Effect of Folic Acid and Antioxidant Vitamins on Endothelial Dysfunction in Patients With Coronary Artery Disease

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OBJECTIVES The purpose of this study was to determine whether lowering homocysteine levels with folic acid, with or without antioxidants, will improve endothelial dysfunction in patients with coronary artery disease (CAD).

BACKGROUND Elevated plasma homocysteine levels are a risk factor for atherosclerosis. Homocysteine may promote atherogenesis through endothelial dysfunction and oxidative stress.

METHODS In a double-blind, placebo-controlled, randomized trial, we used vascular ultrasound to assess the effect of folic acid alone or with antioxidants on brachial artery endothelium-dependent flow-mediated dilation (FMD). Seventy-five patients with CAD (screening homocysteine level ≥9 μmol/liter) were randomized equally to one of three groups: placebo, folic acid alone or folic acid plus antioxidant vitamins C and E. Patients were treated for four months. Plasma folate, homocysteine, FMD and nitroglycerin-mediated dilation were measured before and after four months of treatment.

RESULTS Plasma folate, homocysteine and FMD were unchanged in the placebo group. Compared with placebo, folic acid alone increased plasma folate by 475% (p < 0.001), reduced plasma homocysteine by 11% (p = 0.23) and significantly improved FMD from 3.2 ± 3.6% to 5.2 ± 3.9% (p = 0.04). The improvement in FMD correlated with the reduction in homocysteine (r = 0.5, p = 0.01). Folic acid plus antioxidants increased plasma folate by 438% (p < 0.001), reduced plasma homocysteine by 9% (p = 0.56) and insignificantly improved FMD from 2.6 ± 2.4% to 4.0 ± 3.7% (p = 0.45), as compared with placebo. Nitroglycerin-mediated dilation did not change significantly in any group.

CONCLUSIONS Folic acid supplementation significantly improved endothelial dysfunction in patients with coronary atherosclerosis. Further clinical trials are required to determine whether folic acid supplementation may reduce cardiovascular events. (J Am Coll Cardiol 2000;36:758–65)

There is substantial epidemiologic evidence linking mild hyperhomocysteinaemia with atherosclerosis in the coronary, cerebral and peripheral arteries (1–3). Despite a number of negative prospective trials (4–6), the evidence overall indicates that hyperhomocysteinaemia is an independent risk factor for atherosclerosis (7). Similar to cholesterol, the association between plasma homocysteine and atherosclerosis appears to be graded, starting at values within the “normal” range, rather than having a threshold effect (1,8,9). A recent Norwegian study found a strong and graded relation between plasma homocysteine levels >9 μmol/liter and mortality in patients with coronary artery disease (CAD) (10).

Experimental evidence suggests that homocysteine may promote atherogenesis through its toxic effects on the vascular endothelium (11–13), which is likely mediated through oxidative stress (14). In humans, there is evidence that chronic hyperhomocysteinaemia is associated with impaired endothelium-dependent flow-mediated vasodilation of the brachial artery (15–17).

Folic acid, alone or combined with other B vitamins, is safe and effective in lowering plasma homocysteine levels (18–25). Moreover, antioxidant vitamins, such as vitamins C and E, may have an adjunctive role in preventing homocysteine-mediated oxidative vascular injury (3,26–29). Observational studies suggest that B vitamins may reduce cardiovascular risk (30) and the extent of carotid atherosclerosis (9,31). However, there is no evidence, based on randomized, controlled trials, that folic acid supplementation, alone or with antioxidant vitamins, will reduce the risk of cardiovascular disease. Although large-scale, randomized trials to test this hypothesis are under way (32), smaller intervention trials using surrogate vascular end points may be of value (7,33). Accordingly, this clinical trial aimed to test the hypothesis that lowering plasma homocysteine levels with folic acid, alone or with antioxidant vitamins C and E, would improve impaired endothelium-dependent brachial artery flow-mediated dilation (FMD) in patients with established CAD and normal or mildly elevated levels of plasma homocysteine.

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pressure for 5 min and rapidly deflated, resulting in reactive
mally on the forearm, was inflated to 250 mm Hg of
similar position was used in the follow-up study. Baseline
This position was maintained throughout the test, and a
alization of the anterior and posterior wall–lumen interfaces.
15 cm above the antecubital crease, ensuring optimal visu-
flow-mediated dilation (FMD) was calculated as the per-
FMD = flow-mediated dilation
MDA = malondialdehyde
NMD = nitroglycerin-mediated dilation
NO = nitric oxide

**METHODS**

**Study design and group.** The study was a randomized, double-blind, placebo-controlled, parallel-design trial con-
ducted between March 1998 and February 1999. The protocol was approved by the Research Ethics Committee of the Queen Elizabeth II Health Sciences Centre.

Patients undergoing cardiac catheterization at the Queen Elizabeth II Health Sciences Centre were screened for entry into the trial. Patients between 18 and 75 years of age were eligible if they had angiographically proven CAD, defined as ≥50% stenosis of the lumen diameter in a major epicardial coronary vessel and a screening fasting plasma total homocysteine level ≥9 μmol/liter within four weeks of randomization. Patients were excluded if they had unstable angina or myocardial infarction within two months of screening, the need for coronary artery bypass graft surgery, uncontrolled hypertension, diabetes mellitus, fasting total cholesterol level >7.2 mmol/liter (278 mg/dl), requirements for folic acid supplementation or pernicious anemia. Patients on folic acid, B vitamins, multivitamins or antioxi-
dants within four weeks of randomization were excluded. All patients gave written, informed consent. Of the 166 consecutive patients screened, 91 were excluded (80 had a screening homocysteine level <9 μmol/liter and 11 refused to participate). The remaining 75 patients were enrolled and underwent baseline endothelial function and biochemical assessment before randomization.

**Noninvasive assessment of endothelial function.** Long-
acting vasoactive medications were withheld for 24 h before endothelial function testing, and smokers refrained from smoking on the morning of the test. Patients were studied after a 12-h overnight fast. Endothelium-dependent and endothelium-independent dilations of the brachial artery was assessed noninvasively using a high resolution ultrasound system (Hewlett-Packard SONOS 2500) with a 7.5-MHz linear-array vascular transducer, as previously described (34–36). The brachial artery was imaged longitudinally, 2 to 15 cm above the antecubital crease, ensuring optimal visual-
alization of the anterior and posterior wall–lumen interfaces. This position was maintained throughout the test, and a similar position was used in the follow-up study. Baseline images and pulsed Doppler flow velocity measurements were taken. Next, a pneumatic tourniquet, placed prox-
imaly on the forearm, was inflated to 250 mm Hg of
diameter after nitro-
MDA = malondialdehyde
NMD = nitroglycerin-mediated dilation
NO = nitric oxide

**Effect of Folic Acid on Endothelial Dysfunction**

**Fishbein et al.**

September 2000:758–65

Abbreviations and Acronyms

ACE = angiotensin-converting enzyme
ANOVA = analysis of variance
CAD = coronary artery disease
FMD = flow-mediated dilation
MDA = malondialdehyde
NMD = nitroglycerin-mediated dilation
NO = nitric oxide

angiodynamics. Doppler measurements were obtained immediately after deflation, and repeat brachial artery images were continuously recorded for 120 s to assess the response to reactive hyperemia. After 10 min of rest, brachial artery scans were obtained before (second baseline) and after administration of 0.3 mg of sublingual nitroglycerin to assess endothelium-independent vasodilation. All images were recorded on super VHS videotape for subsequent quantitative analysis.

At each phase (baseline, 1 min after reactive hyperemia, second baseline and 3 min after nitroglycerin), the end-

Dias, Abbott Park, Illinois). Serum lipid and creat-
inine levels were measured with a Beckman Synchron CX7 system (Beckman Coulter, Inc., Fullerton, California).
Plasma MDA, a marker of lipid peroxidation, was measured in duplicate by HPLC and fluorescence detection, as previously described (39).

**Treatment and follow-up.** After assessment of endothelial function at baseline, patients were randomly assigned to one of three groups: placebo group (n = 25); folic acid only group (n = 25)—5 mg/day; folic acid plus antioxidants group (n = 25)—folic acid 5 mg/day plus vitamin C 2 g/day and vitamin E (d-alpha-tocopheryl acetate) 800 IU/day. Treatment assignments were blinded, with matching placebo capsules. The assigned treatment was continued for four months. Compliance was assessed by pill count and was defined as consumption of >80% of the vitamin capsules. Patients were instructed to avoid concomitant “open-label” vitamin or antioxidant use during the study. The study was conducted after folic acid fortification had been introduced in Canada in harmony with the U.S. levels (40). All other medications were held constant throughout the study, if possible. None of the smokers quit during the study. Patients underwent follow-up endothelial function and biochemical measurements after four months.

**Statistical analysis.** The baseline characteristics of the three groups were compared using one-way analysis of variance (ANOVA) for continuous variables and the chi-square test for categoric variables. The primary end point—the effect of treatment on endothelium-dependent FMD and endothelium-independent NMD over time—was assessed by two-way repeated measures ANOVA, with Bonferroni correction where appropriate. As homocysteine, folate and vitamin B12 values were skewed, a logarithmic transformation was performed, and the results were expressed as geometric mean values. Linear regression was used to assess the relation between the change in FMD and the change in homocysteine, folate and MDA. A general linear model with backward elimination was used to assess clinical and biochemical predictors of the change in FMD. Categoric and continuous variables, including baseline homocysteine and folate levels and all the clinical characteristics in Table 1, were included in the model. Two-sided p values <0.05 were considered to indicate statistical significance. Continuous data are expressed as the mean value ± SD, unless stated otherwise.

**RESULTS**

**Characteristics of the patients.** The study group consisted of 75 patients (16 women and 59 men, mean age 59 ± 10 years). The baseline clinical and angiographic characteristics for the three groups of patients are shown in Table 1. The only significant difference among the groups was less angiotensin-converting enzyme (ACE) inhibitor use in the folic acid plus antioxidants group. Lipid-lowering therapy was equally distributed among the groups. Use of ACE inhibitors and lipid-lowering therapy did not significantly change at follow-up.

**Effects on plasma homocysteine, B vitamin, lipid and MDA levels.** Plasma total homocysteine, folate, vitamin B12, serum lipid and plasma MDA levels at baseline and
follow-up are shown in Table 2. There were no significant differences between the three groups at baseline.

Overall, the geometric mean value of the baseline plasma homocysteine level was 12.1 μmol/liter. Only 23% of the patients had hyperhomocysteinemia (>15 μmol/liter) at baseline. As compared with baseline levels, homocysteine levels were reduced by 11% in the folic acid only group (p = 0.001 vs. baseline) and by 9% in the folic acid plus antioxidants group (p = 0.02 vs. baseline) at follow-up. However, the homocysteine-lowering effects of the two active treatment groups were not significantly different from those of the placebo group (p = 0.23 for folic acid vs. placebo; p = 0.56 for folic acid plus antioxidants vs. placebo).

Overall, the geometric mean value of the baseline plasma folate level was 14.5 nmol/liter (6.4 μg/liter). No patient was folate deficient (<6.3 nmol/liter [2.8 μg/liter]) at baseline. The increase in plasma folate levels from baseline was significantly greater in the folic acid only group (475%, p < 0.001) and in the folic acid plus antioxidants (438%, p < 0.001) group as compared with the placebo group.

Plasma vitamin B₁₂ and serum lipids did not change significantly in any group. In contrast, plasma MDA levels significantly fell in the folic acid plus antioxidants group, but remained unchanged in the placebo and folic acid only groups (p < 0.05 vs. placebo group and folic acid only group).

Effects on FMD and NMD. Basal heart rate, blood pressure, end-diastolic brachial artery diameters, brachial artery flow and reactive hyperemia were similar in each group at the baseline and follow-up (Table 3).

Baseline FMD was similarly impaired in the three groups (Table 3). Flow-mediated dilation did not change in the placebo group. In contrast, FMD significantly improved from 3.2 ± 3.6% to 5.2 ± 3.9% in the folic acid only group (Table 3; p = 0.003 vs. baseline). In the folic acid plus antioxidants group, there was a trend toward improvement in FMD (2.6 ± 2.4% to 4.0 ± 3.7%; p = 0.14 vs. baseline). When using two-way (treatment group vs. time) repeated measures analysis of variance, there were significant treatment effects (p < 0.01) for FMD and NMD. No significant interaction effects were observed for either FMD or NMD.

### Table 2. Effects on Plasma Homocysteine, Plasma Folate, Plasma Vitamin B₁₂, Serum Lipids and Plasma Malondialdehyde Levels

<table>
<thead>
<tr>
<th>Variable</th>
<th>Placebo Group (n = 25)</th>
<th>Folic Acid Only Group (n = 25)</th>
<th>Folic Acid Plus Antioxidants Group (n = 25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma homocysteine (μmol/liter), geometric mean (95% CI)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>12.1 (11.0–13.4)</td>
<td>12.3 (11.0–13.7)</td>
<td>11.8 (10.3–13.6)</td>
</tr>
<tr>
<td>Follow-up</td>
<td>11.8 (10.5–13.3)</td>
<td>10.9 (9.8–12.2)</td>
<td>10.8 (9.3–12.5)</td>
</tr>
<tr>
<td>Plasma folate (nmol/liter),* geometric mean (95% CI)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>14.7 (12.9–16.8)</td>
<td>13.8 (11.8–16.1)</td>
<td>14.9 (13.3–16.8)</td>
</tr>
<tr>
<td>Follow-up</td>
<td>15.6 (13.2–18.4)</td>
<td>79.2 (49.5–126.7)</td>
<td>80.1 (53.5–119.9)</td>
</tr>
<tr>
<td>Plasma vitamin B₁₂ (pmol/liter),† geometric mean (95% CI)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>218 (201–238)</td>
<td>227 (204–253)</td>
<td>229 (208–252)</td>
</tr>
<tr>
<td>Follow-up</td>
<td>213 (192–238)</td>
<td>225 (200–252)</td>
<td>208 (188–231)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/liter),‡ arithmetic mean (95% CI)</td>
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<td></td>
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</tr>
<tr>
<td>Baseline</td>
<td>5.2 (4.9–5.5)</td>
<td>5.3 (4.9–5.6)</td>
<td>5.4 (4.9–5.9)</td>
</tr>
<tr>
<td>Follow-up</td>
<td>5.2 (4.9–5.5)</td>
<td>5.4 (5.0–5.7)</td>
<td>5.4 (5.0–5.9)</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/liter),§ arithmetic mean (95% CI)</td>
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<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>3.3 (3.1–3.6)</td>
<td>3.5 (3.1–3.8)</td>
<td>3.5 (3.0–3.9)</td>
</tr>
<tr>
<td>Follow-up</td>
<td>3.2 (2.9–3.5)</td>
<td>3.5 (3.1–3.8)</td>
<td>3.4 (3.1–3.8)</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/liter),¶ arithmetic mean (95% CI)</td>
<td></td>
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</tr>
<tr>
<td>Baseline</td>
<td>0.9 (0.8–1.0)</td>
<td>0.9 (0.8–1.0)</td>
<td>0.9 (0.8–1.0)</td>
</tr>
<tr>
<td>Follow-up</td>
<td>1.0 (0.9–1.1)</td>
<td>1.0 (0.8–1.1)</td>
<td>1.0 (0.9–1.1)</td>
</tr>
<tr>
<td>Triglycerides (mmol/liter),§ arithmetic mean (95% CI)</td>
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<tr>
<td>Baseline</td>
<td>2.5 (1.9–3.1)</td>
<td>2.1 (1.7–2.4)</td>
<td>2.3 (2.0–2.7)</td>
</tr>
<tr>
<td>Follow-up</td>
<td>2.3 (1.9–2.8)</td>
<td>2.2 (1.8–2.6)</td>
<td>2.3 (1.9–2.7)</td>
</tr>
<tr>
<td>Malondialdehyde (nmol/ml),§ arithmetic mean (95% CI)</td>
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</tr>
<tr>
<td>Baseline</td>
<td>0.83 (0.71–0.96)</td>
<td>0.89 (0.76–1.02)</td>
<td>0.94 (0.80–1.08)</td>
</tr>
<tr>
<td>Follow-up</td>
<td>0.83 (0.70–0.96)</td>
<td>0.89 (0.76–1.02)</td>
<td>0.82 (0.68–0.95)</td>
</tr>
</tbody>
</table>

*To convert values for folate to μg/liter, divide by 2.266. †To convert values for vitamin B₁₂ to ng/liter, divide by 0.7378. ‡To convert values for total cholesterol, LDL cholesterol and HDL cholesterol to mg/dl, divide by 0.02586. §To convert values for triglycerides to mg/dl, divide by 0.01129. ¶p < 0.05 vs. placebo group. #p < 0.05 vs. placebo group and folic acid only group.

CI = confidence interval; HDL = high density lipoprotein; LDL = low density lipoprotein.
measures ANOVA, we found no significant treatment group effect \((p = 0.34)\), but a highly significant time effect \((p = 0.0004)\). Overall, there was a significant interaction between treatment group and time \((p = 0.036)\), indicating a significant difference between the three treatment groups with regard to the change in FMD. The improvement in FMD was significantly greater in the folic acid only group as compared with the placebo group \((p = 0.04\) folic acid only vs. placebo), whereas the improvement in the folic acid plus antioxidants group was not significantly different from that in the placebo or folic acid only group \((p = 0.45)\) for folic acid plus antioxidants vs. placebo, \(p > 0.5\) for folic acid plus antioxidants vs. folic acid). Similar results were obtained after adjusting for differences in baseline ACE inhibitor use by analysis of covariance. Endothelium-independent NMD remained unchanged in each group (Table 3).

Predictors of improvement in FMD. The improvement in FMD in the folic acid group was significantly correlated to the reduction in plasma homocysteine levels \((r = 0.5, p = 0.01)\), but not to the change in plasma folate or MDA. In contrast, there was no significant relation between the change in endothelial function and changes in plasma homocysteine, folate or MDA in the folic acid plus antioxidants group. A general linear model was used to determine predictors of improvement in endothelial function. Treatment group \((p = 0.04)\), previous revascularization \((p < 0.03)\) and previous myocardial infarction \((p < 0.03)\) were significant predictors of a change in endothelial function. In contrast, gender, smoking, hypertension, medications (including lipid-lowering therapy or ACE inhibitors) and baseline levels of homocysteine and folate were not significant predictors.

Follow-up, side effects and compliance. During follow-up, the only cardiovascular events were unstable angina in three patients (one in each group). In general, the study

### Table 3. Hemodynamic Data, Brachial Artery End-Diastolic Diameters and Flow, Flow-Mediated Dilation and Nitroglycerin-Mediated Dilation

<table>
<thead>
<tr>
<th></th>
<th>Placebo Group ((n = 25))</th>
<th>Folic Acid Only Group ((n = 25))</th>
<th>Folic Acid Plus Antioxidants Group ((n = 25))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Basal hemodynamic data</strong></td>
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<td></td>
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<tr>
<td>Heart rate (min⁻¹)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Baseline</td>
<td>(4.27 ± 0.91)</td>
<td>(4.44 ± 0.98)</td>
<td>(4.38 ± 0.77)</td>
</tr>
<tr>
<td>Follow-up</td>
<td>(4.26 ± 0.91)</td>
<td>(4.36 ± 0.87)</td>
<td>(4.34 ± 0.77)</td>
</tr>
<tr>
<td><strong>Systolic blood pressure (mm Hg)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>(132 ± 17)</td>
<td>(132 ± 20)</td>
<td>(134 ± 16)</td>
</tr>
<tr>
<td>Follow-up</td>
<td>(133 ± 17)</td>
<td>(130 ± 18)</td>
<td>(138 ± 17)</td>
</tr>
<tr>
<td><strong>Diastolic blood pressure (mm Hg)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>(79 ± 10)</td>
<td>(81 ± 10)</td>
<td>(81 ± 11)</td>
</tr>
<tr>
<td>Follow-up</td>
<td>(79 ± 12)</td>
<td>(79 ± 9)</td>
<td>(84 ± 9)</td>
</tr>
<tr>
<td><strong>Brachial artery measurements</strong></td>
<td></td>
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<tr>
<td>Basal end-diastolic diameter (mm)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Baseline</td>
<td>(4.4 ± 0.1)</td>
<td>(4.4 ± 0.98)</td>
<td>(4.38 ± 0.77)</td>
</tr>
<tr>
<td>Follow-up</td>
<td>(4.4 ± 0.91)</td>
<td>(4.36 ± 0.87)</td>
<td>(4.34 ± 0.77)</td>
</tr>
<tr>
<td>Basal brachial artery flow (ml/min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>(138 ± 68)</td>
<td>(155 ± 89)</td>
<td>(151 ± 123)</td>
</tr>
<tr>
<td>Follow-up</td>
<td>(141 ± 68)</td>
<td>(168 ± 84)</td>
<td>(164 ± 107)</td>
</tr>
<tr>
<td><strong>Reactive hyperemia (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>(312 ± 140)</td>
<td>(305 ± 144)</td>
<td>(343 ± 194)</td>
</tr>
<tr>
<td>Follow-up</td>
<td>(394 ± 226)</td>
<td>(390 ± 286)</td>
<td>(355 ± 275)</td>
</tr>
<tr>
<td><strong>Flow-mediated dilation (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>(2.7 ± 3.3)</td>
<td>(3.2 ± 3.6)</td>
<td>(2.6 ± 2.4)</td>
</tr>
<tr>
<td>Follow-up</td>
<td>(2.9 ± 3.7)</td>
<td>(5.2 ± 3.9)</td>
<td>(4.0 ± 3.7)</td>
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<tr>
<td><strong>Nitroglycerin-mediated dilation (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>(12.5 ± 7.0)</td>
<td>(11.7 ± 6.2)</td>
<td>(13.8 ± 7.8)</td>
</tr>
<tr>
<td>Follow-up</td>
<td>(13.5 ± 6.0)</td>
<td>(12.9 ± 4.8)</td>
<td>(14.9 ± 9.2)</td>
</tr>
</tbody>
</table>

*p = 0.003 vs. baseline; p = 0.04 vs. placebo group. Data are presented as the mean value ± SD.

![Figure 1. Mean (±SE) change in FMD after four months of therapy in the three treatment groups: placebo group \((n = 25)\); folic acid only group \((n = 25)\); and folic acid plus antioxidants group \((n = 25)\) \((p = 0.036\) for overall comparison of the three groups). Compared with placebo, folic acid alone significantly improved FMD after four months \((p = 0.04)\). In contrast, the improvement in the folic acid plus antioxidants group was not significantly different from that in the placebo group \((p = 0.45)\) or the folic acid only group \((p > 0.5)\).](image-url)
medication was well tolerated. Side effects were noted in seven patients (two in the placebo group, four in the folic acid group and one in the folic acid plus antioxidants group) and were severe enough to discontinue the medication in four patients (two in the placebo group, one in the folic acid group and one in the folic acid plus antioxidants group). Side effects included abdominal cramps, diarrhea and rash. On the basis of their pill counts, seven patients (9%) were thought to be noncompliant with their study medications (three in the placebo group, two in the folic acid alone group and two in the folic acid plus antioxidants group). Although these noncompliant patients were included in our primary intention-to-treat analysis, excluding them did not change the overall results of the study.

**DISCUSSION**

Our study shows that four months of folic acid supplementation (5 mg/day) significantly improves endothelium-dependent FMD in patients with established CAD and normal or mildly elevated levels of plasma homocysteine.

**Effect of homocysteine on endothelial function.** There is experimental (11–13) and clinical (15–17,27,41,42) evidence suggesting that homocysteine causes endothelial dysfunction, which is a key step in the development and progression of atherosclerosis (43). Normally, the vascular endothelium plays an integral role in preventing atherosclerosis through the production of nitric oxide (NO) (44). Experimentally, homocysteine causes endothelial cell damage (11), impaired release of NO (12), increased inactivation of NO (26) and impaired endothelium-dependent vasomotor function (13). Endothelium-dependent FMD, which is dependent on the release of endothelium-derived NO, is impaired in children with homocysteinuria (15) and adults with mild to moderate elevations of homocysteine (16,17). In addition, experimentally raising plasma homocysteine levels with an oral methionine load leads to an acute, transient impairment of FMD in healthy subjects (27,29,41,42).

**Effect of folic acid supplementation on endothelial function.** Given its reproducibility, measurement of FMD is useful for studying the potential reversibility of endothelial dysfunction with various antiatherogenic treatments (35,36). Our patients had established coronary atherosclerosis and several risk factors, all associated with abnormal FMD (34–36). Regardless of the underlying cause, folic acid supplementation significantly improved endothelial dysfunction in our patients. The improvement was modest and, on average, did not restore vascular reactivity to the normal range (45). However, this degree of improvement is similar to the effects seen with statins (46) and ACE inhibitors (47) in patients with CAD. As statins and ACE inhibitors are associated with reduced cardiovascular events in large-scale trials, the degree of improvement in endothelial function seen with folic acid in our study may have clinical relevance.

In contrast to our findings, two previous studies failed to demonstrate improvement in endothelial function with folic acid supplementation in patients on dialysis (48,49). These conflicting results likely reflect differences in their study group, which had end-stage renal disease and marked hyperhomocysteinemia, without established atherosclerosis. In contrast, two recent studies have determined that short-term folic acid supplementation could improve endothelial function in patients with familial hypercholesterolemia and normal homocysteine levels (50) and in healthy subjects with hyperhomocysteinemia (51). Our results extend these vascular benefits of folic acid to patients with established CAD with normal or mildly elevated homocysteine levels.

**Effect of folic acid on homocysteine lowering.** In our study, folic acid alone (5 mg) produced a modest 11% reduction in homocysteine levels after four months. Previous studies have shown that folic acid in doses of 0.4 to 10 mg can lower homocysteine levels to varying degrees (18–25). Our results contradict the 23% reduction seen in the Homocysteine Lowering Trialsists’ meta-analysis (23), but are in keeping with the 11% to 14% reduction seen when patients with CAD consumed fortified breakfast cereals (24). The limited homocysteine lowering seen in our study was likely related to the relatively low levels of homocysteine and relatively high folic acid levels at baseline (23) and to the confounding effect of folate fortification (23,33,52). When designing our study in 1996, the ideal dose of folic acid was unknown (32). We chose a daily dose of 5 mg on the basis of its efficacy in healthy subjects (18) and vascular patients (20,22), as there were concerns about the efficacy of low dose folic acid (0.4 mg) in patients with CAD (2). Further evidence suggests that the homocysteine-lowering effects of low dose folic acid (<0.5 mg) are similar to higher doses (>3 mg) (23,25,32).

**Potential mechanisms underlying the improvement in endothelial function.** The improvement in endothelial function seen with folic acid was likely mediated through multiple mechanisms. Given that this improvement correlated with the reduction in homocysteine levels, we hypothesize that lowering homocysteine levels prevented homocysteine-induced endothelial injury, impaired NO release or inactivation of NO, or all of these. However, folic acid likely has beneficial effects on NO availability that are independent of its homocysteine-lowering effect. For example, folic acid can reverse endothelial dysfunction in experimental hyperhomocysteinemia (42), and 5-methyltetrahydrofolate, the active form of folic acid, can restore endothelial function in familial hypercholesterolemia (53) without lowering homocysteine levels. Folic acid may increase NO formation by stimulating the recycling of tetrahydrobiopterin, an essential cofactor of endothelial NO synthase (54). Alternatively, folates can prevent the oxidative degradation of NO by reducing superoxide generation from NO synthase or xanthine oxidase (53). Thus, folic acid may restore endothelial function by modulating NO formation or degradation, or both.
Effect of folic acid with antioxidant vitamins on endothelial function. There is experimental evidence that homocysteine-induced endothelial injury is mediated through oxidative stress (11,12,14,26). In vitro studies suggest that homocysteine generates reactive oxygen species, including superoxide anion and hydroxyl radical, which may cause endothelial dysfunction through lipid peroxidation or by reducing the availability of NO, or both (11,12,14,26). Thus, it was surprising that the folic acid plus antioxidants did not significantly improve endothelial function, whereas folic acid alone did, despite the beneficial effects of combined therapy on lipid peroxidation. However, as the two responses on FMD were not significantly different from each other (p > 0.5 for folic acid plus antioxidants vs. folic acid alone; Fig. 1), we cannot conclude that the folic acid–antioxidants combination was less effective than folic acid alone. Importantly, our study was not sufficiently powered to indicate that folic acid was superior to the combination therapy. In contrast to our findings, recently it has been determined that pretreatment with vitamin C, alone (27,29) or with vitamin E (28), can prevent acute impairment in endothelial function in experimentally induced hyperhomocysteinemia. Moreover, long-term vitamin C supplementation has been shown to significantly improve FMD in patients with CAD (55). Although our discordant findings may simply reflect an insufficient sample size, the possibility of an unfavorable interaction between vitamins C and E and folic acid, or a prooxidant effect, cannot be excluded. It is also important to recognize that vitamin E has failed to have beneficial effects on endothelial function in patients with coronary atherosclerosis (56,57), and recently, two important mega-trials have failed to show a reduction in cardiovascular events with vitamin E (58,59).

Study limitations. First, many patients were smokers, taking lipid-lowering therapy or ACE inhibitors, which could affect FMD. However, further analysis suggests that these factors, including the unequal distribution of baseline ACE inhibitor use, did not influence our results. Second, our findings are limited to patients with established coronary atherosclerosis and “average” homocysteine levels. Therefore, these results cannot be extrapolated to the general population or to subjects with marked hyperhomocysteinemia. Finally, we studied peripheral artery function as a surrogate vascular end point, and thus our findings cannot be directly applied to other vascular beds and may not imply clinical benefit. However, endothelial dysfunction is often a generalized process (35), and endothelial dysfunction of the brachial artery is closely related to coronary endothelial dysfunction (35) and the extent of coronary atherosclerosis (60). Therefore, our findings may have relevance to the coronary circulation. By improving endothelial dysfunction and NO bioavailability, folic acid may potentially modify the atherogenic process and reduce cardiovascular events (61).

Conclusions and implications. We have shown that four months of folic acid supplementation can significantly improve endothelial dysfunction in patients with established coronary atherosclerosis. These results provide further support for the potential therapeutic role of folic acid in CAD. However, one must await the results of large-scale clinical trials to determine whether folic acid will reduce cardiovascular morbidity and mortality in patients with established CAD.

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