European Heart Journal (2007) **28**, 608–612
doi:10.1093/eurheartj/ehl533

Oxidative-stress-mediated arterial dysfunction in patients with peripheral arterial disease

L. Loffredo[1], A. Marcoccia[1], P. Pignatelli[1], P. Andreozzi[1], M.C. Borgia[1], R. Cangemi[1], F. Chiarotti[2], and Francesco Violi[1]*

[1]*IV Divisione di Clinica Medica, Department of Experimental Medicine and Pathology, University of Rome 'La Sapienza', Viale del Policlinico 155, Roma 00161, Italy and* [2]*Department of Cell Biology and Neuroscience, Istituto Superiore di Sanità, Rome, Italy*

Received 18 May 2006; revised 30 December 2006; accepted 18 January 2007; online publish-ahead-of-print 13 February 2007

KEYWORDS
Peripheral vascular disease;
Flow-mediated dilation;
Nitric oxide;
Oxidative stress;
Propionylcarnitine

Aims To investigate the existence of a relationship among flow-mediated dilation (FMD), nitric oxide (NO), and oxidative stress in patients with peripheral arterial disease (PAD), and to assess if the administration of an antioxidant was able to improve arterial dilatation.

Methods and results We performed a cross-sectional study comparing FMD, 8-Hydroxy-2-deoxy-2-deoxyguanosine (8-OHdG), a marker of oxidative stress, and nitrite/nitrate (NOx) serum levels in a population of 25 PAD patients and 40 controls. In the second part of the study, 21 PAD patients were randomly allocated to a treatment sequence of 7 days of i.v. infusion of placebo or 6 g/day propionyl-L-carnitine (PLC) in a cross-over design. Compared with controls, patients with PAD had enhanced 8-OHdG serum levels (2.4 ± 1.2 vs. 4.24 ± 3.11 ng/mL; $P < 0.001$), reduced NOx (17.02 ± 6.11 vs. 11.28 ± 6.02 μM; $P < 0.001$), and lowered FMD (10.34 ± 2.14 vs. 6.69 ± 2.95; $P < 0.001$).

PLC infusion was associated with an increase of FMD [from 6.6 ± 0.6 to 11.1 ± 1.2% (mean ± SE), $P = 0.004$] and NOx (from 14.5 ± 1.4 to 17.1 ± 1.2 μM; +18%, $P = 0.012$) and a decrease of 8-OHdG (from 3.62 ± 0.37 to 2.64 ± 0.32 ng/mL; −27%, $P < 0.001$). No changes were observed after placebo treatment.

Conclusion This study shows that in PAD patients, oxidative stress is implicated in determining reduced FMD.

Introduction

Peripheral arterial disease (PAD) is a clinical setting affecting more than 5% of population older than 60.[1] Despite the low rate of peripheral complications and amputation, PAD is complicated by high rate of coronary and cerebral events. For this reason, PAD is considered a marker for systemic atherosclerosis and its early diagnosis may be helpful for identifying patients at risk for cardiovascular events.[2]

Among patients with PAD, analysis of ankle/arm pressure ratio may help in identifying those who are at higher risk of cardiovascular events.[2,3] More recently endothelial dysfunction has been recognized as another useful marker to stratify the risk of cardiovascular disease not only in patients with coronary heart disease (CHD) but also in PAD patients.[4–6] In particular, Brevetti *et al.*[6] demonstrated that PAD patients having low flow-mediated vasodilatation (FMD) were at high risk to develop cardiovascular complications including CHD and leg amputation.

Even if FMD is prevalently dependent upon release of nitric oxide (NO), a potent vasodilator and anti-aggregating molecule, from vascular wall,[7] the interplay between FMD and NO in PAD has not been fully clarified. In subjects with cardiovascular risk factors, Heiss *et al.*[8] demonstrated that plasma nitroso compounds, a marker of NO generation, inversely correlated with FMD. The mechanism accounting for reduced NO generation and, more in particular, the interplay between NO and oxidative stress was not investigated. Thus, oxidative stress plays a major role in modulating NO bioactivity inasmuch as it greatly contributes to its rapid metabolism via NO inactivation by O_2^-[9]; such interaction leads to formation of peroxinitrite, a potent oxidant molecule present in the atherosclerotic plaque.[9] On the basis of the fact that oxidative stress is enhanced[10] and FMD is reduced in PAD,[11] we speculated that oxidative stress could be responsible for reduced NO bioactivity and eventually arterial dysfunction. Therefore, the first aim of the study was to investigate the existence of a relationship among oxidative stress, NO, and FMD in patients with PAD. The second aim of the study was to assess if the administration of an antioxidant was able to improve arterial dilatation in PAD patients.

* Corresponding author: Tel: +39 06 4461933; fax: 39 06 49970893.
E-mail address: francesco.violi@uniroma1.it

Methods

The study has been divided in two parts. In the first part, we performed a cross-sectional study comparing FMD, oxidative stress, as assessed by serum levels of 8-Hydroxy-2-deoxy-2-deoxyguanosine (8-OHdG),[12] and NO generation, as assessed by serum levels of nitrite/nitrate (NOx), in a population of PAD and controls.

In the second part, we performed an interventional trial in PAD patients to assess if the infusion of an antioxidant, propionyl-L-carnitine (PLC), was able to improve endothelial dysfunction.

Cross-sectional study

The study has been carried out in 25 consecutive patients, aged between 40 and 80, presenting for symptoms of intermittent claudication (for at least 6 months) in the ambulatory of our Division between September 2004 and October 2005. Forty consecutive subjects presenting themselves for metabolic screening, without clinically relevant atherosclerotic disease, were enrolled in the same period as control. These patients had no clinical history of cardiovascular disease, had normal ECG, and echocardiography.

Every PAD patient to be enrolled in the study had:

(1) claudication (defined as leg pain on walking, disappearing within 10 min of standing, of presumed atherosclerotic origin) and
(2) ankle/brachial index (ABI) that was assessed as ankle/arm systolic blood pressure ratio by Doppler ultrasonography <0.80 on the worst leg at rest.

All subjects underwent a full medical history, physical examination, 12-lead ECG, laboratory test, and measurement of ABI. Patients had to be in stable conditions without abrupt changes of ABI ($>20\%$) in the last month before the enrolment. Subjects were excluded from the study if they had liver insufficiency, serious renal disorders (serum creatinine >2.8 mg/dL) and myocardial infarction, coronary revascularization, peripheral vascular surgery or percutaneous intervention procedure, unstable angina, acute cerebro-vascular disease or deep venous thrombosis or if they were current smokers.

Informed written consent was obtained from all subjects: the study was conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee.

To evaluate the presence of atherosclerotic disease in PAD patients and controls, all subjects enrolled in the study underwent ultrasonographic scans of the carotid arteries for intima–media thickness (IMT) evaluation; controls were excluded from the study if they had an IMT >0.9 mm.

The number of patients initially assessed for inclusion into the study was 35; after initial assessments four patients were excluded from the study for an ABI >0.8, one patient for serum creatinine >2.8 mg/dL, four patients for CHD, and one patient for current smoking; four subjects enrolled for cross-sectional study refused to participate in the interventional study.

Interventional study with PLC

Twenty-one of 25 PAD patients accepted to participate in the interventional study that was performed between January and March 2006. They were randomly allocated to a treatment sequence with i.v. infusion of placebo or PLC (6 g/day) in a double-blind cross-over design. There was a 1 month-washout between the two phases of the study. Every infusion was carried out over a 12 h period for 7 days. All patients have been hospitalized during each period of the study. FMD, oxidative stress, and NOx were measured at baseline and 12 h after the last infusion of placebo or PLC.

Randomization and blinding

A person, not involved in the study, assigned codes to the study treatments, randomly allocated the selected participants to a treatment sequence with i.v. infusion of placebo or PLC (6 g/day), and kept the key in a sealed envelope. The randomization was carried out by a procedure based on a random numeric sequence. The authors were unaware of treatment allocation.

ABI measurement

ABI was calculated with the patient placed in the supine position, measuring the higher systolic pressure of the anterior or posterior tibial artery in each limb and dividing this pressure with the highest brachial systolic pressure.[3] In patients with diabetes, we performed toe pressure determinations.

Measurement of carotid intima–media thickness

Longitudinal ultrasonographic scans of the carotid artery were obtained on the same day as the studies of the brachial artery reactivity and included the evaluation of the right and left common carotid arteries 1 cm proximal to the carotid bulb. In each examination, the same operator used different scanning angles to identify the greatest IMT, defined as the distance between the junction of the lumen and intima and that of the media and adventitia. Three measurements of IMT were obtained from the right and left carotid arteries, respectively, and were averaged to determine the mean IMT for both sides combined. The coefficient of variation was 2.6%.

Flow-mediated vasodilatation

Ultrasound assessment of endothelial dependent and independent FMD of brachial artery was investigated according to the recently reported guidelines.[13] Briefly, the study was performed in a temperature-controlled room (22°C) with the subjects in a resting, supine state between the hours of 8 a.m. and 10 a.m.; brachial artery diameter was imaged using a 7.5-Mhz linear array transducer ultrasound system (Siemens) equipped with electronic callipers, vascular software for two-dimensional imaging, colour and spectral Doppler, and internal electrocardiogram; the brachial artery was imaged at a location 3–7 cm above the antecubital crease; to create a flow stimulus in the brachial artery, a sphygmomanometric cuff was placed on the forearm; the cuff was inflated at least 50 mmHg above systolic pressure to occlude artery inflow for 5 min; all vasodilatation measurements were made at the end of diastole; FMD was expressed as a change in post-stimulus diameter evaluated as a percentage of the baseline diameter.

NO_2/NO_3 and 8-OHdG serum levels

Between 8 and 9 a.m. a blood sample was taken from each patient who had fasted at least 12 h. Samples were immediately centrifuged at 3000 rpm for 15 min at 4°C, and the supernatant was collected and stored at −80°C until measurement.

NO serum levels were evaluated through the measurement of metabolic end products (Calbiochem nitritenitrate assay), i.e. nitrite and nitrate (NOx), using enzymatic catalysis coupled with Griess reaction as reported by Verdon *et al.*[14] Intra- and inter-assay coefficients of variation were 2.2 and 4.0%, respectively.

Serum levels of 8-OHdG were analysed using a commercial competitive enzyme-linked immunosorbent assay for quantitative measurement of the oxidative DNA adduct 8-hydroxy-2deoxyguanosine, (Bioxytech 8-OHdG-EIA, OXIS Health Products, Portland) following the manufacturer's protocol. Samples were submitted to filtration to separate interfering substances (cut off molecular weight 10 000). Absorbance was read at 450 nm wavelength and the concentrations of 8-OHdG were determined by comparison with the standard curve. The lower detection limit of 8-OHdG in this assay was 0.15 ng/mL. Intra- and inter-assay coefficients of variation have been determined at multiple points on the standard curve and were 2.1 and 4.5%, respectively.

Statistical analysis

Sample size determination

As reported earlier, for the cross-sectional study, we recruited all the patients ($n = 25$) attending the ambulatory of our Division between September 2004 and October 2005, who respected the inclusion/exclusion criteria. The number of controls ($n = 40$) was computed with respect to a two-tailed Student's *t*-test for independent groups, considering as (i) clinically relevant difference in FMD to be detected between patients and controls $|\delta| \geq 2.5\%$, (ii) standard deviations homogeneous between groups SDs = 2.95%, (iii) type I error probability $\alpha = 0.05$ and power $1 - \beta = 0.90$.

As regards the interventional cross-over study, we computed the minimum sample size with respect to a two-tailed one-sample Student's *t*-test, considering as (i) clinically relevant difference for FMD variation to be detected between PLC and control treatments $|\delta| \geq 2.5\%$, (ii) standard deviation of the paired differences SD = 3.0%, (iii) type I error probability $\alpha = 0.05$ and power $1 - \beta = 0.90$. This resulted in $n = 18$, which was increased to $n = 21$ in order to keep the degrees of freedom for the assessment of treatment effect in the statistical analysis at the required level.

Statistical methods

Categorical variables are reported as counts (percentage) and continuous variables as means ± SD unless otherwise indicated. Independence of categorical variables was tested by χ^2-test. Comparisons between PAD patients and controls were carried out by Student's *t*-test and were replicated as appropriate with nonparametric tests (Kolmogorov–Smirnov (z) test in case of non-homogeneous variances as verified by Levene's test).

The cross-over study data were analysed for the assessment of treatment and period effects, by performing a split-plot ANOVA with one between-subject factor (treatment sequence) and two within-subject factors (period 1 vs. 2; pre- vs. post-treatment).[15] The full model was considered, allowing for the assessment of all main effects and 5-way interactions. Pairwise comparisons were corrected by the Bonferroni test; results were expressed as means ±SE.

Bivariate analysis was performed with Pearson's linear regression test.

A value of $P < 0.05$ was considered statistically significant. All analyses were carried out with SPSS-13.0 software (SPSS Inc.).

Results

Cross-sectional study

Clinical characteristics of PAD patients and controls are reported in *Table 1*. As expected, PAD patients had higher prevalence of risk factors for cardiovascular disease, including hypertension and diabetes, compared with controls. Dyslipidaemia was not different between patients and controls likely because many PAD patients were treated with statins.

Compared with controls, patients with PAD had a lower FMD (10.34 ± 2.14 vs. 6.69 ± 2.95%; $P < 0.001$); also, PAD patients had enhanced oxidative stress, as documented by elevated serum levels of 8-OHdG (4.24 ± 3.11 vs. 2.4 ± 1.2 ng/mL; $P < 0.001$), and reduced NOx serum levels (11.28 ± 6.02 vs. 17.02 ± 6.11 μM; $P < 0.001$).

Simple linear regression analysis showed that NOx was correlated with FMD ($r = 0.45$; $P = 0.02$) and 8-OHdG ($r = -0{,}672$; $P < 0.001$).

Table 1 Clinical characteristics of patients with PAD and controls

Variables	Controls ($n = 40$)	PAD ($n = 25$)	*P*-value
Mean age (years)[a]	64.2 ± 11.2	63.7 ± 7.9	0.846
Males/Females	30/10	21/4	0.583
Hypertension, % (*n*)	37.5 (15)	96 (24)	**< 0.001**
Diabetes mellitus, % (*n*)	0 (0)	20 (5)	**< 0.001**
Dyslipidaemia, % (*n*)	30 (12)	48 (12)	0.231
Medication, % (*n*)			
ACE-inhibitors	25 (10)	48 (12)	0.102
Oral anti-diabetic drugs	7.5 (3)	16 (4)	0.507
Insulin	0 (0)	4 (1)	0.811
Statin	7.5 (3)	40 (10)	**0.004**
Ex-smokers, % (*n*)	30 (12)	64 (16)	**0.015**
FMD (%)[a]	10.34 ± 2.14	6.69 ± 2.95	**< 0.001**
NO_X (μM)[a]	17.02 ± 6.11	11.28 ± 6.02	**< 0.001**
8-OHdG (ng/mL)[a]	2.4 ± 1.2	4.24 ± 3.11	**< 0.001**
ABI[a]	1.2 ± 0.11	0.52 ± 0.12	**< 0.001**

[a]Data are expressed as mean ± SD.
Statiscally significant values are expressed in bold.

Interventional study

After placebo infusion, no change of brachial basal diameter was observed (from 4.58 ± 0.11 to 4.59 ± 0.11 mm; P = n.s.); conversely after PLC infusion, a significant increase of brachial basal diameter was observed (from 4.6 ± 0.10 to 4.89 ± 0.11 mm; $P < 0.001$).

Because drugs that produce increases in baseline diameter lead to smaller estimates of FMD,[16] we calculated FMD as an absolute change in diameter (expressed as percentage variation) in order to minimize this problem.

From the ANOVA performed on cross-over study data, we found neither significant effect of period nor significant interactions between period and treatment and/or pre–post infusion ($0.47 < P < 0.96$ for all effects). On the contrary, we observed significant effects of treatment [$F(1,19) = 7.69$, $P = 0.012$], pre-post infusion [$F(1,19) = 14.14$, $P = 0.0013$], and two-way interaction treatment × pre–post infusion [$F(1,19) = 9.98$, $P = 0.005$]. The latter indicates that the variation pre–post infusion was different between the two treatments. In particular, the pairwise comparisons showed that FMD significantly increased in the PLC group (before vs. after infusion: 6.6 ± 0.6 vs. 11.1 ± 1.2%; $P = 0.004$), while no significant change was observed in the placebo group (before vs. after infusion: 6.8 ± 0.6 vs. 7.4 ± 0.7%; $P = 0.645$) (*Figure 1*).

Finally, we analysed NOx and 8-OHdG serum levels in 10 out of the 21 patients enrolled for the interventional study. Clinical characteristics of this subgroup were similar to that of the entire population participating in the interventional study (not shown). After placebo infusion, no changes of NOx (from 14.8 ± 1.1 to 13.9 ± 1.2 μM; $P = 0.566$) and 8-OHdG (from 3.52 ± 0.33 to 3.45 ± 0.35 ng/mL; $P = 0.347$) serum levels were observed. Conversely, in patients after PLC infusion an increase of NOx (from 14.5 ± 1.4. to 17.1 ± 1.2 μM; $P = 0.012$) and a

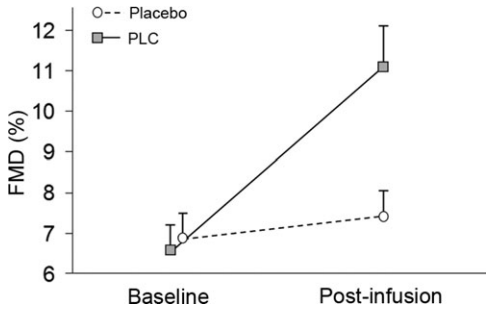

Figure 1 FMD before and after infusion of placebo or PLC.

decrease of 8-OHdG (from 3.62 ± 0.37 to 2.64 ± 0.32 ng/mL; $P < 0.001$) were found.

Discussion

Previous studies have shown reduced FMD in different series of PAD patients including those undergoing peripheral vascular revascularization or those with less severe PAD not requiring vascular surgery.[4,6] FMD is an important predictor of poor outcome, as PAD patients with low FMD are at high risk of subsequent vascular accidents.[6]

The mechanism accounting for reduced FMD in PAD patients has not been fully clarified. FMD is prevalently dependent upon endothelial release of NO,[7] and several studies demonstrated a significant direct correlation between reduced FMD and circulating levels of nitroso compounds that reflect systemic NO bioactivity.[8,17] However, the mechanism accounting for reduced NO bioactivity was neither clarified nor was it investigated to determine if oxidative stress could play some role in reducing NO bioactivity. Therefore, we first investigated the behaviour of FMD in PAD population, then we tried to analyse the role of NO bioactivity and its interplay with oxidative stress.

In accordance with previous reports,[11] our PAD population had reduced FMD, which was significantly associated with PAD, independent from the coexistence of risk factors, which may potentially affect FMD. To measure NO generation, we analysed the plasma concentration of nitrate and nitrite, which are formed *in vivo* upon NO oxidation.[18,19] Nitrite, in particular, is one of the major oxidative metabolites of NO and greatly contributes to the circulating pool of bioactive NO.[19] Patients with PAD had significantly lower NOx serum levels compared with controls suggesting, in accordance with previous report,[20] that NO generation is reduced in this clinical setting. The significant association between NOx and FMD in PAD population is novel and extends previous data indicating that circulating levels of NO-derived compounds are predictors of FMD.[8,17]

Thus, NO is rapidly inactivated by superoxide anion to give formation of peroxynitrite, an important oxidant species detected in the atherosclerotic plaque.[9] Also, oxidative stress may affect NO generation by inhibiting eNOS.[7] Using different markers of oxidative stress, such as serum isoprostanes, and the titre of antibodies against oxidized LDL, previous studies demonstrated a significant increase of oxidative stress in PAD patients.[10] The present study reinforces these data as serum levels of 8-OHdG, a marker of oxidative stress,[12,21] were significantly higher in patients with PAD compared with controls and suggests that it may be implicated in reducing NO bioactivity. The cross-sectional study seemed to support this hypothesis as serum levels of 8-OHdG and NOx were inversely correlated, thereby suggesting that oxidative stress could be implicated in eliciting arterial dysfunction via reducing NO generation.

To further explore this hypothesis, we performed an interventional study with LPC, a molecule that possesses antioxidant property via inhibition of arachidonic acid-induced NADPH oxidase activation.[22] Owing to the low bioavailability of oral LPC,[23–25] we preferred the intravenous route to investigate if LPC had antioxidant property and was able to influence FMD. The interventional trial showed that LPC infusion was associated with enhanced arterial vasodilatation as documented by the significant increase of arterial diameter compared with placebo. Such vasodilating effect was associated with a reduction of 8-OHdG and an increase of NO generation, so reinforcing the hypothesis that oxidative stress was implicated in arterial dysfunction of PAD patients. These data are consistent with an experimental study performed in spontaneously hypertensive rats in which PLC infusion was associated with amelioration of arterial dysfunction and increased serum levels of NOx.[26]

The intrinsic mechanism through which PLC inhibited oxidative stress *in vivo* can only be a matter of speculation. We have previously shown that PLC inhibits *in vitro* platelet NADPH oxidase activation.[22] This enzyme is also present in the vascular wall where it has a key role in arterial dysfunction via the production of superoxide anion.[27] Further study is, therefore, necessary to investigate if the vasodilating effect of carnitine is dependent on inhibition of vascular NADPH oxidase activation.

Our study has potential limitations that should be acknowledged. Patients with PAD have several risk factors that may contribute to lower FMD independently from PAD. Even if small sample size limits definite conclusions, it is of interest that PAD is significantly associated with low FMD. Measurement of NOx as markers of NO generation have been studied in atherosclerotic patients and provided divergent results.[28–32] This may depend on the fact that NOx is influenced by many exogenous and endogenous factors including dietary nitrate uptake, inhalation of atmospheric gaseous nitrogen oxides, salivary formation, and renal function.[33] Even if we cannot exclude that these factors may have influenced NOx, our data are in accordance with previous ones showing low NO generation in PAD patients[20] and are consistent with a relationship between circulating levels of nitrite and nitrate and FMD.[8,17] The mechanism through which oxidative stress inhibits NO generation and in turn FMD was not investigated. Even if it is arguable that oxidative stress affects NO synthase and/or NO degradation, further study is necessary to deeply explore the underlying mechanism.

In conclusion, this study shows that in PAD patients there is an imbalance between oxidative stress and NO, which may be responsible for reduced FMD. This hypothesis is supported by interventional study with an antioxidant that ameliorated arterial dysfunction. Owing to the relevance of arterial dysfunction in predicting poor outcome, our findings warrant further study to investigate the potential usefulness of antioxidants that prevent NADPH oxidase activation in PAD population.

Acknowledgement

This study was supported by a grant from the University of Rome 'La Sapienza' (Ateneo 2003).

Conflict of interest: none declared.

References

1. Dormandy J, Mahir M, Ascady G, Balsano F, De Leeuw P, Blombery P, Bousser MG, Clement D, Coffman J, Deutshinoff A. Fate of the patient with chronic leg ischaemia. A review article. *J Cardiovasc Surg (Torino)* 1989;**30**:50–57.
2. Hirsch AT, Haskal ZJ, Hertzer NR, Bakal CW, Creager MA, Halperin JL, Hiratzka LF, Murphy WR, Olin JW, Puschett JB, Rosenfield KA, Sacks D, Stanley JC, Taylor LM Jr, White CJ, White J, White RA, Antman EM, Smith SC Jr, Adams CD, Anderson JL, Faxon DP, Fuster V, Gibbons RJ, Hunt SA, Jacobs AK, Nishimura R, Ornato JP, Page RL, Riegel B. ACC/AHA 2005 Practice Guidelines for the management of patients with peripheral arterial disease (lower extremity, renal, mesenteric, and abdominal aortic): a collaborative report from the American Association for Vascular Surgery/Society for Vascular Surgery, Society for Cardiovascular Angiography and Interventions, Society for Vascular Medicine and Biology, Society of Interventional Radiology, and the ACC/AHA Task Force on Practice Guidelines (Writing Committee to Develop Guidelines for the Management of Patients With Peripheral Arterial Disease): endorsed by the American Association of Cardiovascular and Pulmonary Rehabilitation; National Heart, Lung, and Blood Institute; Society for Vascular Nursing; TransAtlantic Inter-Society Consensus; and Vascular Disease Foundation. *Circulation* 2006;**113**:e463–e654.
3. Hiatt WR. Medical treatment of peripheral arterial disease and claudication. *N Engl J Med* 2001;**344**:1608–1621.
4. Gokce N, Keaney JF Jr, Hunter LM, Watkins MT, Menzoian JO, Vita JA. Risk stratification for postoperative cardiovascular events via noninvasive assessment of endothelial function: a prospective study. *Circulation* 2002;**105**:1567–1572.
5. Gokce N, Keaney JF Jr, Hunter LM, Watkins MT, Nedeljkovic ZS, Menzoian JO, Vita JA. Predictive value of noninvasively determined endothelial dysfunction for long-term cardiovascular events in patients with peripheral vascular disease. *J Am Coll Cardiol* 2003;**41**:1769–1775.
6. Brevetti G, Silvestro A, Schiano V, Chiariello M. Endothelial dysfunction and cardiovascular risk prediction in peripheral arterial disease: additive value of flow-mediated dilation to ankle-brachial pressure index. *Circulation* 2003;**108**:2093–2098.
7. Cooke JP. Flow, NO, and atherogenesis. *Proc Natl Acad Sci USA* 2003;**100**:768–770.
8. Heiss C, Lauer T, Dejam A, Kleinbongard P, Hamada S, Rassaf T, Matern S, Feelisch M, Kelm M. Plasma nitroso compounds are decreased in patients with endothelial dysfunction. *J Am Coll Cardiol* 2006;**47**:573–579.
9. Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res* 2000;**87**:840–844.
10. Mueller T, Dieplinger B, Gegenhuber A, Haidinger D, Schmid N, Roth N, Ebner F, Landl M, Poelz W, Haltmayer M. Serum total 8-iso-prostaglandin F2alpha: a new and independent predictor of peripheral arterial disease. *J Vasc Surg* 2004;**40**:768–773.
11. Brevetti G, Silvestro A, Di Giacomo S, Bucur R, Di Donato A, Schiano V, Scopacasa F. 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. *J Vasc Surg* 2003;**38**:374–379.
12. Higashi Y, Sasaki S, Nakagawa K, Matsuura H, Oshima T, Chayama K. Endothelial function and oxidative stress in renovascular hypertension. *N Engl J Med* 2002;**346**:1954–1962.
13. Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, Deanfield J, Drexler H, Gerhard-Herman M, Herrington D, Vallance P, Vita J, Vogel R. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. *J Am Coll Cardiol* 2002;**39**:257–265.
14. Verdon CP, Burton BA, Prior RL. Sample pretreatment with nitrate reductase and glucose-6-phosphate dehydrogenase quantitatively reduces nitrate while avoiding interference by NADP+ when the Griess reaction is used to assay for nitrite. *Anal Biochem* 1995;**224**:502–508.
15. Hills M, Armitage P. The two-period cross-over clinical trial. *Br J Clin Pharmacol* 1979;**8**:7–20.
16. Moens AL, Goovaerts I, Claeys MJ, Vrints CJ. Flow-mediated vasodilation: a diagnostic instrument, or an experimental tool? *Chest* 2005;**127**:2254–2263.
17. Kleinbongard P, Dejam A, Lauer T, Jax T, Kerber S, Gharini P, Balzer J, Zotz RB, Scharf RE, Willers R, Schechter AN, Feelisch M, Kelm M. Plasma nitrite concentrations reflect the degree of endothelial dysfunction in humans. *Free Radic Biol Med* 2006;**40**:295–302.
18. Wang X, Tanus-Santos JE, Reiter CD, Dejam A, Shiva S, Smith RD, Hogg N, Gladwin MT. Biological activity of nitric oxide in the plasmatic compartment. *Proc Natl Acad Sci USA* 2004;**101**:11477–11482.
19. Lundberg JO, Weitzberg E. NO generation from nitrite and its role in vascular control. *Arterioscler Thromb Vasc Biol* 2005;**25**:915–922.
20. Boger RH, Bode-Boger SM, Thiele W, Creutzig A, Alexander K, Frolich JC. Restoring vascular nitric oxide formation by L-arginine improves the symptoms of intermittent claudication in patients with peripheral arterial occlusive disease. *J Am Coll Cardiol* 1998;**32**:1336–1344.
21. Wu LL, Chiou CC, Chang PY, Wu JT. Urinary 8-OHdG: a marker of oxidative stress to DNA a risk factor for cancer atherosclerosis and diabetics. *Clin Chim Acta* 2004;**339**:1–9.
22. Pignatelli P, Lenti L, Sanguigni V, Frati G, Simeoni I, Gazzaniga PP, Pulcinelli FM, Violi F. Carnitine inhibits arachidonic acid turnover, platelet function, and oxidative stress. *Am J Physiol Heart Circ Physiol* 2003;**284**:H41–H48.
23. Mancinelli A, Longo A, Nation RL, Evans AM. Disposition of L-carnitine and its short-chain esters, acetyl-L-carnitine and propionyl-L-carnitine, in the rat isolated perfused liver. *Drug Metab Dispos* 2000;**28**:1401–1404.
24. Harper P, Elwin CE, Cederblad G. Pharmacokinetics of bolus intravenous and oral doses of L-carnitine in healthy subjects. *Eur J Clin Pharmacol* 1988;**35**:69–75.
25. Sahajwalla CG, Helton ED, Purich ED, Hoppel CL, Cabana BE. Multiple-dose pharmacokinetics and bioequivalence of L-carnitine 330-mg tablet versus 1-g chewable tablet versus enteral solution in healthy adult male volunteers. *J Pharm Sci* 1995;**84**:627–633.
26. Herrera MD, Bueno R, De Sotomayor MA, Perez-Guerrero C, Vazquez CM, Marhuenda E. Endothelium-dependent vasorelaxation induced by L-carnitine in isolated aorta from normotensive and hypertensive rats. *J Pharm Pharmacol* 2002;**54**:1423–1427.
27. Zalba G, San Jose G, Moreno MU, Fortuno MA, Fortuno A, Beaumont FJ, Diez J. Oxidative stress in arterial hypertension: role of NAD(P)H oxidase. *Hypertension* 2001;**38**:1395–1399.
28. Hata T, Hashimoto M, Kanenishi K, Akiyama M, Yanagihara T, Masumura S. Maternal circulating nitrite levels are decreased in both normal normotensive pregnancies and pregnancies with preeclampsia. *Gynecol Obstet Invest* 1999;**48**:93–97.
29. Cassone Faldetta M, Laurenti O, Desideri G, Bravi MC, De Luca O, Marinucci MC, De Mattia G, Ferri C. L-arginine infusion decreases plasma total homocysteine concentrations through increased nitric oxide production and decreased oxidative status in Type II diabetic patients. *Diabetologia* 2002;**45**:1120–1127.
30. Osanai T, Fujiwara N, Saitoh M, Sasaki S, Tomita H, Nakamura M, Osawa H, Yamabe H, Okumura K. Relationship between salt intake, nitric oxide and asymmetric dimethylarginine and its relevance to patients with end-stage renal disease. *Blood Purif* 2002;**20**:466–468.
31. Akarasereenont P, Nuamchit T, Thaworn A, Leowattana W, Chotewuttakorn S, Khunawat P. Serum nitric oxide levels in patients with coronary artery disease. *J Med Assoc Thai* 2001;**84**(Suppl. 3):S730–S739.
32. Ishibashi T, Matsubara T, Ida T, Hori T, Yamazoe M, Aizawa Y, Yoshida J, Nishio M. Negative NO3- difference in human coronary circulation with severe atherosclerotic stenosis. *Life Sci* 2000;**66**:173–184.
33. Szabo C, Day BJ, Salzman AL. Evaluation of the relative contribution of nitric oxide and peroxynitrite to the suppression of mitochondrial respiration in immunostimulated macrophages using a manganese mesoporphyrin superoxide dismutase mimetic and peroxynitrite scavenger. *FEBS Lett* 1996;**381**:82–86.