Direct Association between C-reactive Protein Serum Levels and Endothelial Dysfunction in Patients with Claudication

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Objectives. To evaluate the relationship between C-Reactive Protein (hsCRP), a serum marker of inflammation, and endothelial dysfunction in patients with intermittent claudication.

Design, Patients and Methods. Cross-sectional study with stratified sampling on dependent variables of age, genre, hypertension, hyperlipidemia, diabetes, smoking status and ankle-brachial index (ABI) to select 156 patients from a target population of 4,100 patients with claudication. We assessed the flow-mediated arterial dilation (FMAD) as a reporter of endothelial function and plasma levels of hsCRP and fibrinogen.

Results. Patients with a FMAD \(<3\% (range for the lowest 5\% of healthy subjects) had increased levels of plasma hsCRP (6.3 vs 2.3 mg/L; \(p<0.05\)) and fibrinogen (351 vs 302 mg/L; \(p<0.05\)) in comparison to those with FMAD \(>3\%\). There was a negative correlation between hsCRP and FMAD (\(r = -0.465; p<0.05\)).

Conclusion. Impaired endothelial dysfunction is association with increased plasma concentrations of inflammatory markers, and both may have a role in the aetiopathogenesis of peripheral arterial disease.

Keywords: C-reactive protein; Inflammatory etiology of atherosclerosis; Peripheral arterial occlusive disease; Inflammation markers.

Introduction

We are increasingly aware that inflammation plays a major role in the pathogenesis of peripheral arterial disease (PAD).1 Atherosclerosis can be considered as a chronic inflammatory disease of the arterial system.2 Moreover, recognizing the inflammatory nature of atherosclerosis has important clinical implications. Only 50% of the patients with PAD present with hyperlipidemia,3 which is the main objective of preventive treatment; and the prevalence of classical risk factors such as diabetes, smoking and high blood pressure is only slightly higher in patients diagnosed with coronary arterial disease (CAD) or PAD, compared with patients of the same age without these diseases.4 C-Reactive Protein (CRP) and fibrinogen are non-specific plasma markers in the acute phase of inflammation and have been of special interest over the past few years for several reasons. These reasons include their potential direct role in the pathogenesis of non-specific circulatory inflammation,5,6 their role as disease markers7,8,9 and disease progression markers1 and their use for monitoring potential treatments.10 However, it has not yet been established by which the mechanisms the high protein levels (CRP and fibrinogen) in the acute stage of inflammation are involved in the generation, development and severity of PAD.

One of the possible mechanisms by which inflammation leads to PAD could be through alteration of the functional properties of the endothelium, triggering a series of changes that are directly related to the onset, progression and clinical severity of atherosclerosis. As our group has shown,11 endothelial function can be evaluated in a reliable and simple way by determining the arterial vasodilator response to a pharmacological or physical stimulus, such as sustained ischaemia, through the measurement of nitric oxide levels in the brachial artery.12,13

Previous studies have noted the existence of a relationship between classical cardiovascular risk factors...
(CVRF) (age, hypertension, diabetes, hyperlipidemia and smoking) and reduced endothelium-mediated arterial reactivity, measured in the brachial artery, even in the absence of symptomatic atherosclerotic disease. Moreover, there is a positive correlation of peripheral endothelial function, measured in the brachial artery, with that in the coronary circulatory bed. Further reduction of endothelial reactivity is associated with increased cardiovascular risk. Altered endothelium-mediated arterial dilation is related to a chronic low-grade inflammatory status in patients with CAD. Therefore, the vasomotor dysfunction of the brachial artery seems to reliably reflect generalized endothelial dysfunction and is an effective measurement of vulnerability to the development of atherosclerosis. However, there are few data either on endothelium-mediated arterial dilation in PAD or on the potential role of endothelial dysfunction in atherogenesis and its relation to the systemic inflammatory processes.

Objectives

The aim of the present study was to evaluate the possible relationship between CRP and fibrinogen plasma levels and endothelial dysfunction in patients with claudication of the same clinical severity and CVRF burden, enabling us to understand those processes through which inflammation acts at the beginning of and during the progression of PAD.

Patients and Methods

The target population consisted of the 4100 patients with ischaemic claudication cared for by our Angiology and Vascular Surgery Service. This cross-sectional study carried out a stratified sampling with the dependent variables age, gender, hypertension, hyperlipidemia, diabetes, smoking status and the ankle-brachial index (ABI) in order to rule out any confounding factors that could potentially bias the possible association between CRP and fibrinogen plasma levels and endothelial dysfunction. The sample included 156 patients (all male; mean age 64.8 ± 10.2 years) from the outpatient population at Getafe University Hospital, (Madrid, Spain). PAD had been diagnosed according to clinical criteria and an ABI < 0.9 with one or more stenoses of at least 50% in an artery of the lower limbs, shown by duplex or angiography. All of the patients included in the study were over 45 years-old and presented with stage 2 or 3 intermittent claudication according to the Rutherford scale. No patient who had been subjected to a previous revascularization procedure was included in the study. We also excluded from the sample all patients diagnosed with any chronic inflammatory disease or acute infectious process and those who had fever physical trauma or had undergone surgery in the previous 45 days.

The sample size necessary to detect the possible differences in plasma CRP as a function of flow-mediated arterial dilation (FMAD) measured in the brachial artery was determined based on the assumption of a standard error of 0.05 and assuming a statistical power of 0.8 for our cross-sectional design. The estimated variances for each of the variables were taken from studies previously published on our population and tables of sample size for analytical studies.

We collected demographic data and reviewed each patient’s clinical record, recording: classical CVRFs, other pertinent disease background, concomitant pathologies, medication and current clinical status. A complete physical check-up was performed at the first visit. Within one week, ABI was determined in the posterior tibial arteries and the dorsalis pedis arteries of both lower limbs for each patient according to the routine protocol of our vascular lab.

Endothelial function was evaluated in the brachial artery using Doppler ultrasound equipment (Technos, Esaote) as described and validated in previous studies and according to recent guidelines. The diameter of the brachial artery was measured after 40 and 70 seconds of reactive hyperemia and compared with basal measurements at rest. Reactive hyperaemia was induced by deflating a pressure cuff placed on the arm at a pressure of 250 mmHg for five minutes. FMAD was expressed as a percentage of the relative change in arterial diameter before placing and after deflating the cuff. All the measurements were taken in the morning, after 12 hours of fasting without medication, in a quiet room and after 10 minutes of rest.
Determination of hyper-sensitive CRP (hsCRP)

Peripheral blood samples were obtained from each patient through venous puncture, and CRP plasma levels were determined by automated hyper-sensitive immune-assay (Roche Diagnostics GMBH) with a low limit detection of 0.2 mg/L and with a variation coefficient of 4.2% in 4 mg/L and of 6.3% in 1 mg/L. In addition, we determined in each patient the plasma lipid profile (total cholesterol, triglyceride, HDL, LDL and VLDL lipoprotein levels), plasma concentrations of homocysteine, fibrinogen, creatinine and glycosylated hemoglobin (HbA1c), as well as a full blood count.

Diagnostic criteria

The CVRFs considered in this study were smoking, hypertension, diabetes and hyperlipidemia. A patient was considered a non-smoker only if the patient had absolutely no history of smoking. The patient was considered to be an ex-smoker if he had given up the habit at least 6 months prior to inclusion into the study. A patient was classified as hypertensive when arterial systolic/diastolic blood pressure values were >140/90 mmHg or the patient was taking medication for high blood pressure. A patient was considered to have Diabetes Mellitus if he presented with basal glycaemia of over 6.5 mmol/L or was being treated with oral anti-diabetic drugs or insulin.

Hyperlipidemia was considered to be present if the plasma concentration of total cholesterol was over 6.2 mmol/L, triglyceride levels were over 2.25 mmol/l or the patient was taking lipid-reducing drugs.

CAD was considered to be present when the patient’s clinical record included any episode of myocardial infarction, or a positive coronary angiogram/isotope scan. Cerebro-vascular disease was considered present when the patient had a history of previous ischaemic event or a stenosis of over 50% documented by duplex or angiography in at least one carotid artery.

Statistical analysis

We used non-parametric tests for analyzing the values of hsCRP and fibrinogen, since their distribution was not normal. The Kolmogorov-Smirnov Test was used to check the normal distribution of FMAD in our population and then analysis was carried out using the Student T-test.

A multi-variate analysis was carried out for CVRFs, plasma markers of acute inflammation and for anti-inflammatory, platelet aggregation inhibitor, statins and angiotensin enzyme converter inhibitors (AECI) medication to control for confounders among the groups in each analysis. Correlation analysis was carried out with the rho-Spearman uni-variate test between hsCRP and FMAD, to disclose any possible correlation between these two variables. We used the prevalence ratio to obtain the power of association between hsCRP and FMAD. It is defined as the ratio of the number of patients whose hsCRP plasma levels were elevated (over 3 mg/L as suggested by the American Heart Association) and in whom a FMAD under 3% was detected (3% being the lowest 5 percentile value of FMAD in healthy subjects under 30 years studied in our laboratory) to the number of patients with hsCRP > 3 mg/L and FMAD over 3%.

Prevalence Ratio of endothelial dysfunction in patients with a chronic inflammatory vascular process

\[
\text{Prevalence Ratio} = \frac{\text{exposed patients}}{\text{non-exposed patients}} = \frac{\text{patients with CRP} > 3 \text{ mg/L and FMAD < 3%}}{\text{patients with CRP} > 3 \text{ mg/L and FMAD > 3%}}
\]

The data are expressed as the mean ± standard deviation (in normally distributed variables) or the median [interquartile range] (in the non-normally distributed variables). All calculations were carried out with SPSS 11.5 (Microsoft) software. Statistical significance was considered with a p-value of <0.05 in 2-sided tests.

Results

From stratified sampling dependent on variables of age, sex, hypertension, hyperlipidemia, diabetes, smoking status and ABI, we obtained a sample of 156 male patients. A homogeneity test was applied to each variable; for no variable was there a lack of homogeneity in distribution. Ischaemic heart disease was present in 30 (20%) and cerebro-vascular disease in 18 (12%) of the patients with intermittent claudication.

To examine the relationship between FMAD and CRP, we divided the patients into 2 groups. We used a cutoff point for FMAD of 3% (lowest 5 percentile in a healthy population). This cutoff point gives us a threshold value for FMAD, with high specificity (S = 96%; p < 0.05) and positive predictive value (PPV = 95%; P < 0.05) for the presence of impaired endothelial function. The demographic characteristics of the two groups are shown in Table 1.
There were no differences in either age, prevalence of CVRF, clinical severity, acute inflammatory markers (other than CRP and fibrinogen), systemic atherosclerotic disease, or in treatment between the 2 groups categorized according to the FMAD threshold (Table 1). However, in the patients with marked endothelial dysfunction there were higher levels of hsCRP (6.3 vs. 2.3 mg/L; \( p < 0.05 \)) and fibrinogen (351 vs. 302 mg/dL; \( p < 0.05 \)) compared with those with FMAD > 3%.

The plasma CRP and fibrinogen levels according to quartile of FMAD are shown in Table 2. This emphasizes how these inflammatory markers increase with deteriorating endothelial function (Fig. 1). Again, in Table 3 we show how FMAD varies according to quartile of plasma CRP concentration, to support the negative relationship between these two variables (Fig. 2).

The relationship between inflammation and endothelial dysfunction was supported further by the negative correlation between FMAD and the plasma levels of hsCRP \( (r = -0.465; p < 0.05) \). (Fig. 3) The strength of relationship between these two variables was assessed by the prevalence ratio, 1.8 [95% CI 1.01–2.3].

**Discussion**

During the past decade, PAD has become recognised as an inflammatory disorder, but neither the processes involved nor the sequence of pathogenetic mechanisms that come together to produce the disease are known in detail.30

Alteration of normal endothelial homeostasis is postulated as one of the inaugural events in the

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**Table 1. Features of patients groups categorized according to FMAD in the brachial artery**

<table>
<thead>
<tr>
<th>Feature</th>
<th>FMAD &lt; 3% (N = 66)</th>
<th>FMAD &gt; 3% (N = 90)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsCRP (mg/L)</td>
<td>6.3 [2.4; 9.1]</td>
<td>2.3 [0.8; 5.2]</td>
<td>0.02</td>
</tr>
<tr>
<td>Fibrinogen (mg/dL)</td>
<td>351 [261; 466]</td>
<td>302 [222; 341]</td>
<td>0.04</td>
</tr>
<tr>
<td>Age (years)</td>
<td>65 ± 12</td>
<td>64 ± 8</td>
<td>0.89</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>36(54)</td>
<td>48(54)</td>
<td>0.93</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>145 ± 15</td>
<td>145 ± 13</td>
<td>0.92</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>83 ± 12</td>
<td>84 ± 10</td>
<td>0.94</td>
</tr>
<tr>
<td>Hyperlipidemia (%)</td>
<td>18(27)</td>
<td>21(23)</td>
<td>0.87</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>5.53 ± 1.57</td>
<td>5.45 ± 0.93</td>
<td>0.88</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.975 ± 1.25</td>
<td>2.1 ± 1.36</td>
<td>0.81</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>21(33)</td>
<td>27(30)</td>
<td>0.79</td>
</tr>
<tr>
<td>Glycemia (mmol/L)</td>
<td>6.88 ± 1.83</td>
<td>6.38 ± 1.16</td>
<td>0.80</td>
</tr>
<tr>
<td>HBA1c (%)</td>
<td>6.2 ± 1.2</td>
<td>6.0 ± 1.2</td>
<td>0.85</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>27(41)</td>
<td>42(46)</td>
<td>0.76</td>
</tr>
<tr>
<td>ABI</td>
<td>0.66 ± 0.19</td>
<td>0.65 ± 0.15</td>
<td>0.78</td>
</tr>
<tr>
<td>Leucocytes (10^3/L)</td>
<td>8231 ± 2312</td>
<td>8005 ± 2110</td>
<td>0.91</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>1.05 ± 0.12</td>
<td>0.97 ± 0.1</td>
<td>0.54</td>
</tr>
<tr>
<td>Homocysteine (μmol/L)</td>
<td>10.88 ± 3.1</td>
<td>12.87 ± 4.5</td>
<td>0.61</td>
</tr>
<tr>
<td>Heart Disease (%)</td>
<td>15(22)</td>
<td>15(16)</td>
<td>0.55</td>
</tr>
<tr>
<td>Cerebro-vascular Disease (%)</td>
<td>12(18)</td>
<td>6(66)</td>
<td>0.23</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-platelet (%)</td>
<td>48(72)</td>
<td>66(74)</td>
<td>0.77</td>
</tr>
<tr>
<td>Statins (%)</td>
<td>18(27)</td>
<td>27(30)</td>
<td>0.78</td>
</tr>
<tr>
<td>ACEi (%)</td>
<td>33(50)</td>
<td>48(53)</td>
<td>0.72</td>
</tr>
<tr>
<td>Nitrates (%)</td>
<td>3(5)</td>
<td>6(7)</td>
<td>0.67</td>
</tr>
<tr>
<td>Beta-blockers (%)</td>
<td>6(9)</td>
<td>6(7)</td>
<td>0.63</td>
</tr>
<tr>
<td>Calcium antagonist (%)</td>
<td>9(14)</td>
<td>12(13)</td>
<td>0.59</td>
</tr>
</tbody>
</table>

**Table 2. Inflammation markers as a function of FMAD quartiles**

<table>
<thead>
<tr>
<th>FMAD Quartiles (%)</th>
<th>p25</th>
<th>p50</th>
<th>p75</th>
<th>p100</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1.6 (N = 59)</td>
<td>1.6–4.5 (N = 39)</td>
<td>4.5–9.1 (N = 39)</td>
<td>&gt;9.1 (N = 39)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsCRP (mg/L)</td>
<td>7.2[1.6;11.8]</td>
<td>5.8[2.1;9.8]</td>
<td>3.1[2.2;6.6]</td>
<td>1.7[0.4;3.4]</td>
<td>0.04</td>
</tr>
<tr>
<td>Fibrinogen (mg/L)</td>
<td>398[321;476]</td>
<td>376[311;431]</td>
<td>313[288;369]</td>
<td>297[212;320]</td>
<td>0.03</td>
</tr>
</tbody>
</table>
inflammatory process of plaque formation, progression and degeneration.\textsuperscript{31,32,33} Cardiovascular risk factors modify endothelial function, even in the absence of clinical evidence for cardiovascular disease.\textsuperscript{14} CRP levels appear to be associated with the clinical severity of PAD (our unpublished data). In this study, such factors were controlled in the design stage, by means of a stratified sampling method. Thus, hypertension, diabetes, hyperlipidemia, smoking, age, gender and ABI are evenly distributed among patients with FMAD < 3\% and those with better endothelial function. We found plasma levels for hsCRP and fibrinogen, two key inflammation markers in endothelial dysfunction,\textsuperscript{2} differed significantly between the two patient subgroups. CRP was inversely correlated with FMAD; thus patients with higher plasma levels of hsCRP and fibrinogen have the lowest percentage values of FMAD. The strength of relationship between hsCRP and FMAD was supported by a significant prevalence ratio of 1.8. The relationship between reduced FMAD and high levels of CRP and fibrinogen was independent of age and other CVRFs. Similarly previous studies have suggested that in PAD the association between altered FMAD and high levels of inflammatory markers is not related to classical risk factors.\textsuperscript{21,23}

Although our findings do not establish any causal relationships, they are consistent with the hypothesis that inflammation contributes to the alteration of endothelial function in PAD. Hence the results of Sinisalo et al.’s,\textsuperscript{18} who conclude that in CAD patients CRP level normalization causes a parallel improvement in endothelial function, become of great interest. Verma et al.\textsuperscript{34} found that brachial artery FMAD was not related to CRP and had a weak relationship with classical CVRFs in a major cohort of healthy individuals. This, together with the already widely proven CRP marker forecasts function for PAD\textsuperscript{6,7,8,24} in healthy subjects, supports the hypothesis that inflammation comes before endothelial dysfunction in the time sequence of pathogenic processes.

Recently, it has been suggested that CRP affects the metabolism of nitric oxide (NO). Experimental in vitro studies and the recent study by Clapp et al.\textsuperscript{35} on an inflammatory endothelial dysfunction model in humans show that CRP has a direct and specific effect on endothelial function by means of a prolonged increase in the production of NO. This increases the expression of GTP-cyclohydrolase-1, the limiting enzyme in the synthesis of the co-factor of NO-synthetase, tetrahydrobiopterin, causing a deregulation of the cell-receptors of NO, and the resulting reduction of its effect.

On the other hand, Vita et al.\textsuperscript{36} suggest that certain CVRFs and pro-inflammatory cytokines activate endothelial cells to express leukocyte adhesion molecules, promoting the adhesion of monocytes and T-lymphocytes, causing an accumulation of leukocytes on the vessel wall.

![Graph of association trend between CRP and FMAD](image)

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**Table 3. FMAD as a function of the hsCRP quartiles**

<table>
<thead>
<tr>
<th>hsCRP Quartiles (mg/L)</th>
<th>p25 (&lt;1.3)</th>
<th>p50 (1.3–5.1)</th>
<th>p75 (5.1–8.9)</th>
<th>p100 (&gt;8.9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMAD(%)</td>
<td>8.7±0.61</td>
<td>7.9±0.45</td>
<td>4.1±0.54</td>
<td>1.76±0.43</td>
</tr>
</tbody>
</table>

**Fig. 2.** Graph of association trend between CRP and FMAD (p < 0.05).

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**Fig. 3.** Correlation between FMAD and the plasma levels of hsCRP. (Spearman correlation coefficient r = -0.465; p < 0.05).

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This study adds to the weight of the association between endothelial function and inflammatory processes in a coherent way. It has internal consistency, since a dose-response relationship was observed (linear correlation between CRP and FMAD). It has external consistency or repeatability, since the results are consistent with those of other studies and finally the results are biologically plausible. However, further prospective studies are required to establish the causality of the relationships we have demonstrated.

Acknowledgements

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The authors have no conflicts of interest.

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