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In Vivo Confirmation of the Role of Statins in Reducing Nitric Oxide and C-Reactive Protein Levels in Peripheral Arterial Disease

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Abstract *Objectives:* Inflammatory and other processes mediating impairment of endothelial function, where there are increased levels of C-reactive protein (CRP) and plasma nitrites, have a part to play in the early stages of peripheral arterial disease (PAD). Our objective was to analyse the effect of statins on the plasma nitrite and CRP levels in PAD.
Material and methods: A prospective study of 30 patients with PAD Fontaine stage II, with no prior treatment with statins, determined high sensitivity (hs)-CRP and lipid profile in the patients. Plasma nitrite levels were determined by colourimetric assay based on the Griess reaction, at baseline and after 1 month of treatment with atorvastatin 40 mg day^{-1}.
Results: A significant reduction in plasma nitrite levels was detected after the treatment with statins (11.88 ± 7.8 μM vs. 5.7 ± 1.8 μM, $p = 0.0001$). There was also a significant reduction in hs-CRP levels (13.58 ± 24.00 vs. 3.93 ± 3.19, $p = 0.02$).

When the patients were stratified according to claudication stage, a significant reduction in nitrite levels was obtained, both in patients with PAD Fontaine stage IIA (9.5 ± 3.3 μM vs. 5.3 ± 1.7 μM, $p = 0.0001$) and in stage IIB (16.6 ± 11.6 μM vs. 6.7 ± 1.8 μM, $p = 0.032$).
Conclusions: Treatment with statins lowers plasma nitrite and CRP levels in patients with PAD. Our data support the effects of statins *in vivo* that have been demonstrated on the endothelium *ex vivo*, suggesting a beneficial effect by acting on the initial processes that trigger the disease, reducing oxidative stress (increase in the bioavailability of nitric oxide as peroxynitrite levels decrease) and curtailing the inflammatory processes which perpetuate the disease.

Endothelial dysfunction is considered to be an early marker for atherosclerosis, preceding evidence of atherosclerotic plaques on angiography or ultrasound scan. Endothelial dysfunction has been attributed to a deterioration in nitric

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oxide (NO) bioactivity and an increase in the formation of oxygen free radicals.[1] NO has several antiatherosclerotic effects, such as inhibition of monocyte migration, inhibition of smooth muscle cell proliferation and inhibition of platelet aggregation.[2–4] In patients with peripheral arterial disease (PAD), an increase in plasma nitrite levels has been observed; this increase can be detected from the first stages of the disease, but has no correlation with the severity.[5]

C-reactive protein (CRP) is a systemic inflammation marker, and its concentration has been associated with the future development of atherothrombotic events, both in patients with known cardiovascular disease and in apparently healthy subjects.[6] A linear association has been demonstrated between the clinical severity of PAD and an increase in high sensitivity (hs)-CRP plasma levels.[7] We know that CRP has a role in the modulation of the harmful effect of oxidised low-density lipoprotein (LDL) on endothelial function, contributing to oxidative stress and the subsequent production of free radicals (superoxide anion). These free radicals are capable of directly inactivating the NO, producing peroxynitrite — which is a cytotoxic, proinflammatory and potent oxidant — and may contribute to damage and endothelial dysfunction and to oxidation of the lipoproteins in atherosclerotic lesions.[8,9]

In studies *in vitro* with endothelial cell cultures and in studies with animals fed diets rich in cholesterol, it has been seen that statins are capable of increasing endothelial nitrous oxide synthase (eNOS) expression, reducing the NO/peroxynitrite ratio (by preventing the NOS from 'uncoupling' and subsequently acting as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase), increasing levels of tetrahydrobiopterin (BH4) in the endothelial cells, preventing the formation of atherosclerotic plaques and reducing CRP levels.[10–14]

Our objective was to analyse the effect produced by treatment with statins on plasma levels of nitrites and CRP in patients with Fontaine stage II PAD.

Material and Methods

A prospective study was carried out which selected 30 patients with Fontaine stage II PAD, confirmed by haemodynamic study (Doppler) and treadmill stress tests, with no previous re-vascularisation and no treatment with statins or contraindications for their use. All patients received treatment with atorvastatin 40 mg daily for 1 month.

Cardiovascular risk factors, treatment and general condition were recorded on inclusion and after 1 month of treatment with statins, and ankle—brachial index (ABI) were measured at rest as per the standard technique in the dorsalis pedis and posterior tibial arteries of both lower limbs.[15] Blood tests were performed at baseline and after treatment with statins, including basic clinical chemistry (blood sugar, renal function, electrolytes, etc.) and lipid profile. Patients were considered to be hypertensive if they presented with systolic blood pressure greater than 140 mmHg and/or diastolic pressure greater than 90 mmHg and/or were on antihypertensive treatment for at least 1 year prior to inclusion in the study.[16] Patients with plasma total cholesterol greater than 6.5 mmol l^{-1}, LDL cholesterol greater than 3.2 mmol l^{-1} or triglycerides greater than 2.25 mmol l^{-1}, or those on lipid-lowering treatment were considered to have dyslipidaemia.[17] Patients were considered diabetic if they presented with baseline blood sugar greater than 120 g dl^{-1} or if they required treatment with hypoglycaemics.[18] Chronic renal failure was defined as serum creatinine greater than 1.5 mg dl^{-1}.[19]

For the determination of plasma nitrite levels, the subjects came to the study having fasted for at least 12 h and without having taken their usual medication during that period. Blood was drawn from an antecubital vein and centrifuged for 10 min at 800*g*, with plasma then being stored at 4 °C. Plasma nitrite concentrations were determined by colourimetric assay based on the Griess reaction.[20] This is a chemical reaction which uses sulfanilamide and *N*-(1-naphthyl)ethylenediamine dihydrochloride (NED) under acidic conditions (phosphoric acid). The system can detect NO_2^- in a variety of biological and experimental fluids, the limit of detection being 2.5 μM (125 pmol). Each sample was analysed in triplicate, taking the mean of the three determinations. The blood tests were repeated in a control group of 10 patients to assess the reproducibility of the test, the coefficient of variation being less than 5%.

A venous blood sample was also taken to determine plasma concentrations of CRP using an automated high-sensitivity immunoassay (Roche Diagnostics), with a lower limit of detection of 0.2 mg l^{-1} and a coefficient of variation of 4.2% in 4 mg l^{-1} and 6.3% in 1 mg l^{-1}.[21]

Statistical analysis

The sample size necessary for the detection of significant differences was calculated on the basis of previous studies which analysed the NO plasma levels and hs-CRP.[5,7] A Student's *t*-test for paired samples was used for these variables with normal distribution. The analysis of normality was carried out using the Kolmogorov—Smirnov and Shapiro—Wilk tests. Spearman's rho test was used for the correlation between variables.

The data are expressed as mean ± standard deviation and the categoricals as percentages. The hs-CRP data are expressed as median ± 25th and 75th percentiles. Statistical significance was assumed for $p < 0.05$.

Results

Thirty patients with Fontaine stage II PAD were recruited. The patient demographics and current treatment are described in Table 1. No adverse reactions to the treatment with statins were recorded.

A significant reduction in plasma levels of total cholesterol, LDL cholesterol, total cholesterol/high-density lipoprotein (HDL) cholesterol ratio and total triglycerides was observed (Table 2).

After 1 month of treatment with statins, a significant reduction in plasma nitrite levels was detected in these patients (11.88 ± 7.8 μM vs. 5.7 ± 1.8 μM, $p = 0.0001$) (Fig. 1).

Table 1 Demographic data and treatment

	Patients	
	n	%
Age (years)	71.37 ± 10.81	(52–94)
Gender		
Male	26	86.7%
Ischaemia stage:		
IIA	20	66.7%
IIB	10	33.3%
Hypertension	22	73.3%
DM	12	40%
Smoking		
Current	12	40%
Ex-smoker	10	33.3%
AMI	4	13.3%
Dyslipidaemia	8	26.7%
CVA	2	6.7%
COPD	9	18%
AGGREG. inhibitor	26	86.7%
ACE inhibitors	14	46.7%
ARA-II	4	13.3%
B-BLOCKER	4	13.3%
Nitrites	2	6.7%
CA-Antagonists	6	20%

DM, diabetes mellitus; AMI, acute myocardial infarct; CVA, Cerebrovascular accident; COPD, chronic obstructive pulmonary disease; AGGREG, aggregation.

Analysis of hs-CRP levels in these patients showed statistically significant reduction after 1 month of treatment with atorvastatin (13.58 ± 24.00 vs. 3.93 ± 3.19, $p = 0.02$) (Fig. 2).

When we adjusted the data at baseline to Fontaine stage as an adjustment variable in order to eliminate a potential confounder (as earlier stage of the disease), we did not find that the stages of claudication were independent of plasma nitrite levels (decreased significantly both in patients with stage IIA ischaemia and in stage IIB; $p = 0.78$). However, it was independent of the hs-CRP levels (hs-CRP levels decreased significantly in the patients with stage IIA ischaemia, but not in those with PAD Fontaine stage IIB, $p = 0.01$).

Table 2 Initial lipid-profile values and values after 1 month of treatment with statins

	Mean ± SD	*P*
Total CHOL baseline	5.39 ± 1.28 mmol l^{-1}	0.0001
Total CHOL after treatment	3.79 ± 0.15 mmol l^{-1}	
LDL CHOL baseline	3.38 ± 1.07 mmol l^{-1}	0.0001
LDL CHOL after treatment	1.85 ± 0.60 mmol l^{-1}	
HDL CHOL baseline	1.34 ± 0.28 mmol l^{-1}	0.12
HDL CHOL after treatment	1.4 ± 0.39 mmol l^{-1}	
CHOL/HDL baseline	4.02 ± 0.86	0.0001
CHOL/HDL after treatment	2.76 ± 0.63	
TGC baseline	1.30 ± 0.53 mmol l^{-1}	0.024
TGC after treatment	1.07 ± 0.43 mmol l^{-1}	
TGC/HDL baseline	2.34 ± 1.35	0.113
TGC/HDL after treatment	1.99 ± 1.21	

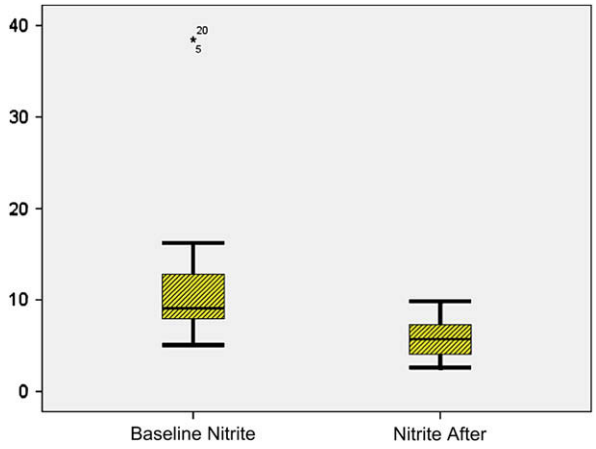

Figure 1 Comparison of plasma nitrite levels (μM) at baseline and post-treatment with statins. *$p < 0.05$.

Discussion

High levels of LDL and oxidative stress lead to endothelial dysfunction. The result is a drop in the bioavailability of endothelial NO, a reduction in the effective lumen of the vessel and an increase in sensitivity to acetylcholine-induced vasoconstriction. The levels of oxidised LDL (ox-LDL) are increased in patients with coronary artery disease or diabetes and are an independent predictive factor for future cardiac events in these patients.[22] Ox-LDLs reduce the expression of eNOS proteins and the bioavailable NO concentration, while at the same time producing an increase in the release of peroxynitrite, reflecting an increase in oxidative stress.[23]

It could be assumed that the ability of statins to reverse these abnormalities is simply the result of a reduction in LDL levels, since an increase in these levels can in itself cause endothelial dysfunction.[24] However, it has been demonstrated that the beneficial effects of statins on the

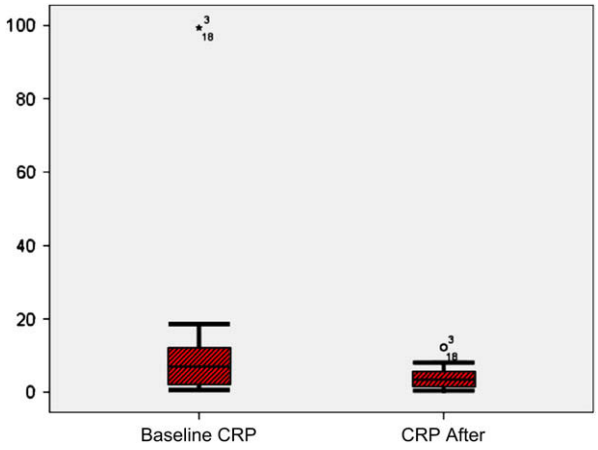

Figure 2 Comparison of hs-CRP levels (mg l^{-1}) at baseline and post-treatment with statins. *$p < 0.05$.

endothelium are independent of the fall in LDL levels.[25] Both properties of statins promote an improvement in endothelial function and in the production of NO.[26,27]

The beneficial effects on endothelial function produced by the statins have been demonstrated in studies with endothelial cell cultures and in animal models subjected to diets rich in cholesterol. The statins increase the bioavailability of NO and reduce levels of peroxynitrite — the main component of oxidative stress — increasing the NO—peroxynitrite ratio, crucial in maintaining endothelial function. This beneficial effect on the function of the enzyme eNOS is produced on both normal endothelium and extensively dysfunctional endothelium.[10] The effect on the release of NO in the endothelial cells produced by the statins is the result of an increase in eNOS expression and reduced eNOS 'uncoupling'. When BH4 levels are low and/or the production of superoxide anion increases, NOS 'uncouples' and behaves like an NADPH oxidase, increasing the production of superoxide anion and hydrogen peroxide more than that of NO, whereby the net balance is a reduction in NO activity.[9,28] The statins are capable of reducing superoxide anion levels through the inhibition of NADPH oxidase.[29] They are also capable of increasing GTP cyclohydrolase I (GTPCH) — the first and rate-limiting enzyme in BH4 synthesis in the endothelial cells. The statins are thus able to increase levels of BH4, an essential cofactor involved in the functioning of eNOS, both via GTPCH and by preventing the oxidation of BH4 to radical BH3, as a result of reducing the levels of peroxynitrites. This then prevents the 'uncoupling' of the NOS, thus breaking the vascular oxidative stress cycle.[10,11,30] In previous studies, increased plasma nitrite levels have been observed in patients with PAD from very early stages.[5] In our study, we corroborated the action of statins on the reduction in plasma nitrite levels and the subsequent improvement in the bioavailability of NO in patients with PAD, finding a significant reduction in plasma nitrite levels in these patients.

Raised CRP levels seem to be an independent predictive factor for cardiovascular events in patients with PAD,[31] and there is a linear correlation between the increase in plasma CRP levels and the severity of the disease in these patients.[7] This finding strongly suggests the existence of an inflammatory substrate in the aetiopathogenesis of PAD. We know that CRP has a role in modulating the harmful effect of ox-LDL on endothelial function, contributing to oxidative stress and the subsequent production of free radicals (superoxide anion) as a result of being able to increase NADPH oxidase expression.[32] These free radicals are capable of destroying the BH4 cofactor and directly inactivating NO, producing peroxynitrite and thereby contributing to the damage and to endothelial dysfunction and to oxidation of the lipoproteins in the atherosclerotic lesions.[8,9] CRP can also directly inhibit the activity of the enzyme GTPCH, reducing BH4 levels and leading to the 'uncoupling' of eNOS and a decrease in the bioavailability of NO.[33] An increase in peroxynitrite levels has been observed when iNOS is expressed.[9,34] Thus, CRP stimulates the production of NO by NOS, increasing its oxidation and nitrosylation and reducing BH4 levels, promoting the formation of free radicals, which, in turn, inactivate the NO produced and destroy BH4, resulting in endothelial dysfunction. In fact, it has been demonstrated in *in vitro* studies that CRP is capable of stimulating the production of NO, independent iNOS stimulation.[34] A reduction in CRP levels has been described in patients with atherosclerosis treated with statins — a reduction which is independent of baseline lipid levels.[14] In our study, we also observed a significant reduction in hs-CRP levels in patients with PAD after 1 month of treatment with statins. The fact that statistical significance was not found in the reduction in hs-CRP levels in patients with Fontaine stage IIB PAD may be due to the small sample size as, being a sub-analysis of the initial group, it is likely that there were too few patients for statistical significance to be achieved.

The REACH Registry[35] is the largest worldwide registry designed to obtain information on cardiovascular risk-factor control and preventive treatment in a population who have, or are at high risk of having, symptoms of atherothrombosis. This is a registry of consecutive patients who have risk factors only (RFO) for atherothrombosis or who have symptomatic vascular disease (VD): coronary heart disease (CHD) and/or cerebrovascular disease (CVD) and/or peripheral artery disease (PAD). Statin therapy rate was 65.2% vs. 65.6% (no significant difference). Significant differences were found among the CHD, CVD and PAD groups as regards statin therapy rate (78.2%, 51.9% and 57.8%, respectively; $p < 0.005$). The results of our work suggest that patients with claudication due to PAD might benefit from statin therapy independent from serum lipid levels due to the effect of atorvastatin in reducing serum nitrite levels and levels of CRP, because current evidence supports that reduction in nitrite levels is associated with increased bioavailability of NO and because reduction of CRP correlates with reduced systemic inflammatory activity.

Our study has potential limitations that should be acknowledged. One is the fact that nitrite levels are influenced by many exogenous and endogenous factors, including dietary nitrate uptake, inhalation of atmospheric gaseous nitrogen oxides, salivary formation and renal function. Even if we cannot exclude that these factors may have influenced nitrite levels, our data are in accordance with previous studies. Although it could seem that the fact that we assessed only the plasma nitrite levels and no other nitrates is a limitation of the study, we know that otherwise, in physiological conditions, more than 70—90% of plasma nitrites derive from the activity of the eNOS.[36] Another limitation is the small size of patient samples in the analysis of subgroups, and this confers a low statistical power to the results of this sub-analysis.

Treatment with statins reduces plasma nitrite and CRP levels in patients with PAD. Our data validate *in vivo* the effects of statins demonstrated *ex vivo* on the endothelium, which produce a beneficial effect by acting on the initial processes which trigger the disease, reducing oxidative stress (increase in bioavailability of NO on decreasing peroxynitrites levels) and curtailing the inflammatory processes which perpetuate the disease.

Conflict of Interest

The authors have no conflict of interest to declare.

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