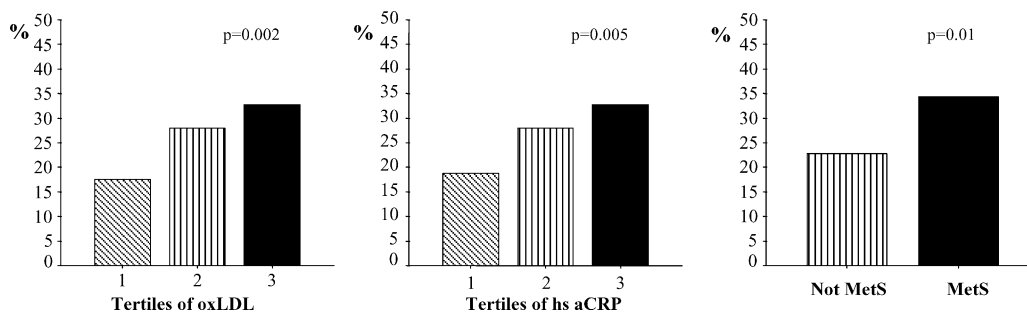


Fig. 1. Occurrence of echolucent femoral plaques in relation to circulating levels of oxLDL, hsCRP and to the MetS (y/n).

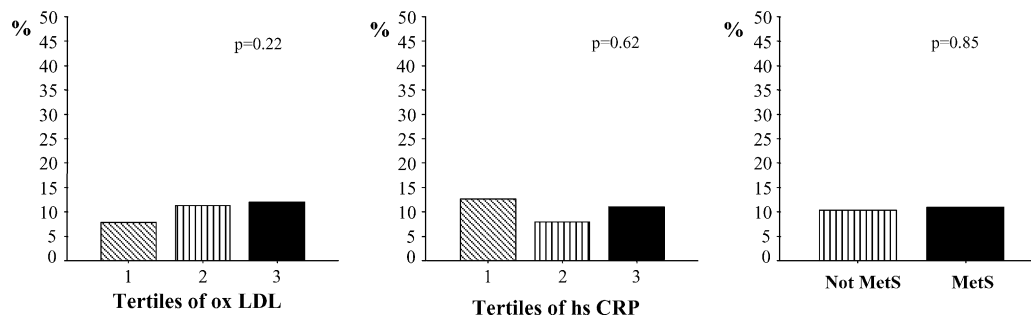


Fig. 2. Occurrence of echogenic femoral plaques in relation to circulating levels of oxLDL, hsCRP and to the MetS (y/n).

are related. 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.

4. Experimental results

Characteristics for the entire study group ($n = 513$) are presented in Table 1. Diabetes occurred in 74 men (14%). The median value in the total study group of hsCRP was 1.32 mg/L. No significant association between oxLDL and current smoking was observed. Subjects with the MetS had higher levels of oxLDL than subjects without the MetS ($p < 0.001$).

The mean value of hsCRP in men with no femoral plaques was 1.24 mg/L, echogenic plaques 1.27 mg/L and echolu-

cent plaques were 1.67 mg/L, respectively. The mean value of oxLDL in men with no femoral plaques was 93.2 U/L, echogenic plaques 100.9 U/L and echolucent plaques were 107.1 U/L, respectively. Significant differences between men with no femoral plaques and echolucent femoral plaques were observed for hsCRP ($p < 0.001$) and oxLDL ($p < 0.05$). There was a trend towards higher frequencies of MetS (y/n) and current smoking in men with echolucent plaques, as compared to subjects with echogenic and no plaques (y/n) ($p = 0.007$ and $p < 0.001$, respectively). There was no significant difference between the subjects with no plaques, echogenic and echolucent plaques in the mean value of LDL cholesterol or systolic blood pressure.

4.1. Risk factors associated with echolucent femoral artery plaques

A higher frequency of echolucent femoral plaques was observed with increasing levels of oxLDL and hsCRP ($p = 0.002$ and $p = 0.005$, respectively; Fig. 1) but not with increasing levels of LDL cholesterol or systolic blood pressure ($p = 0.11$ and $p = 0.59$, respectively). Subjects with the MetS and current smokers had a significantly higher frequency of echolucent plaques, as compared to subjects without the MetS and non-smokers ($p = 0.01$ and $p < 0.001$, respectively; Fig. 1).

4.2. Risk factors associated with echogenic femoral artery plaques

No significant association was observed between circulating levels of oxLDL, hsCRP (Fig. 2) or LDL cholesterol in relation to occurrence of echogenic femoral plaques. Furthermore, no significant association was observed between echogenic plaques, the MetS (Fig. 2), current smoking or systolic blood pressure.

4.3. Multiple logistic regression analysis

In a multiple logistic regression analysis the dependent variable was the presence of echolucent femoral artery plaques and the covariates were tertiles of oxLDL, tertiles of hsCRP, the MetS (y/n) and current smokers (y/n). Tertiles

Table 1
Characteristics

Variable	Study subjects ($n = 513$)
Age (years)	61.6 ± 0.51
BMI (kg/m ²)	27.2 ± 4.40
Waist (cm)	99.5 ± 12.4
SBP (mmHg)	131 ± 19
HDL cholesterol (mmol/L)	1.25 ± 0.35
LDL cholesterol (mmol/L)	3.74 ± 0.94
Serum triglycerides (mmol/L)	1.39 (0.42–8.91)
Blood glucose (mmol/L)	5.4 ± 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 femoral artery	
No, n (%)	305 (60)
Small, n (%)	27 (5)
Moderate/large, n (%)	160 (31)
Plaque morphology in the femoral artery	
Echogenic	51 (10)
Echolucent	127 (25)

Data are presented as the mean value ± S.D., 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.

of oxLDL and current smokers (y/n) turned out to be independent associated with echolucent femoral artery plaques (OR = 1.48 (95% CI 1.13–1.93), $p = 0.004$; OR = 2.57 (95% CI 1.50–4.39), $p = 0.0006$).

4.4. Risk factors associated with hsCRP levels

In a univariate analysis, oxLDL and systolic blood pressure were significantly associated with hsCRP ($r = 0.10$, $p < 0.05$; $r = 0.20$, $p < 0.001$, respectively). In addition, the frequency of the hsCRP values above median were higher in subjects with the MetS and in current smokers, as compared to subjects without the MetS and non-smokers ($p < 0.001$ and $p < 0.05$, respectively).

In a multiple logistic regression model with the hsCRP (above/below median) as a dependent variable and tertiles of oxLDL, current smokers (y/n), MetS (y/n) and echolucent femoral plaques (y/n) as covariates, only the MetS was independently associated with hsCRP levels above median (OR = 3.4 (95% CI 2.2–5.3), $p < 0.001$). To further elucidate which component in the MetS predicted higher hsCRP levels, we included tertiles of oxLDL, current smokers (y/n), echolucent femoral plaque (y/n), tertiles of systolic blood pressure and tertiles of WHR in the model. Doing that only high WHR, as a measure of obesity, turned out to be independently associated with hsCRP levels above median (OR = 2.7 (95% CI 2.1–3.5), $p < 0.001$).

5. Discussion

The main results of the present study support our hypothesis that occurrence of echolucent but not echogenic femoral plaques was associated with increasing plasma levels of oxLDL and hsCRP. Subjects with the MetS and current smokers also had higher frequency of echolucent plaques, as compared to subjects without the MetS and non-smokers. In a multiple logistic regression analysis oxLDL and current smokers turned out to be independently associated with echolucent femoral plaques after taking into account LDL cholesterol, blood pressure, hsCRP and occurrence of the MetS. There was no association between echogenic plaque morphology and the previously mentioned variables.

5.1. Plaque histology and echogenicity in relation to CV events

The value of plaque morphology, assessed by B-mode ultrasound predicting cardiovascular events is now widely accepted [23–25]. In a histopathological study by Schulte-Altdorneburget et al. plaque specimens were examined and compared with B-mode ultrasound where echolucent types of plaques rarely showed fibrosis but showed a high proportion of lipids and/or recent haemorrhage [26]. In a clinical prospective study, Griffin et al. showed that total plaque burden and presence of echolucent femoral plaques were found

to be independent predictors of risk of cardiovascular events [27]. In addition, we have previously shown that the risk of having a CV event was to a large extent confined to those men with an echolucent plaque in the femoral artery at baseline [28].

5.2. Echolucent plaque formation in relation to oxLDL

The role of modified LDL in atherogenesis has widely been studied [29]. The most extensively studied of these is LDL oxidation [30,31]. We have previously shown that high levels of circulating oxLDL is a predictor of progressive subclinical atherosclerosis in healthy middle-aged men [32].

Holvoet et al. demonstrated in animal models that the oxidation of LDL cholesterol indeed occurs in the arterial wall and not in the blood [33,34]. In an ex vivo study it was showed that plaque oxLDL was nearly 70 times higher than plasma oxLDL and it was found that high plasma oxLDL and plaque levels of oxLDL were correlated with the vulnerability to rupture of atherosclerotic lesions [35].

The source of oxLDL in plasma could be the direct release of oxLDL from ruptured or permeable plaques, ischemic injury to damaged cell membranes or a turnover of oxLDL in newly formed or progressing lesions in the arterial tree. This is in line with our results indicating that high levels of circulating oxLDL in plasma is independently associated with the echolucent plaque phenotype that has previously been shown to be more vulnerable to rupture.

Interestingly, oxLDL seems to be the common denominator for the occurrence of echolucent plaques both in the femoral and the carotid artery (results on carotid atherosclerosis are as yet unpublished observations). In a recent report, statins significantly lowered the levels of circulating oxLDL independent of their lipid-lowering effects [36] suggesting oxLDL even to be an important biomarker in monitoring the effects of medication in atherosclerosis.

5.3. The role of inflammation (hsCRP) in atherogenesis

In multivariate analyses, only the MetS was independently associated with hsCRP levels above median, even after taking into account echolucent femoral plaques. Hence, to further elucidate which component in the MetS was associated with hsCRP levels above median, we included blood pressure and WHR, as a measure of obesity in the model. In this case only WHR was significantly associated with hsCRP levels above median.

It has consistently been shown that hsCRP is a well established biomarker of CV risk [37–39]. Furthermore, it has recently been shown that CRP lowering by statins decrease event rate in patients with acute coronary syndrome, independently of the LDL lowering effect [40]. In addition to that, reduction of hsCRP has been shown to be associated with a lower progression rate of atherosclerosis in the coronary arteries, as assessed by intravascular ultrasound [9]. How-

ever, the results of the present study showed that the most important factor for determining hsCRP was the MetS and not atherosclerosis per se. Especially WHR, as a measure of central obesity, turned out to be an independent predictor of hsCRP. In the same model the occurrence of echolucent plaques was not associated with high hsCRP levels. These results suggest that high hsCRP levels, at least in a population-representative sample on middle-aged Swedish men, mainly are driven by obesity than by the presence of echolucent atherosclerotic plaques in the vascular wall. It is well known that adipose tissue produces a wide range of cytokines and factors that to a large extent contribute to the microinflammation seen in patients with diabetes and the MetS [41,42]. Our results corroborate these findings.

The limitation of the present study is that it is cross-sectional and only included 61-year-old Caucasian men. It should also be noted that in this cohort of men the numbers of subjects with echogenic plaque was much lower as compared to the number of subjects with echolucent plaques. Therefore, the risk for type II errors regarding the non-significant results between risk factors and echogenic plaques should be considered.

In conclusion, the main results of the present study are that echolucent but not echogenic femoral plaques were associated with oxLDL, hsCRP, MetS and current smoking. oxLDL and current smoking were independently associated with echolucent plaques after taking into account other well established CV risk factors and hsCRP. The most important predictor for high levels of hsCRP was found to be with the MetS, especially WHR as a measure of obesity.

The results of the present study support our hypothesis that circulating oxLDL is a marker of an unstable plaque phenotype in vivo in man. However, to elucidate causality prospective as well as further mechanistic studies are clearly needed. Larger population studies are needed as well to elucidate if the occurrence of echolucent plaques combined with elevated levels of oxLDL might be a useful tool to identify individuals at high risk for developing cardiovascular diseases.

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