Conclusions: Endothelial dysfunction is an event which takes place in the first stages of the disease. It has an inflammatory nature. Its lack of correlation with the clinical severity, also occurring in BAFMD, lends weight to the hypothesis that physiological conditions, 70% of the nitrites in plasma presents has a linear association with the elevation of the hsCRP plasma levels [6]. It has been demonstrated that endothelial dysfunction, measured by BAFMD, is decreased in patients with PAD and this reduction does not correlate with the severity of the disease. This would suggest to us that endothelial dysfunction is an early process in the onset of PAD [7]. Our objective is to analyse the role played by NO (the main molecule deriving from the endothelium which is involved in vascular homeostasis) in the severity of PAD. We aim to determine its relationship to inflammation and the endothelial dysfunction existing in these patients; since there is evidence that inflammatory mediators, concomitantly with endothelial dysfunction, are closely involved in the pathogenesis of PAD.

1. Introduction

The endothelium is responsible for maintaining a balance between vasodilation and vasoconstriction, between inhibition and stimulation of smooth muscle cell proliferation and migration, and between thrombogenesis and fibrinolysis. When this balance is broken and there is an endothelial dysfunction, damage is caused to the arterial wall. Endothelial dysfunction is considered to be an early sign of atherosclerosis, before evidence of atherosclerotic plaque shows up on angiogram or ultrasound scan. Endothelial dysfunction has been attributed to a reduction in nitric oxide (NO) bioactivity and an increase in oxygen free radical formation [1]. Nitrites are the product of the oxidation of the NO derived from the endothelium. Under physiological conditions, 70%–90% of the nitrites in plasma stem from endothelial nitric oxide synthase (eNOS) activity [2].

The measurement of brachial artery flow-mediated dilation (BAFMD) is one of the most reliable indirect methods for measuring endothelial dysfunction. This test is based on the ability of endothelial cells to detect changes in shear stress [3].

C-reactive protein (CRP) is a systemic marker of inflammation, and high concentrations have been associated with the potential development of atherothrombotic events both in patients with known cardiovascular disease and in apparently healthy subjects [4]. It has also recently been suggested that CRP may affect the NO pathway [5], which may be of interest in terms of the association between endothelial dysfunction and atherosclerosis.

There are data demonstrating that the clinical severity with which peripheral arterial disease (PAD) presents has a linear association with the elevation of the hsCRP plasma levels [6]. It has been demonstrated that endothelial dysfunction, measured by BAFMD, is decreased in patients with PAD and this reduction does not correlate with the severity of the disease. This would suggest to us that endothelial dysfunction is an early process in the onset of PAD [7]. Our objective is to analyse the role played by NO (the main molecule deriving from the endothelium which is involved in vascular homeostasis) in the severity of PAD. We aim to determine its relationship to inflammation and the endothelial dysfunction existing in these patients; since there is evidence that inflammatory mediators, concomitantly with endothelial dysfunction, are closely involved in the pathogenesis of PAD.
2. Material and methods

A cross-sectional study was carried out in which two groups of patients were selected: group A included patients with Fontaine stage II PAD (intermittent claudication) confirmed by haemodynamic study (Doppler) and treadmill exercise testing; group B included patients with Fontaine stage III–IV PAD (daily rest pain and/or focal tissue necrosis) demonstrated haemodynamically and/or by an imaging technique (angiography, magnetic resonance angiography, eco-Doppler) who had not previously undergone revascularisation.

Cardiovascular risk factors, treatment and clinical condition at the time of inclusion were all recorded, and the ankle-brachial index (ABI) was measured at rest according to the standard technique in dorsalis pedis artery and posterior tibial artery of both lower limbs. Laboratory determinations were carried out which included basic clinical chemistry (blood glucose, renal function, electrolytes) and lipid profile. Patients were defined as being hypertensive if they had been diagnosed as such (systolic blood pressure $>$140 mmHg and/or diastolic blood pressure $>$90 mmHg) and/or had been on anti-hypertensive treatment for at least one year prior to inclusion in the study. Patients with plasma total cholesterol $>$250 mg/dl, LDL-cholesterol $>$160 mg/dl or triglycerides $>$200 mg/dl, or on lipid-lowering treatment were defined as having dyslipidaemia. Patients were considered to be diabetic if they had baseline blood glucose $>$120 g/dl or required treatment with hypoglycaemics. Chronic renal failure was considered as serum creatinine $>$1.5 mg/dl.

For the determination of nitrite levels in plasma, the subjects had to fast (including not taking their usual medication) for at least 12 h before coming to the study. Blood was taken from an antecubital vein prior to carrying out the BAFMD. The blood was centrifuged for 10 min at 800 g and the plasma was then stored at 4°C. The plasma nitrite concentration was measured by colorimetric analysis using the Griess reaction [8]. This is a chemical reaction which uses sulfanilamide and N-1-naphthylethylenediamine dihydrochloride (NED) under acid conditions (phosphoric acid). This system is capable of detecting NO$_2^-$ in a variety of biological and experimental fluids, and has a limit of detection of 2.5 $\mu$M (125 pmol). Each sample was analysed in triplicate, and the mean of the three determinations was taken. The extraction was repeated in a control group of 20 patients to evaluate the reproducibility of the test; the coefficient of variation was $<$5%.

A venous blood sample was also taken for determination of the plasma concentrations of CRP using highly sensitive, automated immunoassay (Roche Diagnostics), with a lower limit of detection of 0.2 mg/l and a coefficient of variation of 4.2% in 4 mg/l and 6.3% in 1 mg/l [9].

Following the blood samples, the ultrasound analyses were performed after a 10-min rest period in the supine position. The brachial artery was located 3–5 cm proximal to the antecubital fossa and a longitudinal image was obtained with a 10 MHz linear transducer, adjusting the depth and the gain in order to optimise visualisation of the media-intima interfaces of the anterior and posterior walls of the artery. Three measurements were taken of the diameter of the brachial artery, coinciding with the final diastolic point of the Doppler curve, and the mean was calculated. A blood pressure cuff was placed distal to the measurement site and inflated to a pressure of 250 mmHg for 5 min. The pressure was then released, obtaining a new longitudinal image at 60 s, at which point the measurements of the artery diameter at the end-diastolic point of the Doppler curve were repeated. The environmental conditions of the room did not vary between subjects while administering the test. When the blood flow in an arterial segment is occluded, the resulting hypoxia causes vasodilation in the distal vascular bed, reducing vascular resistance. Then, on releasing the occluded segment, there is an increase in blood flow, heightening the shear pressure exercised on the endothelium, boosting the expression of eNOS and causing the release primarily of NO, with the subsequent relaxation of the smooth muscle cells in the vascular wall, resulting in vasodilation [3].

All the images were recorded by one single observer. The measurements were taken by an independent observer, who was blind to the circumstances for which the examinations were being performed and to the patients’ characteristics. The BAFMD was defined as the difference between the baseline diameter and the post-ischaemia diameter, regarding with the baseline diameter and expressed as a percentage. The technique had previously been validated in our laboratory [7, 10].

$$\text{BAFMD} = \frac{\text{Post-ischaemia diameter} - \text{Baseline diameter}}{\text{Baseline diameter}} \times 100$$

A group of healthy subjects were analysed: $<$30 years of age; no cardiovascular risk factors; no chronic conditions and/or long-term treatments; normal vascular examination; and ABI $>$0.9. As with the first group, nitrite levels in plasma and hsCRP were determined and BAFMD was measured.

2.1. Statistical analysis

The sample size necessary to obtain significant differences was calculated on the basis of previous studies which analysed the following end-points: NO levels in plasma; hsCRP; and BAFMD [9, 10]. Student’s $t$-test was used for the end-points with normal distribution and the Mann–Whitney $U$-test for those in which the distribution was not normal. Whenever the same test was applied more than once within the same data setting, Bonferroni corrections were applied. The Kolmogorov–Smirnov and Shapiro–Wilk tests were used for the analysis of normality. The $\chi^2$-test was used for categorical variables and the Spearman’s $\rho$-test for the correlation between variables.

The data are expressed as mean $\pm$ S.D. and the categoricals as percentages. The hsCRP data are expressed as median (percentiles 25 and 75). Statistical significance was assumed for $P<0.05$.

3. Results

Fifty patients were included in group A and 32 patients in group B. The clinical characteristics and the treatment the
patients were taking at the time of the study are presented in Table 1. No significant differences were found between the two groups with respect to cardiovascular risk factors or concomitant treatments. Forty-one healthy volunteers were recruited as a control group.

Analysing the BAFMD in groups A and B, we observed that there were no statistically significant differences between the two (4.7 ± 4.2% vs. 4.3 ± 2.8%, P = 0.1, 95% CI = [−1.25; 2.05]). On comparison of these results with those obtained in the healthy control group, statistically significant differences were observed. The patients with PAD presented a lower BAFMD percentage (10.3 ± 4.09% healthy vs. 4.7 ± 4.2% group A, P = 0.001, 95% CI = [4.09; 7.21]; vs. 4.3 ± 2.8% group B, P = 0.001, 95% CI = [4.3; 7.6]) (Fig. 1).

No statistically significant differences were found in the nitrite levels in plasma between groups A and B (25.2 ± 24.4 μM vs. 21.8 ± 19.8 μM, P = 0.38, 95% CI = [−6.6; 13.4]). However, the nitrite levels in plasma were significantly higher in the groups of patients with PAD than in the control group of healthy subjects (25.2 ± 24.4 μM group A vs. 12.7 ± 11.1 μM healthy, P = 0.005, 95% CI = [3.95; 20.05]; 21.8 ± 19.8 μM group B, P = 0.015, 95% CI = [1.6; 16.3]) (Fig. 2).

The hsCRP values were statistically higher in the group of patients with stage III–IV PAD (group B) than in the patients with stage II PAD (group A) and the group of healthy controls (8.2 [4.34–14.1] group A vs. 29.2 [15.5; 39.8] group B, P = 0.001, 95% CI = [−24.2; −17.8]; 1.3 [0.12–2.8] healthy vs. 8.2 [4.34–14.1] group A, P = 0.003, 95% CI = [−8.3; −5.7]; 1.3 [0.12–2.8] healthy vs. 29.2 [15.5–39.8] group B, P = 0.013, 95% CI = [−31.2; −24.8]) (Fig. 3).

A weak reverse correlation was found between the nitrite levels in plasma and the BAFMD (r = −0.3; P = 0.0001). No correlation was found between the hsCRP levels and the nitrites, or between the BAFMD and the hsCRP levels (Fig. 4).

4. Discussion

The endothelium seems to be responsible for the balanced relationships involved in the functioning of the vascular wall. When this balance is upset, the regulation of vascular homeostasis is lost, causing endothelial dysfunction, defined as functional deterioration of the endothelium characterised by vasospasm, vasoconstriction, abnormal coagulation mechanisms, abnormal fibrinolysis and an increase in vascular cell proliferation. A reduction in the BAFMD has been demonstrated in patients with hypercholesterolaemia and hypertension [11], diabetics [12, 13] and smokers [14], even in early stages of atherosclerosis with no anatomical evidence, and in patients with PAD [15].

---

Table 1
Demographic data and treatment

<table>
<thead>
<tr>
<th>Topic</th>
<th>Group A</th>
<th>Group B</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>71.37 ± 10.81 (52–94)</td>
<td>68.3 ± 9.03 (40–89)</td>
<td>0.116</td>
</tr>
<tr>
<td>Gender</td>
<td>Male 47 (94%)</td>
<td>27 (84.4%)</td>
<td>0.635</td>
</tr>
<tr>
<td>Hypertension</td>
<td>28 (36%)</td>
<td>24 (75%)</td>
<td>0.975</td>
</tr>
<tr>
<td>DM</td>
<td>18 (36%)</td>
<td>18 (56.3%)</td>
<td>0.389</td>
</tr>
<tr>
<td>Smoking</td>
<td>Current 28 (56%)</td>
<td>5 (15.6%)</td>
<td>0.116</td>
</tr>
<tr>
<td>Former</td>
<td>28 (56%)</td>
<td>13 (40.6%)</td>
<td>0.389</td>
</tr>
<tr>
<td>Cardiopathy</td>
<td>12 (24%)</td>
<td>4 (12.5%)</td>
<td>0.165</td>
</tr>
<tr>
<td>Dyslipidaemia</td>
<td>27 (54%)</td>
<td>17 (53.1%)</td>
<td>0.841</td>
</tr>
<tr>
<td>CVA</td>
<td>3 (6%)</td>
<td>1 (3.1%)</td>
<td>0.24</td>
</tr>
<tr>
<td>CRF</td>
<td>1 (2%)</td>
<td>1 (3.1%)</td>
<td>0.726</td>
</tr>
<tr>
<td>COPD</td>
<td>9 (18%)</td>
<td>9 (28.1%)</td>
<td>0.132</td>
</tr>
<tr>
<td>Anti-aggreg.</td>
<td>32 (64%)</td>
<td>22 (68.8%)</td>
<td>0.354</td>
</tr>
<tr>
<td>ACE inhibitors</td>
<td>20 (40%)</td>
<td>12 (37.5%)</td>
<td>0.153</td>
</tr>
<tr>
<td>Calcium antagonists</td>
<td>10 (20%)</td>
<td>8 (25%)</td>
<td>0.34</td>
</tr>
<tr>
<td>β-Blockers</td>
<td>6 (12%)</td>
<td>4 (12.5%)</td>
<td>0.894</td>
</tr>
<tr>
<td>Nitrates</td>
<td>7 (14%)</td>
<td>2 (6.3%)</td>
<td>0.064</td>
</tr>
<tr>
<td>Statins</td>
<td>20 (40%)</td>
<td>6 (18.8%)</td>
<td>0.073</td>
</tr>
<tr>
<td>Oral anticoagulants</td>
<td>2 (4%)</td>
<td>3 (9.4%)</td>
<td>0.052</td>
</tr>
<tr>
<td>B-Agonists</td>
<td>3 (6%)</td>
<td>3 (9.4%)</td>
<td>0.261</td>
</tr>
</tbody>
</table>

Fig. 1. Comparison of the BAFMD results according to the clinical severity of the PAD. *P < 0.05 between group A and healthy subjects and between group B and healthy subjects.

Fig. 2. Comparison of the nitrite levels in plasma according to the clinical severity of the PAD. *P < 0.05 between group A and healthy subjects and between group B and healthy subjects.
with no significant differences according to the severity of the disease being found in the BAFMD [7, 10]. Moreover, elevated hsCRP seems to be an independent predictor of cardiovascular events in patients with PAD [16]. In this study we corroborated these results, finding a reduction in BAFMD in patients with PAD with respect to healthy subjects. However, we found no significant differences in the BAFMD with respect to the clinical severity of this disease, which would support the hypothesis that endothelial dysfunction is a process which begins in the early stages of PAD.

Vascular tone is maintained by the release of numerous vasodilator and vasoconstrictor substances. NO is the principal vasodilator released by the endothelium and is generated in endothelial cells as a result of the enzymatic activity of eNOS, which is continuously expressed. Our study demonstrates the association of the increased plasma values of nitrates in patients suffering PAD that, as far as we know, it is the first time that relationship is shown.

There is an inducible isoform of NOS (iNOS), which is stimulated by cytokines and produces much larger quantities of NO than the other isoforms [17]. In order to work, these enzymes require cofactors, including tetrahydrobiopterin (BH4) and NADPH. The importance of NO in atherogenesis was suggested after studies in mice with an apolipoprotein E (apoE) deficiency, in which the atherosclerotic lesions developed spontaneously when eNOS was eliminated. However, in studies with apoE/iNOS double-knockout mice, there was a reduction in the formation of the atherosclerotic plaque. These findings suggest that eNOS-derived NO may ‘protect’ the vascular wall from atherosclerosis, while the NO deriving from iNOS may promote the formation of atherosclerotic lesions [17, 18].

Once released into the lumen of the vessel, the NO deriving from the endothelium is oxidized or participates in nitrosylation reactions. The NO activity is the result of the balance between its production by NOS and its inactivation by oxygen free radicals.

We know that CRP is able to stimulate the production of NO by NOS. As already mentioned, NOS needs the cofactor BH4 in order to function. One of the pathways by which BH4 is synthesized, is through regeneration from a reduced form of BH2 through the pterin pathway. This pathway depends on a normal cellular redox state with oxidative stress impairing the recycling of BH4. When BH4 levels are low, the NOS ‘uncouples’ and behaves like an NADPH oxidase, increasing the production of superoxide anion and hydrogen peroxide more than that of NO; the net balance is therefore a reduction in NO activity [17, 19]. We also know that CRP participates in the modulation of the deleterious effect of oxidized LDL on endothelial function, encouraging oxidative stress and, therefore, the production of free radicals (superoxide anion). These free radicals are able to destroy the cofactor BH4 and directly inactivate the NO, producing peroxynitrite, which is cytotoxic, proinflammatory and a potent oxidant, and may contribute to endothelial damage and dysfunction and to oxidation of the lipoproteins in atherosclerotic lesions [18, 19]. An increase in the levels of peroxynitrite have been observed when iNOS is expressed [20]. Although cytokines (such as γ-interferon and tumour necrosis factor-α) and inflammatory mediators (such as lipopolysaccharides) can increase the transcription of GTP cyclohydrolase (de novo pathway of BH4 synthesis), peroxynitrites may oxidize BH4 to radical BH3, ‘uncoupling’ NOS and perpetuating the cycle of vascular oxidative stress. Oxidized LDL also increases the production of endothelin [19]. High levels of endothelin-1 have been found in patients with arteriosclerosis, both in coronary disease [21, 22] and PAD [23, 24]. In our study, as occurred with BAFMD, we found no relationship between the nitrite levels in plasma, as estimator of NO production, and the severity of the PAD. This leads us to think that the
loss of the physiological function of NO as a homeostatic signal by which the endothelium acts, occurs in the first stages of PAD and that this is perpetuated through a vicious circle (self feed-back) which leads to a reduction in BAFMD, as estimator of endothelial dysfunction.

Prior studies in patients with PAD have shown moderately raised plasma levels of hsCRP and also, that this is associated with a higher risk of developing cardiovascular events. In previous studies, we have seen that elevated hsCRP has a linear association to the clinical severity with which PAD presents [6]. In this study, we not only found this linear association between clinical severity and elevated hsCRP, but we also found that, although weak, there seems to be a reverse correlation between hsCRP levels and BAFMD values in these patients.

Therefore, the CRP stimulates the production of NO by NOS, increasing NO oxidation and nitrosylation and reducing the levels of BH4, encouraging the formation of free radicals. These free radicals, in turn, inactivate the NO which has been produced and destroy the BH4, resulting in endothelial dysfunction. In fact, it has been demonstrated in vitro studies, that CRP is capable of stimulating the production of NO, independent of iNOS stimulation [25]. In our study, we observed increased levels of hsCRP in the patients with PAD and, also there was a linear correlation between these levels and the clinical severity degree. This finding suggests the existence of an inflammatory substrate in the aetiopathogenesis of PAD. Both CRP, as principal indicator of systemic inflammation, and other cytokines (IL-6, TNF-α, etc.), may be responsible for the loss of balance in the endothelial NO system and the subsequent endothelial dysfunction. The reverse correlation between BAFMD and the hsCRP levels found in this study, although weak, implies a relationship between inflammation and endothelial dysfunction in the aetiopathogenesis of PAD, with the loss of the homeostatic function of NO as a key step in the origins of the disease, but not in its progression. There are a lot of in vitro studies and data in animal experimental research that show the relationship of NO in both processes: inflammation and endothelial dysfunction and the oxidative stress leading to the development of atherosclerosis [25]. Our results may explain in which way the NO could act as a link between them being that it is involved in both [19].

The cross-sectional nature of our study doesn’t allow us to establish the cause and effect relationship of this process. We prove the plasma levels of nitrites are elevated in the PAD, independently of the clinical severity of the disease. This finding is plausible, as we described, and fits among other pieces of the puzzle that, step by step, allows us to better know how the atherosclerosis occurs.

5. Conclusions

There are increased plasma levels of nitrites in patients suffering of PAD. The fact that no correlation was found between the elevated nitrite levels in plasma and the clinical severity of the PAD, as occurred with the no worsening of BAFMD, supports the hypothesis that endothelial dysfunction is something which occurs in the first stages of the disease, perpetuating itself in a vicious circle sustained by an inflammatory base in whose pathway NO also plays a key role. The presence of elevated levels of CRP reconfirms the hypothesis of the sustained inflammatory nature of PAD.

References


