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Hyperhomocysteinaemia and endothelial dysfunction in young patients with peripheral arterial occlusive disease

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Abstract. Hyperhomocysteinaemia, defined as an abnormally high plasma homocysteine concentration after an oral methionine load, is common in young (≤ 50 years) patients with peripheral arterial occlusive disease. It is thought to predispose to atherosclerosis by injuring the vascular endothelium. Treatment with pyridoxine and/or folic acid may lower plasma homocysteine levels. In mildly hyperhomocysteinaemic patients with peripheral arterial occlusive disease, we studied the effect of daily treatment with pyridoxine (250 mg) plus folic acid (5 mg) on homocysteine metabolism (i.e. plasma concentrations in the fasting state and after methionine loading, in 48 patients) and on endothelial function (in 18 patients). Endothelial function was estimated as the plasma concentrations of the endothelium-derived proteins, von Willebrand factor (vWF), thrombomodulin (TM), and tissue-type plasminogen activator (tPA). At baseline, fasting homocysteine levels were above normal in 24 of the 48 patients (50%); post-load levels, by definition, were above normal in 100% of patients. After 12 weeks of treatment, fasting and post-load levels were normal in 98 and 100% of patients, respectively. Endothelial function was assessed in 18 patients who completed 1 year of treatment. At baseline, median vWF (235%) and TM (57.1 ng mL^{-1}) levels were above normal. At follow-up, vWF levels had decreased to 170% ($P = 0.01$) and TM levels had decreased to 49 ng mL^{-1} ($P = 0.04$). tPA levels were normal at baseline and did not change. Endothelial dysfunction is present in young patients with peripheral arterial occlusive disease and hyperhomocysteinaemia. Pyridoxine plus folic acid treatment normalizes homocysteine metabolism in virtually all patients, and appears to ameliorate endothelial dysfunction.

Keywords. Atherosclerosis, endothelial dysfunction, hyperhomocysteinaemia.

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Introduction

Mild hyperhomocysteinaemia is an independent risk factor for peripheral arterial occlusive disease [1–4]. Studies in animals and *in vitro* indicate that high plasma concentrations of homocysteine, derived from demethylation of dietary methionine, may predispose to atherosclerosis by injuring the vascular endothelium, which results in endothelial dysfunction [5–9]. Pyridoxine and/or folic acid supplementation have been shown to reduce plasma homocysteine concentrations in mildly hyperhomocysteinaemic patients with peripheral occlusive disease [10], but it is not known to what extent such treatment normalizes homocysteine metabolism as estimated by plasma homocysteine concentrations in the fasting state and after an oral methionine load. It is also unknown whether endothelial dysfunction is present in these patients and, if so, whether treatment aimed at normalizing homocysteine metabolism improves endothelial function.

We assessed endothelial function in young patients with peripheral arterial occlusive disease and hyperhomocysteinaemia as defined by an abnormal plasma concentration after methionine loading [1]. A gold standard for endothelial dysfunction is not available; we therefore measured plasma levels of three endothelium-derived proteins involved in the regulation of haemostasis, i.e. von Willebrand factor (vWF), thrombomodulin (TM) and tissue-type plasminogen activator (tPA), because *in vitro* and *in vivo* data suggest that endothelial injury is associated with increased plasma levels of these proteins [11–18]. In addition, we investigated the effects of treatment with pyridoxine plus folic acid on homocysteine metabolism and endothelial function.

Patients and methods

Patients

Between November 1991 and April 1993, a total of

Table 1. Clinical characteristics of patients with peripheral arterial occlusive disease and hyperhomocysteinaemia

<i>n</i> (M/F)	48 (17/31)
Age (years)	43.9 (1.1)
Blood pressure (mmHg)	138/87 (20/20)
Smokers	32 (67%)
Serum cholesterol (mmol L ⁻¹)	6.3 (1.2)
Diabetes mellitus	2 (4%)
Serum creatinine (μmol L ⁻¹)	85 (21)

Data are mean values (SD) unless otherwise indicated.

205 consecutive patients presenting with peripheral arterial occlusive disease and aged 50 years or younger were recruited from the Department of Vascular Surgery at the Free University Hospital, Amsterdam. Symptomatic peripheral arterial occlusive disease was defined as intermittent claudication and/or ischaemic ulceration, rest pain and gangrene, and/or amputation for ischaemia, and was confirmed by Doppler and/or angiographic studies. After obtaining each patient's informed consent, clinical and laboratory data were collected.

Blood pressure was measured after 15 min of supine rest without altering antihypertensive regimens. Diabetes was defined according to WHO criteria, and known diabetes duration was recorded. Patients were classified as non-smokers (those who did not smoke cigarettes, cigars, or a pipe) or smokers (all others). Drug use was also recorded. After an overnight fast, blood was drawn for measurement of serum total cholesterol (measured enzymatically), creatinine (modified Jaffé reaction), glucose (glucose oxidase method), and endothelial function parameters (as specified below), and an oral methionine loading test was performed to detect hyperhomocysteinaemia. The clinical data were collected and laboratory assays performed by personnel 'blinded' to the presence of treatment with pyridoxine plus folic acid.

Methionine loading test

Plasma levels of homocysteine were determined in the fasting state and 6 h after an oral methionine load (0.1 g kg⁻¹). Plasma homocysteine levels were measured as total homocysteine by using HPLC with fluorescence detection [19]. Normal fasting and post-load values measured in our laboratory are <18 and <51 μmol L⁻¹, respectively, in men (*n* = 21), <15 and <49 μmol L⁻¹, respectively, in premenopausal women (*n* = 56), and <19 and <69 μmol L⁻¹, respectively, in postmenopausal women (*n* = 20). Deficiencies of vitamin B₁₂, pyridoxine and folic acid were excluded by measuring serum levels. Normal values are >120 pmol L⁻¹ for vitamin B₁₂, >17 nmol L⁻¹ for pyridoxine, and >3.4 nmol L⁻¹ for folic acid.

A total of 205 patients were tested in this way; 48 (23%) of them had hyperhomocysteinaemia as defined by an abnormal post-load plasma concentra-

tion [1]. Table 1 shows the clinical features of these patients. All had normal renal and hepatic function.

Main outcome measures

The 48 hyperhomocysteinaemic patients were all treated with pyridoxine (250 mg daily) plus folic acid (5 mg daily). The effect on fasting and post-load homocysteine levels was studied after 6 weeks and, in patients in whom the post-load plasma concentration at 6 weeks was not in the normal range, again 6 weeks later. Parameters of endothelial function, and the effect of treatment on these parameters, were investigated in all patients who, in June 1993, had completed at least 1 year of treatment (*n* = 18). This period was chosen to allow a reasonable amount of time for the effect of treatment on endothelial function to become manifest.

Endothelial function. The plasma concentrations of von Willebrand factor antigen (vWF) [20], thrombomodulin (TM; Asserachrom Thrombomodulin, Diagnostica Stago, Asnières, France) [18], and tissue-type plasminogen activator antigen (tPA; Imulyse tPA, Biopool, Umeå, Sweden) [21] were measured by enzyme-linked immunosorbent assays. The plasma vWF level is expressed as a percentage of normal pooled plasma, the antigen level of which is defined as 100% (normal range, 50–150% [13]). For TM and tPA, normal ranges are 16.5–47.5 ng mL⁻¹ and 1.84–9.80 ng mL⁻¹, respectively, as obtained in a control group (*n* = 21) matched for age with the 18 hyperhomocysteinaemic patients in whom TM and tPA were measured. All blood samples were taken between 08.00 and 09.00 hours, after an overnight fast. We were careful to avoid acute increases in the concentrations of these proteins associated with exercise, smoking, prolonged venous occlusion, hypoglycaemia, and acute illness. The intra- and interassay version of the vWF, TM and tPA assays is less than 10%; the day-to-day variability of vWF within individuals is about 10%; that of tPA is about 15% (unpublished data).

Statistical analysis

Data are given as mean values (SD) or as median (range), unless otherwise indicated. Parametric and non-parametric tests were used as appropriate. Paired tests were used to compare baseline with post-treatment data. We used univariate analysis to study the relationship between endothelial function and possible determinants thereof, including fasting and post-load homocysteine levels as well as 'classic' risk factors for atherosclerotic vascular disease (age, sex, smoking habits, systolic and diastolic blood pressure, and serum cholesterol; diabetes was not included in the analysis because there was only one diabetic among the 18 patients in the endothelial function study). The relationship of these 'classic' risk factors with endothelial function parameters was assessed

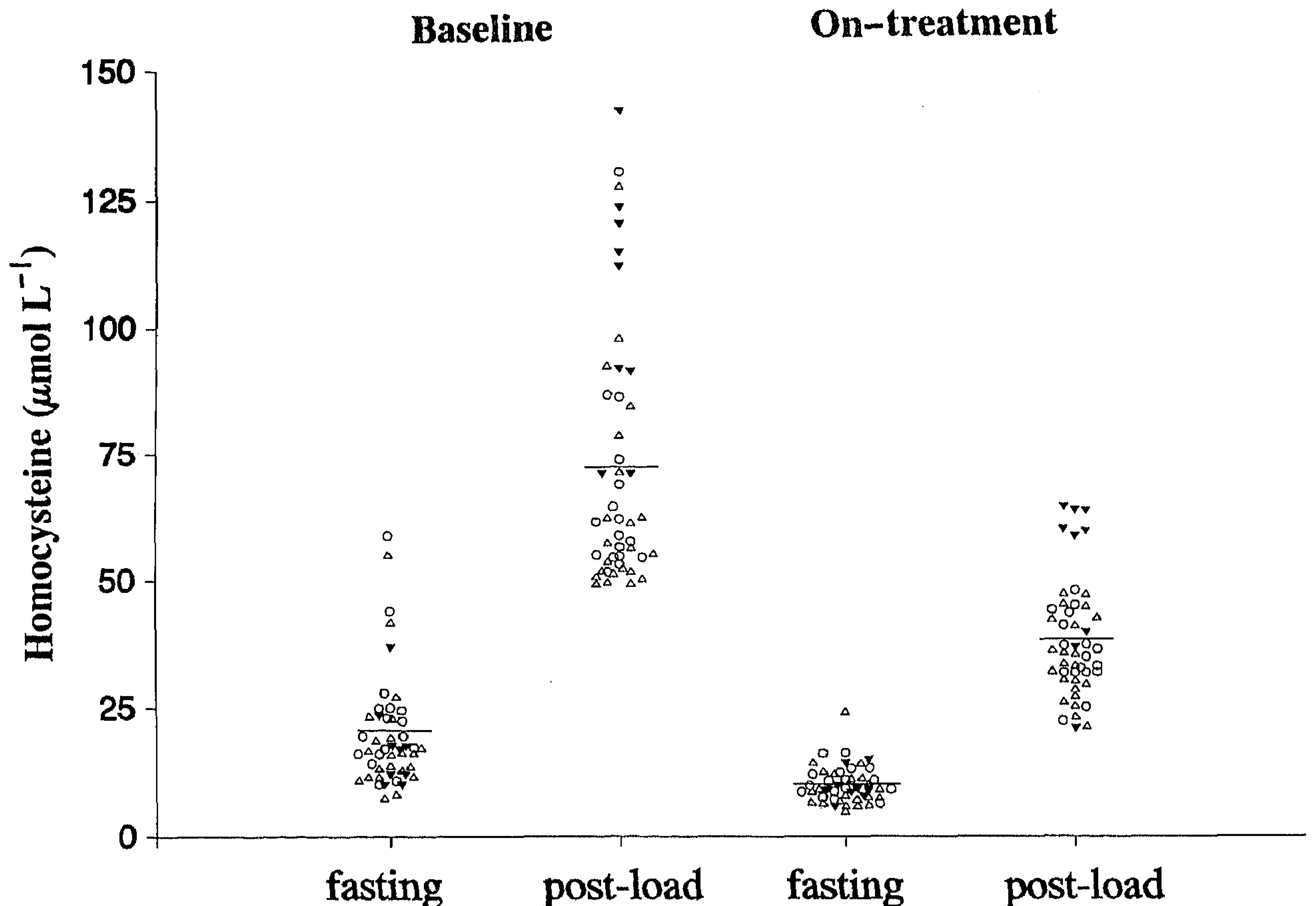


Figure 1. Plasma homocysteine concentrations (fasting and after methionine loading) before and during treatment (6 weeks in 45 patients and 12 weeks in three patients) with pyridoxine plus folic acid: O, men; Δ , premenopausal women; ∇ , postmenopausal women.

both at baseline and at follow-up (i.e. at 1 year). In addition, we studied the relationship between homocysteine levels (fasting and post-load) and endothelial function parameters at baseline and between homocysteine levels after treatment (i.e. at 6 weeks) and endothelial function parameters at follow-up (i.e. at 1 year). All testing was two-tailed, with a value of 0.05 taken to be the level of significance.

Results

The patients reported no adverse effects of the pyridoxine plus folic acid treatment. No new vascular events occurred during follow-up, either in the 48 patients followed for 6–12 weeks or in the subgroup of 18 patients followed for ≥ 1 year. Mean (SD) fasting and post-load plasma homocysteine levels were 20.7 (14.7) and 73.8 (22.3) $\mu\text{mol L}^{-1}$ before treatment, and 9.6 (3.6) and 36.8 (9.1) $\mu\text{mol L}^{-1}$ after treatment ($P < 0.001$ for both comparisons; Fig. 1). By definition, post-load homocysteine concentrations before treatment were abnormal in all patients. In contrast, fasting concentrations were within the normal range in 24 of the 48 patients (50%). After 6 weeks of treatment, the fasting and

post-load homocysteine concentrations were within the normal range in 47 (98%) and 45 (94%) of 48 patients, respectively. After 12 weeks, these figures were 47 (98%) and 48 (100%).

Table 2 shows the clinical characteristics of the 18 patients who completed at least 1 year of treatment (and in whom endothelial function was assessed).

At baseline, vWF and TM levels were above normal, but tPA levels were in the normal range. At follow up, plasma vWF concentrations had decreased from median 235 to 170% ($P = 0.01$), and plasma TM concentrations had decreased from 57.1 to 49.0 ng ml^{-1} ($P = 0.04$). Plasma tPA levels had not changed (6.9 ng ml^{-1} at baseline vs. 6.5 ng ml^{-1} at follow-up [$P = 0.91$]; Table 2, Fig. 2).

With regard to the classic risk factors, significant relationships were observed at baseline (but not at follow-up) between age and tPA ($r = 0.57$, $P = 0.01$), serum cholesterol and vWF ($r = 0.48$, $P = 0.04$), and smoking habits and TM (smokers had lower TM levels than non-smokers (49 [29–72] vs. 70 [48–113] ng mL^{-1} ; $P = 0.03$).

The fasting homocysteine plasma levels at baseline and after 6 weeks of treatment were not related to endothelial function parameters at baseline and

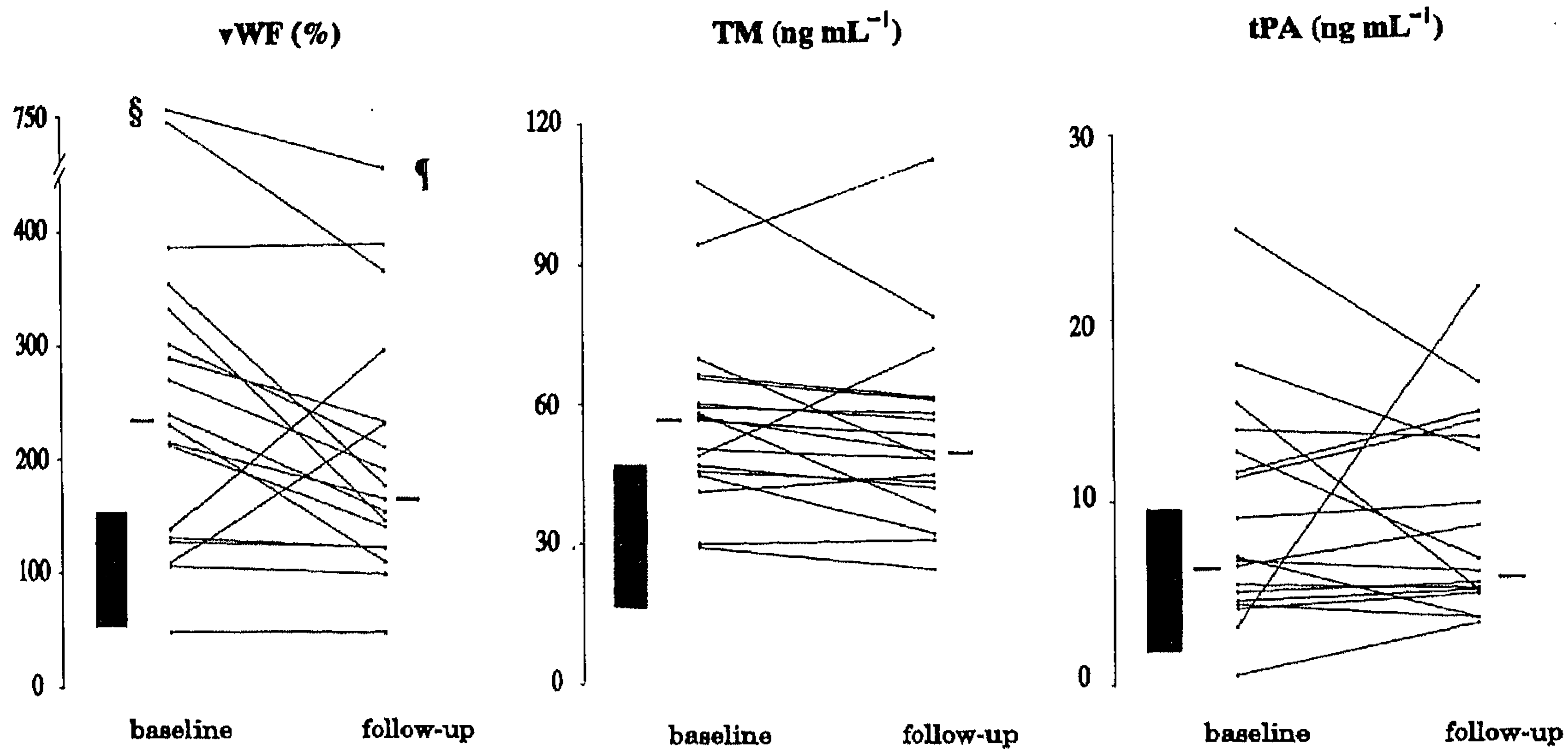


Figure 2. Endothelial function parameters before and after 1 year of treatment with pyridoxine plus folic acid: vWF, von Willebrand factor; TM, thrombomodulin; tPA, tissue-type plasminogen activator, §, ¶ = values not to scale, of +716 and +727 (§), and +454 (¶). The black rectangles indicate the normal ranges.

follow-up, respectively, except for the homocysteine level at 6 weeks and the tPA level at follow-up ($r = 0.44$, $P = 0.06$). In contrast, the post-load homocysteine plasma levels at baseline and after 6 weeks of treatment showed trends towards significant relationships with the endothelial function parameters at baseline and follow-up, respectively: vWF (baseline: $r = 0.36$, $P = 0.14$; follow-up: $r = 0.69$, $P = 0.001$, Fig. 3), TM (baseline: $r = 0.73$, $P = 0.001$; follow-up: $r = 0.35$, $P = 0.15$), and tPA (baseline: $r = 0.06$, $P = 0.8$; follow-up: $r = 0.38$, $P = 0.12$).

Discussion

In keeping with earlier reports [1–4], our results

Table 2. Characteristics of the hyperhomocysteinaemic patients who completed 1 year of treatment

	Baseline	Follow-up
<i>n</i> (M/F)	18 (6/12)	
Age (years)	43.6 (1.2)	44.6 (1.2)
Follow-up duration (months)		12.1 (12–13)
Blood pressure (mmHg)	142/91 (18/26)	141/86 (18/10)
Serum cholesterol (mmol L ⁻¹)	6.3 (0.2)	6.0 (0.4)
Smoking (Y/N)	13/5	11/7
Diabetes mellitus	1 (5.6%)	1 (5.6%)
von Willebrand factor (%)	235	170*
Thrombomodulin (ng mL ⁻¹)	57.1	49†
Tissue-type plasminogen activator (ng mL ⁻¹)	6.9	6.5

Data are mean values (SD) unless otherwise indicated. * $P = 0.01$; † $P = 0.04$.

indicate a high prevalence of mild hyperhomocysteinaemia among young patients with peripheral arterial occlusive disease. Such patients show evidence of endothelial dysfunction as estimated by vWF and TM plasma concentrations. Endothelial dysfunction is thought to play an important role in atherogenesis. Treatment with pyridoxine plus folic acid normalizes homocysteine metabolism in virtually all patients, both in terms of the fasting homocysteine plasma level and the level after an oral methionine load.

Treatment with pyridoxine and folic acid is based on their involvement in homocysteine metabolism. Furthermore, both agents have been shown to lower the grossly elevated homocysteine plasma levels observed in classic homocystinuria, an inborn error of metabolism characterized by premature atherosclerosis and venous and arterial thromboembolism [4]. Classic homocystinuria is usually caused by homozygous deficiency of cystathionine synthase, an enzyme involved in the conversion of homocysteine to

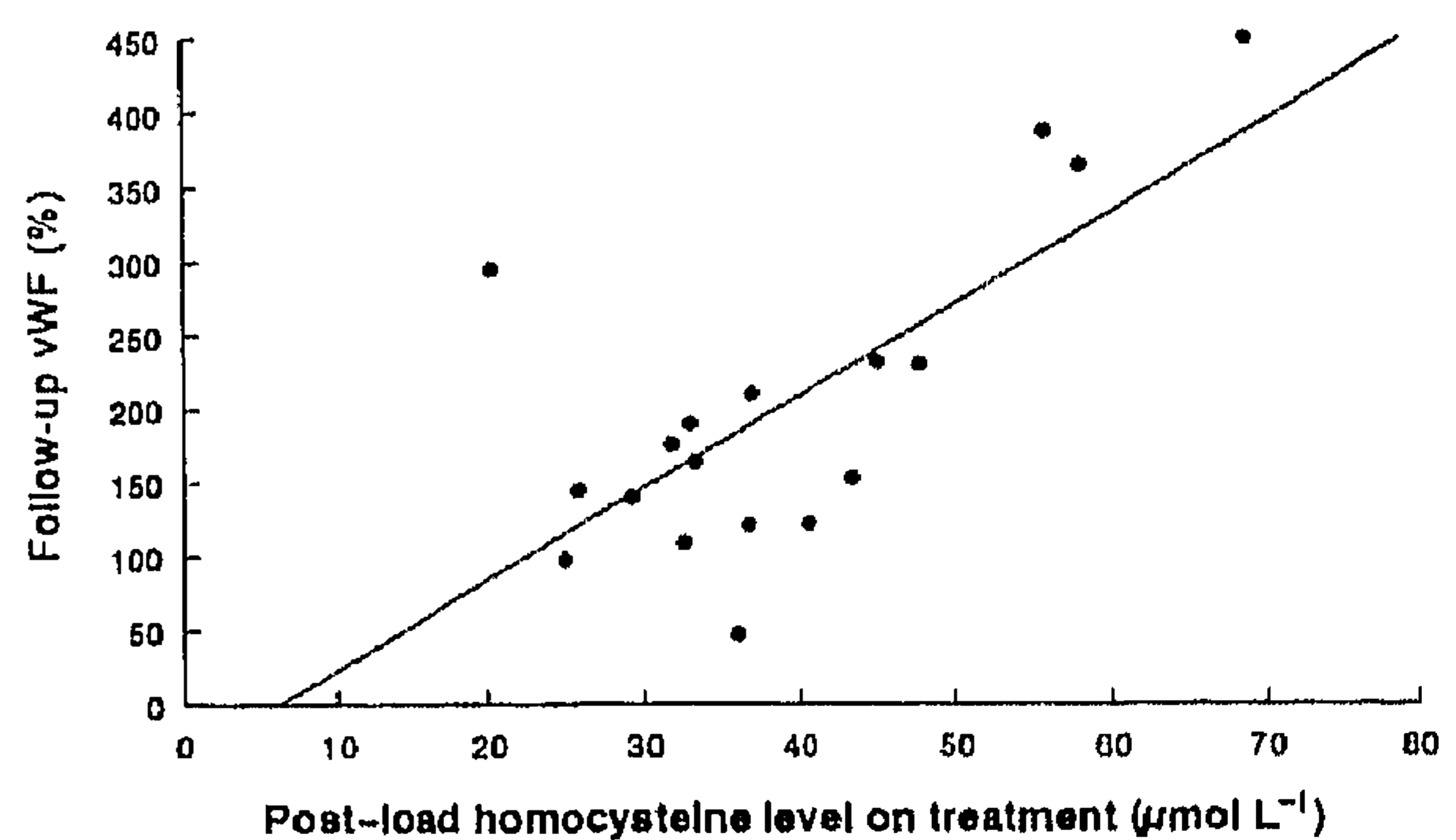


Figure 3. von Willebrand factor after 1 year of treatment with pyridoxine plus folic acid as a function of the post-methionine load plasma homocysteine concentration: $r = 0.69$; $P < 0.001$.

cystathionine. The active form of pyridoxine, pyridoxal phosphate, is a cofactor in this reaction. Its homocysteine-lowering effect is thought to be due to stimulation of cystathionine synthase activity. In contrast, folic acid reduces the plasma homocysteine concentration by enhancing its remethylation to methionine [4]. Therefore, the effects of these treatment modalities can theoretically be expected to be additive.

Our patients were selected for having mild hyperhomocysteinaemia, which may be due to heterozygous cystathionine synthase deficiency [1] or to other metabolic defects [4,22]. Reliable and convenient methods to measure such deficiencies directly are not available at present. Instead, methionine loading, which stresses the pathways involved in homocysteine metabolism, can be used as a diagnostic test to uncover abnormalities of homocysteine handling. It is not clear which estimate of homocysteine metabolism, fasting [3,23] or post-load [1,2] level should be chosen to guide treatment, because it is not known which is the better predictor of atherosclerotic disease. We therefore measured both, and observed no major differences in the metabolic efficacy of pyridoxine plus folic acid treatment, whether expressed as fasting or as post-load homocysteine concentrations.

Importantly, we found such treatment to be associated with improvement, although not normalization, of endothelial function, suggesting that hyperhomocysteinaemia-associated endothelial dysfunction might be reversible. Homocysteine, a highly reactive sulphur-containing amino acid, is thought to damage endothelial cells by several mechanisms, e.g. generation of hydrogen peroxide [7] and depletion of nitric oxide-mediated detoxification of homocysteine [9]. In addition, as hyperhomocysteinaemia is often due to genetic defects in the enzymes that regulate homocysteine metabolism [1,2,4,22], and because these defects are also present in endothelial cells [8], the endothelium of these individuals may be particularly vulnerable to homocysteine toxicity [6]. Endothelial dysfunction is a central feature of current models of atherogenesis [24]. Increased vWF and TM plasma concentrations probably reflect ongoing endothelial injury [11–16]. Furthermore, high plasma vWF levels have been shown to predict a poor cardiovascular prognosis in survivors of myocardial infarction [12] and in patients with non-insulin-dependent diabetes mellitus [13]. The nature of the link between atherosclerosis and vWF and TM is not known with certainty. High vWF and TM are probably markers of the presence of endothelial injury and the process of atherogenesis. In addition, high plasma vWF levels may have functional significance because vWF enhances platelet adhesion and coagulation, the latter by stabilizing factor VIII. TM, a membrane-bound protein, contributes to the inhibition of thrombin generation, thereby establishing an important local control of the coagulation cascade. Elevated TM plasma levels may reflect decreased

binding to the cell membrane [15], thus allowing enhanced thrombin activity or, alternatively, they may result from a homocysteine-induced increase in synthesis and turnover [25]. The precise mechanism by which hyperhomocysteinaemia increases vWF and TM plasma concentration is unknown. *In vitro* studies have suggested that homocysteine might decrease vWF secretion [26] and TM expression [27], findings that indicate major differences between the *in vitro* and *in vivo* situation.

The normal plasma level of tPA, an important regulator of fibrinolysis, suggests that endothelial function was not altered in this respect. However, interpretation is limited by the fact that we studied a relatively small group of patients. In addition, we did not measure tPA's inhibitor, PAI-1, which together with tPA is thought to determine fibrinolytic capacity.

Other limitations of our study should also be considered. First, the treatment was neither randomized nor controlled. It appears unlikely, however, that the substantial effect of treatment on homocysteine levels was a change finding, as such levels are known to be quite stable over time, whereas other conditions known to affect homocysteine metabolism were excluded [4]. In addition, although a placebo-controlled trial would obviously be the most preferable study design, our experience with symptomatic hyperhomocysteinaemic patients suggests that many such patients would not consent to such a trial in view of the perceived safety and efficacy of vitamin therapy [4]. Second, we cannot be certain that the changes in endothelial function parameters were induced by the pyridoxine plus folic acid treatment, although this interpretation is supported by the strong correlation between post-treatment homocysteine and vWF levels (Fig. 3). However, the observed correlations between homocysteine levels and endothelial function parameters, although theoretically plausible, should be considered preliminary until they are confirmed in a larger group. As other cardiovascular risk factors did not change significantly during follow-up, changes in factors such as smoking habits are an unlikely explanation for the observed decreases in vWF and TM. Furthermore, vWF levels are relatively stable over periods of up to 3 years in (diabetic) patients remaining free of cardiovascular disease [13], suggesting that regression to the mean is also an unlikely explanation of the changes in vWF. Third, notwithstanding the promising effects of pyridoxine plus folic acid supplementation on endothelial function parameters, the clinical efficacy of the proposed treatment needs to be investigated in terms of prevention of new vascular events.

In conclusion, endothelial dysfunction is present in young patients with peripheral arterial occlusive disease and mild hyperhomocysteinaemia. Pyridoxine plus folic acid treatment normalizes homocysteine metabolism in the majority of patients, and appears to ameliorate endothelial dysfunction.

In young patients with coronary artery or cerebrovascular disease, the effect of pyridoxine plus folic acid on homocysteine metabolism was similar to that reported here [28].

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