Autoantibodies against oxidized low density lipoproteins in patients with stable angina, unstable angina or peripheral vascular disease

Pathophysiological implications

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Background Antibody antioxidized low density lipoproteins (oxLDL) might play a role both in atherogenesis and in the pathogenesis of acute coronary syndromes.

Methods and Results Antibody titres to oxLDL and levels of C-reactive protein were compared in unstable angina, stable angina or peripheral artery disease. Antibody titres to LDL oxidated by CuSO₄ for 2, 4 and 18 h (Cu-oxLDL-Ab₂-₄-₁₈) or by peroxidase (HRP-oxLDL-Ab) were assessed by ELISA. Cu-oxLDL-Ab₂-₄-₁₈ were consistently higher in peripheral artery disease than in unstable angina (P < 0·001, P < 0·001, P = 0·01, respectively) or in stable angina (P < 0·001, P = 0·01, P = ns) but similar in unstable and stable angina. Accordingly, HRP-oxLDL-Ab were higher in peripheral artery disease than in unstable angina (P < 0·001) or stable angina (P = 0·04) but similar in unstable and stable angina. The number of arterial stenoses was higher in peripheral artery disease than unstable and stable angina (P < 0·01). Cu-oxLDL-Ab and HRP-oxLDL-Ab correlated with the severity of atherosclerosis (P < 0·01, R = 0·4; P = 0·02, R = 0·3 respectively). Conversely, C-reactive protein levels were higher in unstable than in stable angina (P < 0·001) or in peripheral artery disease (P < 0·03) but similar in stable angina and peripheral artery disease and did not correlate with the severity of atherosclerosis.

Conclusion The autoimmune response to oxLDL is likely to play an important role in atherogenesis but not in precipitating acute coronary syndromes.


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Key Words: Lipoproteins, antibodies, inflammation, coronary artery disease, peripheral artery disease.

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Introduction

Several studies have shown that acute coronary syndromes are associated with the activation of inflammatory cells, including monocytes¹,², neutrophils¹,³,⁴ and lymphocytes⁵, that are detectable in blood samples obtained from peripheral veins³–⁵ or from the coronary sinus¹ and in tissue specimens from unstable athero-sclerotic plaques²,⁶,⁷. This inflammatory outburst does not appear to be secondary to ischaemia–reperfusion⁴,⁸, plaque disruption⁹ or thrombin generation¹⁰. Therefore, it might be caused by a primary, yet unknown, inflammatory stimulus. Previous studies failed to demonstrate Cytomegalovirus replication in the unstable atherosclerotic plaque¹¹ or higher levels of the antibody anti-Helicobacter pylori in patients with unstable angina compared to patients with stable angina¹², thus suggesting that these infectious agents are unlikely to play an important role in the activation of inflammatory cells associated with acute coronary syndromes. Furthermore, the pilot observations that antibiotic treatment active against Chlamydia pneumoniae could reduce the...
risk of short- and medium-term complications of acute coronary syndromes\cite{13,14}, were not confirmed by more recent trials on a larger scale\cite{15}, thus suggesting that the overall pathogenetic role of *Chlamydia pneumoniae* also might be limited.

The triggers of the inflammatory response associated with acute coronary syndromes, however, may not necessarily be an infectious agent. Oxidized low density lipoproteins (oxLDL) represent a key component of the atherosclerotic plaque and a recent study has shown that increased plasma levels of malondialdehyde-modified LDL are higher in patients with acute coronary syndromes than in patients with stable angina\cite{16}. Furthermore, the titre of autoantibodies to oxLDL is an independent predictor of the progression of carotid atherosclerosis\cite{17}. In this study we addressed the question whether the humoral immune response to oxLDL plays a role in atherogenesis and in the pathogenesis of acute coronary syndromes; to this end we compared the titre of autoantibodies to oxLDL in patients with unstable angina, in patients with stable angina and in patients with severe peripheral artery disease.

**Methods**

**Patient population**

A total of 106 patients were studied: 58 patients with unstable angina (Group 1), 28 with chronic stable angina (Group 2) and 20 with peripheral artery disease (Group 3).

**Group 1.** Fifty-eight consecutive patients (38 men, aged 64 ± 9 years) admitted to our Coronary Care Unit with Braunwald class IIIIB unstable angina, defined as more than two episodes or one episode of angina at rest lasting >20 min during the preceding 24 h, with a diagnostic ST-segment shift during chest pain and no evidence of myocardial infarction detected with enzymatic markers (creatine kinase). All patients underwent coronary angiography.

**Group 2.** Twenty-eight consecutive patients (23 men, aged 60 ± 8 years) with chronic stable angina, defined as effort angina with at least a 1 year’s stable pattern of symptoms, admitted to our Institute to undergo elective coronary angiography.

**Group 3.** Twenty consecutive patients (12 men, aged 63 ± 8 years) with Leriche-Fontaine stage IIIB-III peripheral artery disease with claudication after <100 m walking, admitted for revascularization by percutaneous procedure by Rotablator or surgery. All patients underwent peripheral angiography.

Exclusion criteria for all patients were left bundle branch block invalidating the ST segment analysis, left ventricular ejection fraction <30%, valvular heart disease, recent surgery (<3 months) including coronary artery bypass surgery, recent trauma (<3 months), recent myocardial infarction (<3 months), known malignancies, known haematological and immunological disorders, and any other inflammatory condition likely to be associated with acute phase response. Specific exclusion criteria for Groups 1 and 2 were history, clinical or Doppler evidence of peripheral artery disease. Specific exclusion criteria for Group 3 were history of angina or myocardial infarction, ECG evidence of a previous myocardial infarction, echocardiographic evidence of regional wall motion alterations and evidence of trophic limb lesions to rule out necrosis as a potential cause of inflammation.

For all three groups of patients, stenoses detected at angiography were considered significant if they reduced the internal lumen diameter >70%. Cigarette smoking was defined as current smoking status or cigarette withdrawal <2 months; hypertension as blood pressure >140/90 mmHg or current treatment with antihypertensive drugs; diabetes as fasting serum glucose levels >126 mg % or current use of antidiabetic drugs; presence of a family history of ischaemic heart disease as presence of at least one first degree relative <60 years with ischaemic heart disease.

**Blood sampling and laboratory assays**

The protocol was approved by the Ethics Committee of the Catholic University and all patients gave their written informed consent. Peripheral blood samples were taken on hospital admission.

Antioxidatively-modified LDL IgG antibodies were measured according to methods previously described\cite{18}. Briefly, three different antigens were employed to coat multiwell ELISA plates, namely non-modified native LDL oxidized after serum ultracentrifugation, LDL oxidized with CuSO4 (Cu-oxLDL) for 2, 4 or 18 h and LDL oxidized with the combination of peroxidase and hydrogen peroxide (HRP-oxLDL). This choice was made in order to mimic the formation of some antigenic epitopes formed in vivo during LDL oxidation\cite{19,20}. The quantitation of antioxLDL antibody levels was performed using ELISA techniques and serum diluted 1:20. Results were expressed in arbitrary units (AU) as the difference between the spectrophotometric readings of antimonified and antinaive antigen wells, in order to minimize the possible detection of false positive values due to cross-reactivity with both epitopes.

C-reactive protein was assayed in an ultrasensitive C-reactive protein assay by nephelometry (Dade-Behring, BN-II, Marburg, Germany) with a sensitivity of 0.2 mg · l\(^{-1}\). Troponin T, a specific marker of myocardial necrosis, was measured to rule out the possible role of myocardial cell damage in inducing the inflammatory response. Troponin T was measured by use of a commercial enzyme immunoussay (Boehringer Mannheim).

**Statistical analysis**

Since C-reactive protein values and antibody titres were not normally distributed, they were expressed as median
and interquartile range and were compared using the Kruskal–Wallis test. Discontinuous variables were compared by chi-squared test. Continuous variables are expressed as mean ± SD and were compared using one-way ANOVA. Correlations between variables were carried out using Spearman’s rank correlation test. A probability value of $P<0.05$ was considered statistically significant. All tests were two tailed.

**Results**

Demographic, clinical and angiographic findings of the three groups of patients are shown in Table 1. The three groups were similar in age, sex, prevalence of smoking habit, hypertension, family history of ischaemic heart disease, diabetes and blood cholesterol levels.

![Figure 1](https://example.com/figure1.png)

*Figure 1* Peripheral samples on hospital admission in unstable angina (empty boxes), stable angina (filled boxes) and peripheral artery disease (greyed boxes). Data are shown as median values and interquartile range. Antibody titres are expressed in arbitrary units (AU) as the difference between the spectrophotometric readings of anti-modified and anti-naive antigen wells in the ELISA plates. AU arbitrary units; Cu-LDL: LDL oxidized with CuSO$_4$; 2 h, 4 h and 18 h: oxidation times; HRP-LDL: LDL oxidized with the combination of peroxidase and hydrogen peroxide; CRP: C-reactive protein. *$P<0.001$ vs unstable and stable angina; †$P<0.001$ vs unstable angina; ‡$P=0.01$ vs stable angina; §$P=0.01$ vs unstable angina; ¶$P=0.04$ vs stable angina; **$P<0.03$ vs peripheral artery disease.

**Table 1** Demographic, clinical and angiographic findings

<table>
<thead>
<tr>
<th></th>
<th>Group 1 (n=58)</th>
<th>Group 2 (n=28)</th>
<th>Group 3 (n=20)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age; years, mean ± SD</td>
<td>64 ± 9</td>
<td>60 ± 8</td>
<td>63 ± 8</td>
<td>ns</td>
</tr>
<tr>
<td>Sex; male, n (%)</td>
<td>38 (70)</td>
<td>23 (85)</td>
<td>12 (60)</td>
<td>ns</td>
</tr>
<tr>
<td>Family history of CAD; n (%)</td>
<td>24 (41)</td>
<td>10 (34)</td>
<td>4 (20)</td>
<td>ns</td>
</tr>
<tr>
<td>Smoking; n (%)</td>
<td>21 (39)</td>
<td>7 (26)</td>
<td>10 (50)</td>
<td>ns</td>
</tr>
<tr>
<td>Hypertension; n (%)</td>
<td>30 (55)</td>
<td>24 (44)</td>
<td>11 (55)</td>
<td>ns</td>
</tr>
<tr>
<td>Diabetes; n (%)</td>
<td>17 (31)</td>
<td>10 (37)</td>
<td>11 (55)</td>
<td>ns</td>
</tr>
<tr>
<td>Cholesterol levels; mg . dl$^{-1}$, mean ± SD</td>
<td>211 ± 50</td>
<td>222 ± 46</td>
<td>193 ± 37</td>
<td>ns</td>
</tr>
<tr>
<td>Stenoses; mean ± SD</td>
<td>2.8 ± 1.5</td>
<td>2.8 ± 1.2</td>
<td>5.1 ± 1.9</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

CAD=coronary artery disease.
Accordingly, antibody titres to HRP-oxLDL were higher in Group 3 than in Groups 1 and 2 (0·28 AU [0·19–0·73] vs 0·15 AU [0·10–0·21], P < 0·001 and 0·15 AU [0·08–0·35], P = 0·04, respectively) but similar in Groups 1 and 2 (P = ns). The severity of obstructive atherosclerosis paralleled the levels of antibodies to oxLDL: indeed, patients in Group 3, with the higher levels of autoantibodies, had significantly more arterial stenoses (5·1 ± 1·9%) than patients in Groups 1 and 2 (2·8 ± 1·5 and 2·8 ± 1·2; P < 0·01) (Table 1). Furthermore, a significant correlation was found between levels of antibodies to oxLDL and the number of arterial stenoses in the overall population (for Cu-oxLDL following 2 h of oxidation P < 0·01, R = 0·4; for Cu-oxLDL following 4 h of oxidation P = 0·02, R = 0·3; for Cu-oxLDL following 18 h of oxidation P = ns; for HRP-oxLDL P = 0·02, R = 0·3).

Conversely, C-reactive protein levels were higher in Group 1 than in Groups 2 and 3 (8·7 mg·L⁻¹ [3·7–22·3] vs 2·9 mg·L⁻¹ [2·3–4·7], P < 0·001 and 4·2 mg·L⁻¹ [2·1–7·5], P < 0·03, respectively) but similar in Groups 2 and 3 (P = ns). It is worth noting that the numbers of stenoses were similar in Groups 1 and 2 (P = ns). C-reactive protein levels did not correlate with the number of arterial stenoses (P = ns, R = 0·16). Troponin T was <0·1 mg·dL⁻¹ in all samples.

**Discussion**

This study confirms that unstable angina is associated with systemic evidence of inflammation. Indeed, serum levels of C-reactive protein, a prototypic acute phase reactant, were significantly higher in patients with unstable than in patients with stable angina or with severe peripheral artery disease. More importantly, the titre of antibodies to oxLDL were higher in patients with peripheral artery disease than in patients with ischaemic heart disease, who were likely to present less atherosclerotic burden.

Oxidative modification of LDL alters its structure, allowing LDL to be taken up by scavenger receptors on macrophages, endothelial and smooth muscle cells[19]. Uptake of oxidatively modified LDL by macrophages seems to bypass a negative feedback mechanism. This unrestricted uptake allows increased influx of LDL into macrophages, creating foam cells, the earliest recognized abnormality in atherosclerotic deposition[20]. Indeed, antibodies against epitopes of oxLDL recognize material in atherosclerotic lesions in man, but not in normal arteries[21,22]. Of note, oxLDL extracted from human atherosclerotic plaques exhibit nearly all of the physico-chemical and immunological properties of in vitro ox-LDL[23,24]. More importantly, alteration of LDL by oxidative modification changes the apolipoprotein B-100 structure[25] and this alteration of the protein structure makes LDL more immunogenic[26]. OxLDL are able to induce both the humoral and cellular immune response. Raised serum levels of in vitro oxLDL have been found in both non-hypercholesterolaemic and hypercholesterolaemic patients with arterial hypertension[26], in both diabetic and non-diabetic patients with obstructive coronary atherosclerosis at angiography[27,28], in asymptomatic subjects who show accelerated 2-year progression of carotid atherosclerosis[17,29] and in patients with early onset peripheral artery disease[30]. Furthermore lymphocytes from human atherosclerotic plaques recognize oxLDL in vitro[31]. Taken together, these findings suggest that lipid oxidation and the subsequent induction of an immune response, in addition to the direct proinflammatory effects of oxLDL themselves[32–33], are all mechanisms likely to play an important role in atherogenesis.

Our study confirms and expands these previous findings by showing that in patients with severe peripheral artery disease the titre of antibodies to oxLDL is higher than that observed in patients with coronary artery disease, probably reflecting the greater atherosclerotic burden in the former. Indeed, not only are peripheral arteries larger than coronary epicardial arteries, but our patients with peripheral artery disease had significantly more arterial stenoses than patients with coronary artery disease. However, several factors may affect serum antibody titre. Indeed, the titre of circulating autoantibodies reflects a balance between the amount of antibody generated and released into the circulation and the consumption of that antibody, either specifically (binding to specific antigens) or non-specifically. In addition, antibody production is under genetic control[34]. Nevertheless, the higher titres of antibodies to oxLDL in patients with more extensive atherosclerosis at angiography appears to confirm that the humoral autoimmune response against oxLDL antigens contained in the atherosclerotic plaque plays a role in atherogenesis. Whether it plays a beneficial or a detrimental role cannot be deduced from the results of our study. Of note, previous studies have shown the antiatherogenic effects of immunization with LDL and ox-LDL in animal models of atherosclerosis[35–36], suggesting that an immune response to oxLDL may not always be adverse.

In contrast, C-reactive protein levels were similar in patients with peripheral artery disease and in patients with stable angina, thus suggesting that either C-reactive protein does not correlate with the atherosclerotic burden, or a potential correlation is less detectable that that observed with anti-oxLDL. Our findings do not confirm those of Tataru et al[37] who found C-reactive protein levels slightly higher in patients with ischaemic heart disease and pre-clinical or clinical evidence of peripheral artery disease, than in patients with ischaemic heart disease but without evidence of peripheral artery disease. This weak but significant association between C-reactive protein levels and atherosclerotic burden found by Tataru et al[37] but not in our study may be due to the small number of patients and to lack of assessment of pre-clinical peripheral artery disease which is likely to increase the background noise in our study.

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In our study, however, C-reactive protein levels were higher in patients with unstable angina than in patients with stable angina or peripheral artery disease, thus confirming that coronary instability is frequently associated with an inflammatory outburst. The sudden activation of inflammatory cells in a chronic atherosclerotic plaque may result in its disruption followed by coronary thrombosis[2,7]. The inflammatory stimuli triggering the activation of inflammatory cells in this setting are still elusive[8,9]. The information currently available does not support the notion that the inflammatory outburst associated with acute coronary syndromes is caused by reactivation of infectious agents[1,13]. In this study, we failed to find higher levels of antibodies antioxLDL in patients with unstable angina compared to those with stable angina, despite a similar severity of coronary atherosclerosis at angiography and higher levels of C-reactive protein in the former. Our findings suggest, therefore, that an autoimmune response to oxLDL is an unlikely trigger of the inflammatory outburst associated with acute coronary syndromes. Nevertheless our study cannot exclude the possibility of a direct pro-inflammatory effect of oxLDL on endothelial cells and monocytes[32,33], also supported by the observation of elevated plasma levels of malondialdehyde-modified LDL in patients with acute coronary syndromes[10]. Thus, it remains quite possible that local accumulation of ox-LDL in an atherosclerotic plaque contributes to the inflammatory response that precipitates plaque rupture.

An important limitation of this study is the lack of peripheral angiography in anginal patients and of coronary angiography in patients with peripheral artery disease. Therefore lack of history of peripheral vascular disease in the former and of ischaemic heart disease in the latter does not allow us to exclude subclinical disease. Yet the possible presence of subclinical disease can only increase the background noise, thus preventing us from identifying potential differences among groups. However, it should not affect the significant differences among groups found in the present study. Another limitation is that patients with peripheral artery disease may have plaques that are disrupted with thrombi in spite of apparently stable symptoms; accordingly we demonstrated the presence of ongoing activation of the coagulation system in patients with peripheral artery disease[39]. Once again this potential peripheral instability may increase background noise, but it is unlikely to affect significant differences among groups. Finally, patients with peripheral artery disease had a lower prevalence of a family history of ischaemic heart disease and a higher prevalence of smoking and diabetes than patients with coronary artery disease. These differences, rather than atherosclerosis extent, might account for differences in antibody levels between groups. It is worth noting, however, that the differences in risk factors were not statistically significant.

In conclusion, our study indicates that different inflammatory mechanisms might operate in atherogenesis and in the pathogenesis of acute coronary syndromes. The autoimmune response to oxLDL is likely to play an important beneficial and/or detrimental role in atherogenesis but not in precipitating acute coronary syndromes. The quest for the triggers of the inflammatory outburst associated with acute coronary syndromes continues.

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References


Oxidized LDL and atherosclerosis


