Endothelial dysfunction in peripheral arterial disease is related to increase in plasma markers of inflammation and severity of peripheral circulatory impairment but not to classic risk factors and atherosclerotic burden

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Objective: **We undertook this study to evaluate in patients with peripheral arterial disease (PAD) the relationship of endothelial dysfunction, which is directly related to progression and clinical complications of atherosclerosis, with variables including classic risk factors, inflammation, severity of peripheral circulatory impairment, and atherosclerotic burden.**

Methods: **This cross-sectional study included outpatients seen in an academic angiologic unit. Eighty-eight consecutive patients with PAD (ankle/brachial index [ABI] < 0.90) were studied. The control group consisted of 30 age-matched and sex-matched healthy subjects. Main outcome measures were endothelial function in the form of brachial artery flow-mediated dilation (FMD), plasma levels of C-reactive protein (CRP) and fibrinogen, severity of PAD according to ABI, and atherosclerotic burden, ie, atherosclerosis in one leg or in two or more other sites.**

Results: **Compared with patients with FMD greater than 6.2% (ie, 5th percentile of FMD in control subjects), patients with FMD less than 6.2% had a similar prevalence of classic risk factors but higher median levels of CRP (1.6 vs 6.0 mg/L; $P < .01$) and fibrinogen (200 vs 374 mg/dL; $P < .01$). The two inflammatory markers were negatively correlated with FMD ($P < .01$). ABI was higher in patients with FMD greater than 6.2% than in those with worse endothelial function (0.72 ± 0.15 vs 0.62 ± 16; $P < .01$); there was no difference with respect to atherosclerotic burden. Multivariate analysis showed that the association of CRP, fibrinogen, and ABI with FMD less than 6.2% was unrelated to classic risk factors. In a second model, which included CRP, fibrinogen, and ABI, all three variables were independently related to FMD less than 6.2%.**

Conclusion: **Inflammation and severity of circulatory impairment are implicated in the pathophysiology of dysfunctional endothelium in PAD. (J Vasc Surg 2003;38:374-9.)**

Peripheral arterial disease (PAD) is closely associated with high risk for myocardial infarction and stroke.[1-4] This increased risk, which appears to be independent of classic risk factors[1,4] and is only partly explained by the expected association of PAD with coronary and cerebrovascular disease,[1,2] is strongly related to severity of PAD.[1,4] Moreover, the poor long-term prognosis of PAD is significantly associated with elevated plasma levels of the inflammatory markers C-reactive protein (CRP) and fibrinogen.[5,6] However, the mechanisms that link severity of circulatory failure in the affected limb and levels of acute-phase proteins to cardiovascular risk in PAD are unclear. One of these mechanisms could be impairment in the functional properties of the endothelium, which elicits a series of changes directly related to initiation, progression, and clinical complications of atherosclerosis.[7] Endothelial function may be evaluated by measuring the vasodilator response to pharmacologic or physical stimuli that induce release of nitric oxide from endothelial cells.[8,9] Reduced endothelium-mediated vasoreactivity, as measured in the brachial artery, is associated with classic cardiovascular risk factors, also in the absence of overt atherosclerotic disease,[10] and is related to low-grade inflammation in patients with coronary artery disease.[11,12] Moreover, it correlates well with response in the coronary circulation[13] and is linked to increased cardiovascular risk.[14,15] Therefore brachial artery vasomotor dysfunction appears to reflect generalized endothelial dysfunction and is a measure of susceptibility to development of atheroma. However, data about endothelium-mediated vasodilation in PAD are scarce[16-18]; thus the determinants of endothelial dysfunction in PAD need to be clarified. Accordingly, we measured endothelium-mediated vasodilation in a group of patients with PAD to address the following questions: Is there a relationship between endothelial dysfunction, classic risk factors, and plasma markers of inflammation? Is endothelial dysfunction related to ankle/brachial index (ABI), which is a marker of PAD severity?[19] Is

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endothelial dysfunction related to atherosclerotic burden (measured as presence of atherosclerosis in only one leg or in two or more sites)? Given the key role of endothelial dysfunction in atherogenesis,[7] the answers to these questions may help in clarifying the mechanisms that favor progression of atherosclerosis in a population at high cardiovascular risk.

PATIENTS AND METHODS

This cross-sectional study included 88 consecutive patients (81 men, 7 women; ages, 64.7 ± 10 years) in whom PAD was diagnosed at duplex scanning on the basis of ABI < 0.90 associated with 1 or more stenoses greater than 50% in one of the arteries supplying the legs.

The control group consisted of 30 subjects matched to the patients for age and sex, recruited from outpatients attending our angiologic unit for treatment of venous insufficiency of the lower limb, hospital staff, and their relatives. All control subjects had ABI greater than 0.90, had no symptoms of coronary artery disease (CAD), and had a normal electrocardiogram. Our institutional ethics committee approved the study, and informed consent was obtained from patients and control subjects.

Data collection. Historical and demographic information was collected at the first visit, after which all patients underwent complete clinical and arterial evaluation, according to the routine protocol of our vascular laboratory. In brief, systolic pressure in the right and left posterior tibial arteries and the right brachial artery was measured twice with a Doppler ultrasound scanning probe after the patients had been resting supine for 5 minutes. The average of the two measurements was used to evaluate ABI. The lower ABI value of the two legs was used for correlation with flow-mediated dilation (FMD). Then all patients underwent echo color Doppler scanning of the abdominal aorta, the lower limb, and the carotid arteries. Patients with no history of CAD underwent myocardial scintigraphy to verify the presence or absence of ischemic heart disease. One week later endothelial function was evaluated with measurement of FMD of the brachial artery with a high-resolution ultrasound system (Hewlett-Packard, Andover, Mass), according to recent guidelines.[20] In brief, FMD was assessed by measuring the change in brachial artery diameter after 60 seconds of reactive hyperemia compared with baseline measurements after deflation of a cuff placed around the forearm that had been inflated to 50 mm Hg above systolic blood pressure for 5 minutes. Increase in diameter after sublingual nitroglycerin spray (0.4 mg) was used as a measure of endothelium-independent vasodilation. The response of the vessel diameter to reactive hyperemia and nitroglycerin was expressed as percent change relative to the diameter immediately before cuff inflation and to the diameter immediately before drug administration, respectively. In our laboratory, intraobserver variability for repeated measurements of resting arterial diameter is 0.01 ± 0.02 mm. When reactive hyperemia studies are performed on different days, the between-occasion, within-patient difference for measurement of FMD is 1.5% ± 0.7%. All drugs were discontinued for 18 hours or longer before the study, which was carried out in the morning, after an overnight fast, in a quiet room at a constant temperature of 21°C ± 1°C. All subjects abstained from smoking and intake of caffeine-containing food and beverages for at least 12 hours before the study. Fasting venous blood was obtained after nontraumatic venipuncture 15 minutes before FMD measurement. Plasma CRP was determined with a highly sensitive (hs) assay (Dade Behring Diagnostics, Marburg, Germany). Clottable fibrinogen was estimated according to a functional assay (Clauss) on a Behring BCS coagulation analyzer with Multifibren U (Dade Behring Diagnostics). Total serum triglyceride, cholesterol, and high-density lipoprotein-cholesterol concentrations were measured with commercially available kits.

Diagnostic criteria. A history of cardiovascular disease was considered positive when confirmed with hospital records documenting CAD (previous myocardial infarction, or positive coronary angiogram or positive myocardial scintigram), and carotid vessel disease (CVD; previous stroke or ultrasound-proved stenosis greater than 50% in at least one carotid artery). Hypertension was diagnosed if systolic arterial pressure exceeded 140 mm Hg or diastolic arterial pressure exceeded 90 mm Hg, or if the patient used antihypertensive drugs. Hyperlipidemia was diagnosed if plasma total cholesterol concentration exceeded 240 mg/dL, plasma triglyceride levels exceeded 200 mg/dL, or the patient used lipid-lowering drugs. Diabetes mellitus was diagnosed if plasma fasting glucose concentration exceeded 120 mg/dL or the patient used hypoglycemic agents. To evaluate the relationship between FMD and atherosclerotic burden, patients were classified as follows: patients with unilateral PAD alone; patients with bilateral PAD alone; patients with bilateral PAD plus 1 site, ie, atherosclerosis in coronary or carotid arteries; and patients with bilateral PAD plus 2 sites, ie, atherosclerosis in both coronary and carotid arteries.

Statistical analysis. Data were analyzed with the t test (normally distributed), Mann-Whitney U test (nonnormally distributed), or χ^2 test (categorical variables). Correlations were obtained with the Spearman method. Multivariate logistic regression analysis was used to assess which variables were significantly related to impaired FMD. Data are expressed as mean plus or minus standard deviation (SD; normally distributed) or median plus [25th; 75th] percentile (nonnormally distributed).

RESULTS

No woman in either the PAD or control group was receiving hormone replacement therapy, and, except for one patient with PAD, all were older than 60 years (Table I). According to the diagnostic criteria, CAD was present in 45 (51%), and CVD was present in 24 (27%) of patients with PAD. No control subject had a history of CAD or CVD.

Compared with healthy subjects, patients with PAD had lower FMD (11.4% [9.3; 12.9] vs 7.3% [5.1; 9.5; $P <$ .01) but similar nitroglycerin-mediated vasodilation (12.5% [10.0; 14.1] vs 11.8% [9.6; 13.1]). This confirms

Table I. Clinical characteristics of control subjects and patients with PAD

	Control subjects (N = 30)	*Patients with PAD (N = 88)*
Age (y)	62 ± 8	65 ± 10
Sex (M/F)	27/3	81/7
Hypertension	6 (20)	48 (55)*
Systolic BP (mm Hg)	136 ± 11	145 ± 18*
Diastolic BP (mm Hg)	80 ± 7	82 ± 10
Smoking status		
Current	5 (17)	46 (52)*
Former	10 (33)	35 (40)
Hyperlipidemia	4 (13)	48 (55)*
Cholesterolemia (mg/dL)	221 ± 48	217 ± 46
HDL-cholesterol (mg/dL)	43 ± 8	41 ± 9
Triglyceride level (mg/dL)	175 ± 58	180 ± 135
Diabetes mellitus	0	28 (32)*
Glycemia (mg/dL)	103 ± 12	118 ± 41*
Treatment		
Antiplatelets	0	69 (78)*
ACE inhibitors	4 (13)	36 (41)*
Calcium antagonists	1 (3)	38 (43)*
β-Blockers	1 (3)	11 (12)
Nitrates	0	30 (34)*
Statins	1 (3)	34 (39)*
Oral hypoglycemic agents	0	22 (25)*
Insulin	0	6 (7)

Numbers in parentheses represent percent.
BP, Blood pressure; *HDL*, high-density lipoprotein; *ACE*, angiotensin-converting enzyme.
*$P < .01$ vs control subjects.

Table II. Characteristics of patients with different degrees of brachial artery endothelium-dependent, flow-mediated dilation

	FMD > 6.2% (N = 52)	*FMD < 6.2% (N = 36)*
Age (y)	63 ± 11	67 ± 9
Sex (M/F)	48/4	33/3
Hypertension	25 (48)	23 (64)
Systolic BP (mm Hg)	145 ± 19	144 ± 17
Diastolic DBP (mm Hg)	82 ± 10	83 ± 10
Smoking		
Current	29 (56)	17 (47)
Former	19 (36)	16 (44)
Hyperlipidemia	28 (54)	20 (55)
Cholseterolemia (mg/dL)	218 ± 50	216 ± 39
HDL cholesterol (mg/dL)	40 ± 10	43 ± 8
Triglyceridelevel (mg/dL)	181 ± 134	178 ± 104
Diabetes mellitus	17 (33)	11 (31)
Glycemia (mg/dL)	120 ± 39	115 ± 41
Coronary artery disease	26 (50)	19 (53)
Carotid vascular disease	13 (25)	11 (30)
Previous MI	23 (44)	15 (42)
ABI	0.72 ± 0.15	0.62 ± 0.16*
hs-CRP (mg/L)	1.6 [0.6; 5.4]	6.0 [2.8; 7.7]*
Fibrinogen (mg/dL)	200 [177; 293]	374 [273; 453]*
Treatment		
Antiplatelets	40 (77)	29 (80)
ACE inhibitors	22 (42)	14 (39)
Calcium antagonists	21 (40)	17 (47)
β-Blockers	7 (13)	4 (11)
Nitrates	17 (33)	13 (36)
Statins	19 (37)	15 (42)
Oral hypoglycemic agents	13 (25)	9 (25)
Insulin	4 (8)	2 (6)

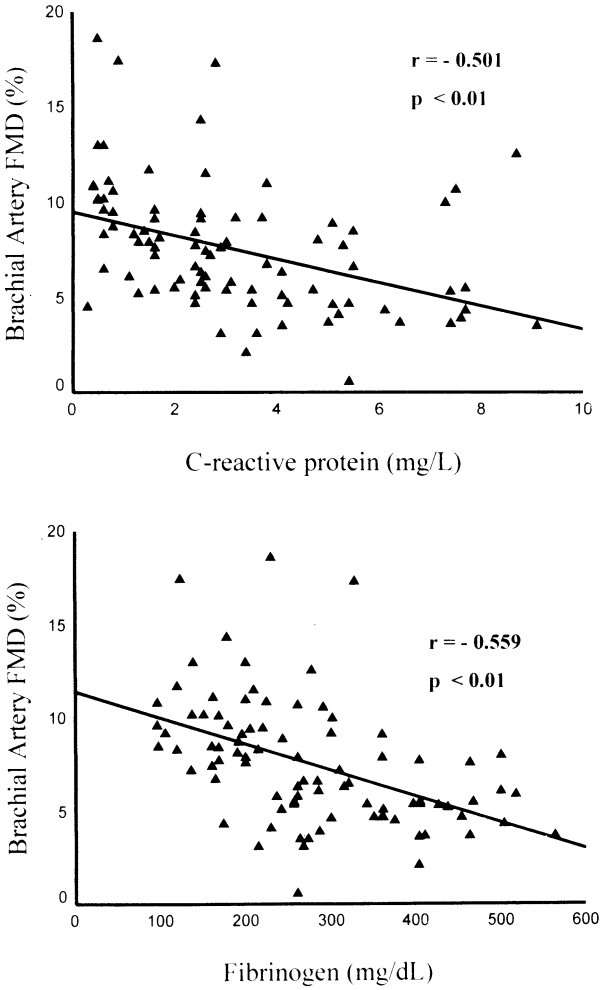
Numbers in parentheses represent percent.
Numbers in brackets represent 25th and 75th percentile.
FMD, Flow-mediated dilation; *BP*, blood pressure; *HDL*, high-density lipoprotein; *MI*, myocardial infarction; *ABI*, ankle/brachial index; *hs-CRP*, high-sensitivity C-reactive protein; *ACE*, angiotension-converting enzyme.
*$P < .01$ vs patients with FMD >6.2%.

that impaired vasoreactivity in PAD depends exclusively on endothelial dysfunction. Plasma levels of the inflammatory marker hs-CRP were higher in patients than in control subjects (2.8 mg/L [1.1; 7.4] vs 0.9 mg/L [0.5; 2.1]; $P < .01$), whereas fibrinogen levels were similar in the two groups: 257 mg/dL [207; 334] in control subjects and 284 mg/dL [210; 404] in the PAD group.

To assess the relationship between FMD and several determinants of endothelial dysfunction, we divided patients with PAD into two groups, using as an arbitrary cutoff point an FMD value of 6.2%, ie, 5th percentile of FMD in the control group. Thirty-six patients had FMD less than 6.2%, and 52 had FMD greater than 6.2% (Table II). Of interest, FMD was still lower in the PAD group with less impaired endothelial function than in the control group (9.1% [7.7; 10.8] vs 11.4% [9.3; 12.9]; $P < .01$).

Relationship between FMD, classic risk factors, and inflammatory markers. There was no difference in age, sex, and prevalence of classic cardiovascular risk factors between the two groups of patients categorized according to FMD (Table II). Conversely, patients with greater endothelial dysfunction had higher plasma levels of both hs-CRP ($P < .01$) and fibrinogen ($P < .01$) compared with patients with FMD greater than 6.2%. The association between inflammatory markers and endothelial dysfunction was confirmed by the finding that FMD negatively correlated with plasma levels of both hs-CRP ($r = -0.501$; $P < .01$) and fibrinogen ($r = -0.559$; $P < .01$) (Fig 1). In contrast, nitroglycerin-mediated vasodilation was unrelated to plasma levels of inflammatory markers (data not shown). Also after adjustment for confounding factors (age, sex, smoking habit, diabetes mellitus, hypertension, hyperlipidemia), plasma levels of hs-CRP and fibrinogen remained predictive of FMD < 6.2%, ie, of greater endothelial dysfunction (Table III, model 1). The same results were obtained when women were removed from the PAD and control groups (data not shown).

Relationship between FMD and ABI. Patients with FMD less than 6.2% had significantly lower ABI than did those with FMD greater than 6.2% (Table II). Moreover, ABI was significantly, albeit weakly, related to FMD ($r = 0.238$; $P < .05$) but not to nitroglycerin-mediated vasodilation (data not shown). ABI, when adjusted for age, sex, and classic cardiovascular risk factors, remained independently associated with greater endothelial dysfunction (Table III, model 1). Stepwise multiple regression analysis including ABI and plasma levels of hs-CRP and fibrinogen,



Correlation of brachial artery flow-mediated dilation (*FMD*) with plasma levels of both high-sensitivity C-reactive protein and fibrinogen.

ie, covariates that showed significant group differences at univariate analysis, indicated that each of the three variables was independently associated with FMD less than 6.2% (Table III, model 2).

Relationship between FMD and atherosclerotic burden. In the group of patients with FMD greater than 6.2%, 13 patients (25%) had isolated monolateral PAD, and 2 patients (4%) had widespread atherosclerosis, ie, bilateral PAD plus 2 sites (Table IV). In the group with FMD less than 6.2%, 4 patients (11%) had isolated monolateral PAD, and 3 patients (8%) had widespread atherosclerosis . Statistical analysis did not show group differences.

DISCUSSION

Impaired endothelial function, measured as reduced brachial artery FMD, has been demonstrated in PAD.[16-18] In our series, 41% of patients had FMD lower than the 5th percentile of the control population. Because brachial artery endothelial function is a measure of systemic vasculature status and a predictor of cardiovascular events,[14,15] our results are consistent with the finding that a large proportion of patients with PAD are at high cardiovascular risk.[1-4]

The main purpose of this study was to investigate the relationship between endothelial dysfunction in PAD and variables potentially able to alter homeostatic properties of endothelial cells. Cardiovascular risk factors impair endothelial function even in the absence of clinical evidence of cardiovascular disease.[10] However, hyperlipidemia, hypertension, smoking, and diabetes mellitus were distributed equally between patients with FMD less than 6.2% and those with better endothelial function. Conversely, plasma levels of hs-CRP and fibrinogen, two markers of inflammation with key roles in endothelial dysfunction,[7] differed significantly between the two subgroups of patients. Both markers negatively correlated with FMD; thus patients with the highest plasma levels of hs-CRP and fibrinogen had the lowest FMD values. Although correlation coefficients provide no information on cause and effect, our findings are consistent with the hypothesis that inflammation contributes to endothelial dysfunction. Of interest, in patients with CAD, normalization of CRP levels parallels improvement in endothelial function.[11] In our study, the association between reduced FMD and high levels of CRP and fibrinogen remained after adjustment for sex, age, and classic risk

Table III. Association of ankle/brachial index, high-sensitivity C-reactive protein, and fibrinogen with brachial artery flow-mediated dilation <6.2%

	Wald χ^2 *(model I)*	*Wald* χ^2 *(model 2)*
ABI	7.80*	7.29*
hs-CRP	4.55*	3.79†
Fibrinogen	6.01*	7.29†

ABI, Ankle-brachial index; *hs-CRP*, high sensitivity C-reactive protein; *FMD*, flow-mediated dilation.
Model 1 tests associations of FMD <6.2% with ABI, hs-CRP, and fibrinogen individually, adjusted for age, sex, smoking habit, hyperlipidemia, hypertension, and diabetes mellitus. Model 2 includes the three variables simultaneously.
*$P < .01$.
†$P < .05$.

Table IV. Atherosclerotic burden in patients with different degrees of brachial artery endothelium-dependent, flow-mediated dilation

	FMD >6.2% (N = 52)		*FMD <6.2% (N = 36)*	
	n	*%*	*n*	*%*
Monolateral isolated PAD (n = 17)	13	25	4	11
Bilateral isolated PAD (n = 14)	7	13	7	19
Bilateral PAD + 1 site (n = 23)	13	25	10	28
Bilateral PAD + 2 sites (n = 5)	2	4	3	8

FMD, Flow-mediated dilation; *PAD*, peripheral arterial disease.

factors. This coincides with the observation that in PAD the association between impaired FMD and elevated levels of other inflammatory markers, ie, circulatory forms of intercellular adhesion molecule–1 and vascular cell adhesion molecule–1, is unrelated to traditional risk factors.[17] We found no difference in prevalence of classic risk factors between patients with worse endothelial function and those with FMD greater than 6.2%. Neunteufl et al[21] found that patients with angiographically proved CAD have greater brachial artery endothelial dysfunction than do those with similar risk profile but with smooth coronary arteries. Together these findings support the concept that the influence of overt atherosclerosis exceeds that of traditional risk factors in affecting endothelial function. Consequently, PAD severity and total atherosclerotic burden could be other factors that influence the degree of endothelial dysfunction. In effect, patients with FMD less than 6.2% had lower ABI than those with FMD greater than 6.2%, even after adjustment for age, sex, and classic cardiovascular risk factors. Oxidative stress, a major determinant of endothelial dysfunction,[22] may be involved in the mechanism linking PAD severity and endothelial function. We did not measure oxidative stress; however, lipid peroxide levels are increased and antioxidant vitamin C levels are decreased in PAD.[23-25] It is conceivable that the lower the ABI, and thus the more severe the ischemic disease in the leg, the greater the oxidative stress and consequently the endothelial dysfunction. On the other hand, oxidative stress and endothelial dysfunction are associated with risk factors for PAD, also in the absence of vascular diseases.[26-28] For example, oxidative stress is one of the first abnormalities detected in children with diabetes, in tandem with impaired endothelial function.[29] Therefore increased oxidative stress could be the cause rather than the consequence of more severe PAD. The cross-sectional nature of this study does not allow us to clarify this point. In any event, the two hypotheses are not mutually exclusive. Oxidative stress and endothelial dysfunction secondary to cardiovascular risk factors could favor development of PAD in susceptible persons, and PAD would contribute to further deterioration in endothelial function via increased oxidative stress.

In multivariate analysis including ABI, hs-CRP, and fibrinogen, we found that all three variables were independently related to FMD less than 6.2%. In addition to being a marker of inflammation, CRP, which induces expression of adhesion molecules and chemokines in human endothelial cells,[30,31] could amplify the inflammatory process and thus endothelial dysfunction. Similarly, fibrinogen may contribute to vascular dysfunction through its proteolytic degradation products,[32,33] which may be found in atheroma.[34,35] Of interest, although in comparison of patients with PAD and control subjects endothelial dysfunction seemed to be unrelated to plasma levels of fibrinogen, in patients with PAD this inflammatory marker was significantly associated with reduced FMD. This apparent inconsistency may be explained by a synergistic action of given levels of fibrinogen with another factor present in patients with PAD but not in control subjects. This additional factor could be damaged endothelium, which when infiltrated by high levels of fibrinogen, as in our patients with FMD less than 6.2%, deteriorates even further.[32-37]

Compared with what we observed in patients with FMD greater than 6.2%, in the subgroup with FMD less than 6.2% the prevalence of isolated monolateral PAD was less than half and the prevalence of widespread atherosclerosis, ie, bilateral PAD plus 2 sites, was double. This suggests that FMD is related to atherosclerotic burden. The lack of difference in the extent of atherosclerosis between the two groups of patients could be due to the relatively small sample size.

Study limitations. Although treatments were discontinued at least 18 hours before the study, we cannot exclude the premise that the long-lasting effect of drugs such as angiotensin-converting enzyme inhibitors and calcium antagonists may have beneficially influenced FMD. However, because the number of subjects taking these drugs was much higher in the PAD group, these treatments did not influence the finding that patients with PAD have lower FMD than control subjects do. At best, longer drug discontinuation would have implied even greater FMD reduction in patients with PAD. In contrast, there was no difference in drug distribution between the two groups of patients, categorized according to FMD (Table II). On the other hand, because of the broad spectrum of drugs and doses administered, we were unable to examine the interaction between treatment status and FMD.

A second limitation of the study is that myocardial scintigraphy was not performed in the control group. This may imply underestimation of the prevalence of CAD, a condition, however, associated with reduced brachial artery FMD.[13] Therefore the lack of this information does not confer bias.

Finally, the measure we used to quantify atherosclerotic burden is less objective than angiographic score. However, at the time of the study, the clinical condition of our patients did not require angiography, and thus the extent of atherosclerotic disease was quantified according to the method previously used by Blann et al.[38]

CONCLUSIONS

A large proportion of patients with PAD have severe endothelial dysfunction that results from a variety of factors. It is interesting that low ABI and high levels of hs-CRP and fibrinogen, which were independently associated with reduced FMD, are also significant predictors of cardiovascular events in patients with PAD.[2,4-6] It is uncertain whether oxidative stress and inflammation per se are modifiable risk factors. However, preliminary data suggest that preventive therapy with angiotensin-converting enzyme inhibitors and statins may work through mechanisms that counteract oxidative stress and inflammation.[39-42] Therefore our data support the need for studies to assess whether these therapies, in addition to modification of classic risk factors, may help to improve endothelial function and reduce cardiovascular risk in PAD.

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