A Randomized Double-Blind Placebo-Controlled Trial of the Effect of Homocysteine-Lowering Therapy With Folic Acid on Endothelial Function in Patients With Coronary Artery Disease

Jeetendra Thambyrajah, BMEDSCI, MRCP,* Martin J. Landray, MRCP,* Heather J. Jones, RGN,* Fiona J. McGlynn, RGN,† David C. Wheeler, MD, FRCP,† Jonathan N. Townend, MD, MRCP*

Birmingham, United Kingdom

OBJECTIVES
This study was designed to determine the effects of folic acid therapy on endothelial function in patients with coronary artery disease (CAD).

BACKGROUND
Hyperhomocysteinemia, a risk factor for CAD, may cause atherosclerosis by oxidative endothelial injury. Folic acid reduces plasma homocysteine, but the effect on adverse vascular events is unknown.

METHODS
In a double-blind placebo-controlled trial, 90 patients (mean age [range] 63 [46 to 79] years, 79 men) with CAD were randomized to either folic acid 5 mg or placebo daily for 12 weeks. Endothelial function was assessed by measuring: 1) flow-mediated endothelium-dependent dilation (EDD) of the brachial artery; 2) combined serum nitrite/nitrate (NOX) concentrations and; 3) plasma von Willebrand factor (vWF) concentration.

RESULTS
At the end of the study, plasma homocysteine was lower in the folic acid group compared with the placebo group (mean [95% confidence interval] 9.3 (8.5 to 10.1) vs. 12.3 (11.3 to 13.4) μmol/l, p < 0.001). Although there were no significant differences in EDD, serum NOX or plasma vWF between the two groups, there was a greater increase in EDD from baseline in the folic acid group compared to placebo (1.2 [0.7 to 1.8] vs. 0.4 [−0.3 to 1.1] %, p = 0.07).

CONCLUSIONS
Folic acid reduced plasma homocysteine and was associated with a trend toward improved endothelial function in patients with CAD. The absence of an unequivocally positive result may have been due to inadequate sample size or chance. This reinforces the need for the results of large randomized controlled trials before the implementation of routine folic acid supplementation. (J Am Coll Cardiol 2001;37:1858–63) © 2001 by the American College of Cardiology
endothelial function. Evidence of such an effect would support a role for folic acid therapy in reducing the progression of atherosclerotic disease in these patients. Endothelial function was assessed by measuring: 1) flow-mediated endothelial-dependent dilation of the brachial artery using high-resolution ultrasound (12); 2) combined plasma nitrite and nitrate concentrations, stable end products of the nitric oxide radical (13); and 3) plasma von Willebrand factor (vWF) concentration, a circulating marker of endothelial injury (14).

**METHODS**

**Subjects.** Patients with unequivocal angiographic evidence of CAD (>50% stenosis in one or more vessels) were screened, and those with a plasma homocysteine concentration >11 μmol/l were invited to participate in this study. Ninety patients, 79 men and 11 women, with mean age of 63 (range 46 to 79) years consented to participate in the study. Exclusion criteria were atrial fibrillation, current or recent (within six months) use of folic acid supplements, B12 deficiency, impaired renal function (serum creatinine >140 μmol/l [1.59 mg/dl]) and recent (within three months) myocardial infarction, coronary revascularization or unstable angina.

**Study design.** Subjects were screened by a doctor-administered questionnaire using prespecified clinical criteria for the presence of cardiovascular risk factors (Table 1). This was confirmed by a review of the hospital case notes. Measurements of waist:hip ratio and body mass index were noted, and after a 10-min period of rest, blood pressure was recorded twice in the sitting position with a standard sphygmomanometer. Brachial artery endothelial function was assessed, and fasting blood samples were obtained for measurement of serum urea and creatinine, lipid profile (total cholesterol, high density lipoprotein cholesterol and triglycerides), serum folate, serum vitamin B12, plasma homocysteine, combined plasma nitrite and nitrate concentrations and plasma vWF concentration at baseline and after 12 weeks of treatment.

Patients were randomly allocated to receive either oral folic acid 5 mg or matching placebo once daily for 12 weeks in a double-blind protocol with all investigators masked to the treatment allocation of the patients. The dose of folic acid was chosen after a meta-analysis of previous studies showed that doses of up to 5 mg lowered plasma homocysteine concentrations by approximately 30% without adverse effects (8). Creatinine clearance was calculated from the Cockcroft-Gault formula (15). Plasma vWF concentration was measured by enzyme-linked immunosorbent assay (Department of Rheumatology, University of Birmingham). Subjects were asked to keep their diet unchanged in order to maintain the usual dietary intake of folate. The study was approved by the local research ethics committee, and written informed consent was obtained from all participants.

**Measurement of plasma homocysteine concentration.** Fasting blood samples were centrifuged within 20 min of collection, and the plasma was frozen at −70°C. Plasma total homocysteine was measured by ion-paired reversed-phase high-performance liquid chromatography with electrochemical detection; the intra-assay and interassay coefficients of variation for the assay are 3.9% and 10.7%, respectively (16).

**Measurement of combined plasma nitrate/nitrite concentration.** The nitric oxide radical has a short half-life, and thus, the plasma concentration of nitrite and nitrate, stable end products of the nitric oxide radical, were used as a surrogate marker of nitric oxide formation. Nitrate was first reduced to nitrite by enzymatic conversion with nitrate reductase. Then, following deproteinization, the combined plasma nitrite and nitrate concentrations were measured using the Griess reaction (13). The intra-assay and intersay coefficients of variation were 6.6% and 9.2% respectively.

**High-resolution ultrasound assessment of brachial artery reactivity.** After the discontinuation of vasoactive medication for 18 h and an overnight fast, endothelial-dependent dilation of the brachial artery, a nitric-oxide-dependent process, was measured by ultrasound using standard techniques (12,17). In each case, endothelium-independent dilation, a reflection of vascular smooth muscle function, was also assessed by measuring the brachial artery response to sublingual glyceryl trinitrate. Subjects were studied in the supine position at an ambient temperature of 20 to 23°C. A single investigator performed all imaging and analysis. A B-mode scan was obtained of the right brachial artery in longitudinal section between 5 cm and 12 cm proximal to the antecubital fossa using a 7.5 MHz phased array transducer attached to a Sigma 44 MHz phased array transducer. The supine position and ultrasound settings were adjusted to opti-
mize the definition of anterior and posterior media-intima interfaces that demarcated the brachial artery diameter. This diameter was calculated as the average of measurements made using an electronic caliper during four cardiac cycles at end-diastole. The brachial artery diameter was measured at a fixed distance from an anatomical marker, such as a bifurcation, and the position of the transducer relative to the medial condyle of the elbow was recorded to ensure that the same part of the artery was assessed on the follow-up visit. All measurements were recorded on super-VHS videotape for subsequent offline analysis. Each study comprised a series of artery diameter measurements as follows: 1) at rest after a 10-min acclimatization; 2) endothelial-dependent dilation 60 s to 90 s after the sudden release of a pneumatic cuff placed on the ipsilateral forearm, distal to the imaged artery, that had been inflated to suprasystolic pressure for 5 min; 3) second resting diameter after a 10-min recovery period; and 4) endothelial-independent dilation 4 min after sublingual administration of 80 μg glyceryl trinitrate spray. Endothelium-dependent and independent dilation were expressed as the percentage change from the mean resting artery diameter, calculated from the average of the first and second resting recordings. The repeatability (intraobserver variability) of this technique was calculated from measurements obtained from 17 subjects by the investigator. The mean (standard deviation) relative difference in the measurements made on two separate occasions was 2.4 (2.1)%, 3.7 (3.9)% and 3.2 (2.5)% for the average baseline diameter, endothelium-dependent dilation and endothelial-independent dilation, respectively (11).

Statistical power and analysis. A power calculation, based on the results of our earlier work in which baseline mean (standard deviation) endothelial-dependent dilation was 3 (3.25) (11), showed that the recruitment of 90 participants (45 in each group) had >80% power to detect an absolute difference in endothelial-dependent dilation (the primary endpoint) of 2% with p < 0.05. Data were analyzed using SPSS for Windows 9.0. Means, and 95% confidence intervals were used to describe continuous variables. Homocysteine, creatinine, triglycerides and serum folate were positively skewed and were therefore log-transformed prior to analysis. Results are shown in natural units. The distributions of discrete and continuous variables between groups were compared using χ² and unpaired t tests, respectively. Differences in markers of endothelial function between the folic acid and placebo groups at the completion of the study were compared with repeated measures analysis of variance. Linear regression was used to assess the association between potential predictor variables and measures of endothelial function. The test results are presented as two-tailed values, and statistical significance was inferred at p < 0.05.

RESULTS

Four patients, two from each group, did not complete the study because they declined to attend for the post-treatment visit. There were no significant differences between the baseline characteristics of the patients in the folic acid and placebo groups who completed the study (Table 2). One patient had a pre-existing deficiency in folate (serum folate concentration <1.5 μg/l), but none of the patients was deficient in vitamin B₁₂. Treatment with folic acid and placebo was well tolerated by all patients, and no side effects were reported or noted.

At the end of the study, serum folate concentration was significantly greater in the folic acid group than the placebo group (mean [95% confidence interval] 23.1 [21.4 to 24.8] μg/l, F = 137.07, p < 0.001, Table 3). The plasma homocysteine concentration in the folic acid group was significantly lower than in the placebo group (9.3 [8.5 to 10.1] vs. 12.3 [11.3 to 13.4] μmol/l, F = 13.10, p < 0.001, Table 3). This represented a 24% reduction in plasma homocysteine in the folic acid group compared with the placebo group.

Baseline endothelial-dependent dilation was similar in the folic acid and placebo groups (3.3 [2.2 to 4.3]% vs. 3.8 [2.6 to 4.9]%, t = 0.64, p = 0.53, Table 2). At the end of the study, there was no significant difference in endothelial-dependent dilation between the folic acid and placebo

| Table 2. Baseline Characteristics of the Folic Acid and Placebo Groups |
|-----------------|-----------|-----------|
|                 | Folic Acid | Placebo   |
| n               | 43        | 43        |
| Age (yrs)       | 63.0 (60.6–65.5) | 63.4 (61.2–65.5) |
| Gender (M:F)    | 37:6      | 38:5      |
| Smoker (never/ex/current) | 13/26/4 | 11/28/4 |
| Hypertension    | 34        | 27        |
| Systolic BP (mm Hg) | 152 (145–159) | 146 (140–152) |
| Diastolic BP (mm Hg) | 89 (84–91)   | 86 (83–89) |
| Hypercholesterolemia | 28        | 25        |
| Total cholesterol (mmol/l) | 4.8 (4.5–5.0) | 4.9 (4.7–5.2) |
| Triglycerides (mmol/l) | 1.44 (1.24–1.66) | 1.48 (1.26–1.75) |
| HDL cholesterol (mmol/l) | 1.1 (1.1–1.2)   | 1.1 (1.1–1.2) |
| LDL cholesterol (mmol/l) | 2.9 (2.7–3.1)   | 3.1 (2.8–3.3) |
| Family history | 27        | 22        |
| Diabetes mellitus | 5        | 4         |
| BMI (kg/m²)     | 28.6 (27.2–30.0) | 27.2 (26.2–28.3) |
| WHR             | 0.96 (0.94–0.98) | 0.97 (0.92–1.01) |
| Creatinine (μmol/l) | 106 (101–112) | 103 (99–108) |
| Creatinine clearance (ml/min) | 74.5 (67.8–81.3) | 72.6 (67.9–77.2) |
| Serum folate (μg/l) | 7.1 (5.9–8.5)   | 6.9 (6.0–8.0) |
| B₁₂ (ng/l)      | 350 (307–392) | 344 (309–378) |
| Homocysteine (μmol/l) | 11.7 (10.6–13.0) | 11.7 (10.7–12.7) |
| Nitrate/nitrite (μmol/l) | 31 (25–38) | 30 (25–35) |
| vWF (IU/dl)     | 161 (140–183) | 161 (136–185) |
| Baseline diameter (cm) | 0.48 (0.47–0.50) | 0.49 (0.47–0.51) |
| EDD (%)         | 3.3 (2.2–4.3) | 3.8 (2.6–4.9) |
| EID (%)         | 14.3 (12.6–16.0) | 16.4 (14.1–18.8) |

Continuous variables are expressed as mean (95% confidence interval). There were no significant differences between the two groups as assessed using chi-squared and unpaired t tests as appropriate. LDL cholesterol was calculated from the Friedewald equation. Creatinine clearance was calculated from the Cockcroft-Gault equation.

BMI = body mass index; BP = blood pressure; EDD = endothelial-dependent dilation; EID = endothelial-independent dilation; F = female; HDL = high density lipoprotein; LDL = low density lipoprotein; M = male; vWF = von Willebrand factor; WHR = waist-hip ratio.
groups (4.5 [3.5 to 5.4]% vs. 4.1 [3.2 to 5.1]%, t = 0.47, p = 0.64). However, endothelial-dependent dilation improved in both treatment groups. This improvement was greater in the folic acid than the placebo group but did not quite reach statistical significance (1.2 [0.7 to 1.8]% vs. 0.4 [−0.3 to 1.1]%, F = 3.40, p = 0.07, Table 3). There was no significant difference in endothelial-independent dilation (14.8 [13.0 to 16.6]% vs. 16.0 [13.9 to 18.2]%, F = 1.81, p = 0.18). In addition, there were no significant differences in either of the other markers of endothelial function; vWF (152 [136 to 169] vs. 150 [136 to 165] IU/dl, F = 0.92) and plasma nitrite/nitrate concentrations (31 [25 to 38] vs. 27 [25 to 38] μmol/l, F = 0.45, p = 0.50) between the two groups (Table 3).

The absolute reduction in plasma homocysteine correlated strongly with the absolute increase in serum folate concentrations (r = −0.372; p < 0.01), but there was no significant correlation with the change in endothelial-dependent dilation, plasma nitrite/nitrate concentration or plasma vWF concentration.

### DISCUSSION

This study has demonstrated that in patients with established CAD, treatment with high-dose oral folic acid resulted in a significant reduction in plasma homocysteine. Although there was no significant difference in endothelial function between the two groups at the end of the study period, there was a greater increase in endothelial-dependent dilation in the folic acid group compared with placebo, which only narrowly failed to reach statistical significance at the 5% level. However, as no unequivocally positive result was obtained it is important to consider whether this was a true lack of effect or whether this may have been due to elements such as inadequate sample size, methodological deficiencies or chance.

As outlined above, this study was designed prospectively in the light of our previous experience with patients with chronic renal failure (11). A retrospective power calculation based on the present study using the results of repeated measures analysis of variance demonstrated an 80% power to detect a difference in endothelial-dependent dilation of 1.4%. The study was not powered to detect smaller improvements in endothelial function, which may still be relevant to the development of atherosclerosis and acute cardiovascular events.

#### Assessment of endothelial function

Endothelial dysfunction is the first detectable physiological abnormality in patients with atherosclerosis and is the primary event in the process of atherogenesis (10). Therapies that reduce adverse cardiovascular events, such as statins and angiotensin-converting enzyme inhibitors, have also been shown to improve endothelial function in patients with CAD (18,19). Thus, measures of endothelial function would be expected to predict the clinical outcomes of homocysteine-lowering treatments. The methods used in this study to measure endothelial function are well described and validated. Ultrasound assessment of endothelial-dependent dilation is an accurate and reproducible technique (20) that correlates with coronary artery endothelial function (as measured by the response to acetylcholine) and the presence of CAD (21). It has also consistently demonstrated subnormal endothelial function in asymptomatic young subjects with a variety of atherosclerotic risk factors (12,22). Elevated plasma levels of circulating vWF are associated with endothelial injury and predict the development and progression of cardiovascular disease (14,23). Plasma nitrite/nitrate concentrations are a surrogate marker of endothelial production of nitric oxide, an important component of the endothelial properties that regulate vascular hemodynamics (13,24). Levels of plasma nitrite/nitrate may be influenced by dietary protein intake, and thus, the lack of specific dietary instructions to our volunteers may have reduced any difference in
this marker between the two groups. However, as the trend was for lower levels of nitrite/nitrate in the folic acid group it is unlikely that a substantial beneficial effect on this marker of endothelial function has been missed.

Potential reasons for the failure to improve endothelial function in the present study. These include an inadequate duration of treatment or insufficient homocysteine lowering. Inadequate treatment duration cannot be excluded, but there are data showing that endothelial function can improve within this time period, for example, after treatment with statins (18,25). The plasma homocysteine concentration of the folic acid-treated group was lowered by 24% into the normal range for a U.K. population, and the magnitude of reduction was similar to that shown in a recent meta-analysis of the effect of folic acid on homocysteine levels (8). Thus, the dose of folic acid would appear to have been adequate, and there is little evidence to suggest that higher doses cause further reductions in plasma homocysteine. It has also been suggested that elevated plasma homocysteine concentrations may not be causally related to atherosclerosis but may be merely a marker of vascular damage (26,27). However, the prospective epidemiological studies are supported by substantial experimental evidence from both endothelial cell culture and animal work showing that homocysteine causes endothelial injury (9,28,29). In addition, in vivo studies have demonstrated impaired endothelial-dependent dilation in healthy subjects with acute experimental hyperhomocysteinemia and in patients with chronic hyperhomocysteinemia (30,31).

Other studies examining the effect of homocysteine-lowering therapy on endothelial function. The present findings are in accord with our previous study of patients with chronic renal failure and severe hyperhomocysteinemia (11) and are also supported by work demonstrating that folic acid did not prevent arterial lesions in pigs with diet-induced hyperhomocysteinemia (32). In contrast, two recent studies have demonstrated a beneficial effect of homocysteine-lowering therapy on endothelial function in patients with CAD. Chambers et al. demonstrated a significant improvement in endothelial-dependent dilation following two months' therapy with B vitamins (folic acid 5 mg/day and vitamin B₁₂ 1 mg/day). The conflicting results may reflect the additional effects of vitamin B₁₂ or differences in the methodology used to analyze the ultrasound scans (33). In the second study patients were randomized to folic acid 5 mg/day, combination therapy with folic acid 5 mg/day, vitamin C 2 g/day and vitamin E 800 IU/day, or placebo for four months. Despite similar changes in folate and homocysteine concentrations in both the actively treated groups, an improvement in endothelial-dependent dilation of borderline significance (p = 0.04), when compared with the placebo group, was demonstrated in the folic acid group but, surprisingly, not in the combined folic acid and antioxidant vitamin group. These results cannot be explained by any known deleterious effect of antioxidant vitamins on endothelial function or interaction with folic acid. More fundamentally, at the end of the study, the homocysteine-lowering effects of the actively treated groups were not significantly different from those of the placebo group (34).

Earlier studies were based on hyperhomocysteinemic subjects without established atherosclerosis. A single dose of folic acid (20 mg) prevented transient endothelial dysfunction induced by experimental acute hyperhomocysteinemia in healthy young volunteers, but the administration of folic acid was neither randomized nor blinded (35). In the second trial 17 healthy subjects with chronic mild hyperhomocysteinemia were studied in a crossover design, and two months of treatment with folic acid (10 mg/day) significantly improved endothelial-dependent dilation of the brachial artery (36). In addition, long-term folic acid (5 mg/day) and vitamin B₁₂ (250 mg/day) therapy in a randomized controlled trial in healthy subjects with a family history of vascular disease was associated with a reduction in the proportion of abnormal exercise electrocardiographic stress tests (37). However, there were no significant improvements in other surrogate measures of subclinical atherosclerosis, and the validity of exercise testing as an end point in patients without overt CAD is open to question (38). The apparent beneficial effects of folic acid in healthy subjects are in contrast to previous work (39,40), including our own (11), in patients with uremia and established atherosclerosis as well as the present study. This discrepancy might be explained by the presence of other cardiovascular risk factors such as hypertension or dyslipidemia, which may contribute to endothelial injury but would not be expected to respond to folic acid. In addition, compared with healthy subjects with hyperhomocysteinemia, the endothelium of patients with atherosclerosis has been subject to chronic injury and might be expected to be severely damaged and therefore less responsive to intervention.

Conclusions. This study has demonstrated that high-dose folic acid corrects hyperhomocysteinemia in patients with CAD, but although endothelial function appeared to improve with folic acid, the result was not statistically significant at a level of 5%. Although this study was adequately powered to detect an absolute difference in endothelial-dependent dilation of 1.4%, it is still possible that a positive result was missed by chance and also that a smaller, but nonetheless potentially clinically significant, improvement in endothelial function might not have been detected. This study reinforces the need for the results of large randomized controlled trials before the implementation of routine folic acid supplementation.

Acknowledgments
We thank the Department of Rheumatology, University of Birmingham, for measuring plasma von Willebrand factor concentration, and the Clinical Investigation Unit, University of Birmingham, for measuring combined nitrite concentrations.
REFERENCES


