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Optimization of Dietary Folate or Low-Dose Folic Acid Supplements Lower Homocysteine But Do Not Enhance Endothelial Function in Healthy Adults, Irrespective of the Methylenetetrahydrofolate Reductase (C677T) Genotype

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OBJECTIVES We sought to study the effect of low-dose folic acid supplementation or optimization of dietary folate intake on plasma homocysteine and endothelial function in healthy adults.

BACKGROUND Elevated homocysteine is associated with cardiovascular disease, but it is not known whether this relationship is causal. Individuals homozygous (TT) for the C677T mutation in the methylenetetrahydrofolate reductase (MTHFR) gene (~12% of the population) have increased homocysteine levels, particularly in association with suboptimal folate intake.

METHODS Healthy subjects (n = 126; 42 of each MTHFR genotype) were included in this cross-over study of three interventions of four months each: 1) placebo plus natural diet; 2) daily 400-μg folic acid supplement plus natural diet; and 3) increased dietary folate intake to 400 μg/day.

RESULTS At baseline, homocysteine was inversely related to plasma folate and was higher in TT homozygotes. For the whole group, plasma folate increased by 46% after dietary folate and by 79% after supplementation, with reductions of homocysteine of 14% and 16%, respectively. Within the genotype, TT homozygotes exhibited the most marked changes in these variables. Brachial artery endothelial function, as determined by a change in end-diastolic diameter in response to increased flow, was not changed by increased folate intake (98 ± 73 μm at baseline, 110 ± 69 μm after a high-folate diet, 114 ± 59 μm after supplementation and 118 ± 68 μm after placebo). Plasma von Willebrand factor antigen was unaltered.

CONCLUSIONS Optimization of dietary folate or low-dose folic acid supplementation reduces plasma homocysteine but does not enhance endothelial function, irrespective of the MTHFR (C667T) genotype. (J Am Coll Cardiol 2001;38:1799–805) © 2001 by the American College of Cardiology

Cardiovascular disease is associated with elevated plasma homocysteine, but it has not been proven to have a causal role (1,2). Major determinants of plasma homocysteine concentration are dietary folate intake and genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) (3–5). The most common genetic cause of moderate hyperhomocysteinemia, occurring in homozygous form in ~12% of the Caucasian population, is MTHFR C677T mutation (gene frequency ~0.32) (6). Activity of MTHFR is reduced, and plasma homocysteine is typically elevated by ~25% (7,8). The combination of TT genotype with reduced folate intake further elevates plasma homocysteine (5).

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It has been proposed that individuals with the TT genotype are more likely to have vascular disease, particularly in the context of suboptimal folate intake (9). However, a recent meta-analysis of MTHFR genotype studies did not confirm this (10). Plasma homocysteine can be lowered by folic acid supplements. Typical folate intake is 250 μg/day in Britain, with a reference nutrient intake of 200 μg/day. Increasing folate intake to 400 μg/day achieves near-maximal homocysteine reduction and has been proposed as the optimal dietary folate intake (4). In the United Kingdom, fortification with folic acid is not mandatory, although there is an increasing trend for food manufacturers to voluntarily fortify breakfast cereal and bread with it. Women planning pregnancy are advised to take a daily 400-μg folic acid supplement to reduce the incidence of neural tube defects. In the United States, universal fortification of grains with folic acid was introduced in 1998, based on evidence that this would reduce the incidence of neural tube defects, and with the additional suggestion that it would improve cardiovascular health by reducing plasma homocysteine (11).

The most plausible mechanism whereby homocysteine

Abbreviations and Acronyms

ANOVA = analysis of variance
FMD = flow-mediated dilation
MTHFR = methylenetetrahydrofolate reductase
NO = nitric oxide
vWF(Ag) = von Willebrand factor (antigen)

could cause vascular disease is by induction of endothelial dysfunction (12). In a healthy artery, when blood flow is increased, endothelial cells are stimulated to release nitric oxide (NO), which induces vasodilation, a response termed "flow-mediated dilation" (FMD). Impairment of FMD, reflecting endothelial dysfunction, occurs with cardiovascular risk factors, including hypertension (13), smoking (14) and diabetes (15). Endothelial damage may also be signified by increased release of endothelial-derived proteins into the plasma, such as von Willebrand factor (vWF) (16).

Endothelial dysfunction has been reported in homocystinuria (17) and hyperhomocysteinemia (18,19). Our group has reported that high-dose folic acid (5 mg/day) enhances endothelial function in healthy subjects with mild hyperhomocysteinemia (20).

The hypothesis to be tested was that increasing folate intake by either optimization of dietary folate or low-dose supplementation with folic acid would both lower homocysteine and enhance endothelial function, the effect being most marked in individuals with the TT genotype for MTHFR. Endothelial function was assessed in a randomized, placebo-controlled intervention trial that recruited subjects according to MTHFR genotype.

METHODS

Subject recruitment (Fig. 1). Healthy men and women aged 18 to 65 years were recruited from blood donor sessions and by workplace screening in South Wales. Exclusion criteria were hypertension (diastolic >100 mm Hg), diabetes, smoking, supplementation with folic acid and vitamins B_6 or B_{12} and pregnancy. The subjects provided informed, written consent, and the study was approved by the Local Research Ethics Committee (Bro Taf Health Authority).

After completion of an eligibility questionnaire, suitable volunteers (n = 634) were screened for MTHFR (C677T) genotype. Forty-two individuals with each genotype (CC, CT and TT) entered the study.

Subject entry. Names (n = 126) and randomization code, but not MTHFR genotype, were relayed to the trial coordinator, so that the study was double-blinded for genotype. Subjects were entered into the study over a period of 18 months. Subjects, as a whole group and as subgroups of 12, were stratified for the MTHFR genotype and balanced for intervention order. This avoided possible bias caused by entering individuals with the more common genotypes (CC and CT) first and the less common genotype (TT) later.

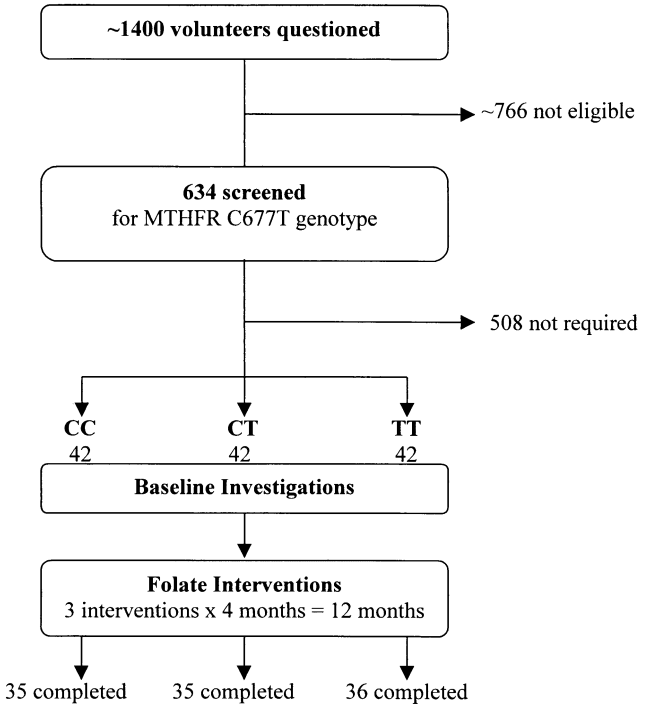

Figure 1. Flow chart detailing the volunteer recruitment procedure. After determination of the methylenetetrahydrofolate reductase (MTHFR) C677T genotype, equal numbers of subjects with each genotype (CC, CT and TT) were allocated to the study.

Study design. The study was a cross-over study of Latin square design incorporating three interventions each of four months duration. After baseline assessment, the subjects participated until completion of each of the three interventions. Each visit was made at the same time of day and followed an 8-h fast (except for water). The subjects had a dietary assessment to estimate folate intake, provided a fasting blood sample and underwent assessment of brachial artery endothelial function (FMD). Endothelium-independent responses to nitrates were not assessed, to maintain good compliance among the subjects. Dietary advice was given as appropriate to the intervention.

Interventions. INTERVENTION 1: PLACEBO PHASE. The subjects continued their usual diet, including foods containing natural folate, but excluding foods specifically fortified with (synthetic) folic acid, and were provided with a matched placebo so that intervention 1 could not be distinguished from intervention 2. Folate intake was expected to be ~200 μg/day.

INTERVENTION 2: SUPPLEMENT PHASE. The subjects followed the same diet as in intervention 1 and were provided with a daily 400-μg folic acid tablet. Total folate intake was expected to be ~600 μg/day.

INTERVENTION 3: DIETARY FOLATE PHASE. The subjects increased their dietary folate intake using both foods naturally high in folate and folic acid–fortified foods available

from general retail outlets (mainly breads and breakfast cereals). The subjects were later reimbursed for the fortified foods. The aim was to increase folate intake to ~400 μg/day.

The period of each intervention was four months. The study was double-blinded for interventions 1 and 2. For intervention 3, requiring a change of diet, the subjects and nutritionist were aware of the intervention, but the vascular scientist was not (i.e., single-blinded).

Dietary and compliance assessment. Full details of the dietary methods will be reported elsewhere. In brief, at the start of each intervention, the nutritionist provided advice on the ensuing four-month period and explained the diet diary and food-frequency questionnaire from which dietary folate intake was estimated. Surplus tablets were collected to monitor compliance.

Determination of the MTHFR (C677T) genotype. Deoxyribonucleic acid was extracted from inner-cheek buccal cells, collected using a cytobrush, and the genotype was determined by heteroduplex analysis (3).

Blood analysis. Venous blood was centrifuged within 10 min of collection, and plasma was stored at −70°C. All four samples from an individual were assayed in the same batch to avoid inter-batch measurement imprecision. Total homocysteine and folate were measured using Abbott IMX methods (Abbott Diagnostics, Abbott Park, Illinois0. Von Willebrand factor was assayed using an enzyme-linked immunosorbent assay–based method.

Vascular protocol. Flow-mediated dilation was assessed in the brachial artery (21). Brachial artery end-diastolic diameter was measured using high-resolution (±3 μm) ultrasonic vessel wall tracking (Vadirec Medical Systems, Arnhem, Netherlands); blood flow was measured by continuous wave Doppler imaging; and blood pressure was measured by photoplethysmography (Finapres, Ohmeda, Madison, Wisconsin). After baseline measurements, a wrist cuff was inflated to suprasystolic blood pressure, distal to the point of vascular measurement. Inflation of this cuff for 5 min induced ischemia in the hand. Cuff release stimulated reactive hyperemia in the hand, resulting in a secondary increase in blood flow *upstream* along the brachial artery. Blood flow and pressure and end-diastolic diameter were recorded at 40-s intervals for 240 s after cuff release and at 6, 8 and 10 min until recovery to baseline values. The maximal absolute change in end-diastolic diameter (μm) observed for each individual subject during the first 200 s after cuff release was taken as the measure of FMD.

Vascular assessments were carried out by a trained vascular research officer (C. H. P.) in a purposely built, temperature-controlled room. Reproducibility studies carried out on separate occasions in healthy subjects demonstrated an inter-day coefficient of variation of 2.8% for measurement of baseline diameter and 12.8% for measurement of FMD.

Statistics. The primary analyses were those evaluating differences in effect between the three intervention regimens. Three-way analysis of variance (ANOVA) was performed (SPSS, version 9.0), with models on subject, period and treatment, and differences were made between pairs of regimens derived. The interventions were tested against baseline values by the paired *t* test. Confirmatory nonparametric tests were used on account of skewed distributions. All correlations reported are nonparametric (Spearman). A p value <0.05 was considered significant.

Table 1. Characteristics of Study Subjects at Baseline

Variable		Mean ± SD
Age at randomization (years)		39 ± 12
Gender (male/female)		53/73
Systolic blood pressure (mm Hg)		118.4 ± 15.4
Diastolic blood pressure (mm Hg)		69.8 ± 11.1
Heart rate (beats/min)		63.4 ± 8.9
Plasma folate (μg/l)		7.8 ± 3.4
Split by MTHFR genotype	CC	9.1 ± 3.5
	CT	7.6 ± 3.1
	TT	6.7 ± 3.3
Plasma total homocysteine (μmol/l)		10.2 ± 4.2
Split by MTHFR genotype	CC	8.8 ± 2.4
	CT	9.3 ± 2.5
	TT	12.5 ± 5.7
Flow-mediated dilation (μm)		98 ± 73
Split by MTHFR genotype	CC	94 ± 67
	CT	93 ± 68
	TT	106 ± 85
von Willebrand factor antigen (U/ml)		1.15 ± 0.38
Split by MTHFR genotype	CC	1.07 ± 0.30
	CT	1.16 ± 0.41
	TT	1.21 ± 0.42

MTHFR = methylenetetrahydrofolate reductase.

RESULTS

Baseline characteristics (Table 1). Plasma folate and homocysteine were inversely related ($r_s = -0.50$, $p < 0.001$) for the whole group. Plasma folate and homocysteine levels were very different (highly significant) between the three genotypes by one-way ANOVA, with TT-genotype subjects having the lowest and CC-genotype subjects having the highest plasma folate levels ($F = 5.66$, $p = 0.004$ and $F = 11.36$, $p < 0.001$, respectively), and CT-genotype subjects having intermediate values, and vice versa for homocysteine. Flow-mediated dilation was not related to plasma folate ($r_s = -0.044$, $p = 0.62$), homocysteine ($r_s = 0.018$, $p = 0.84$) or the MTHFR genotype ($p = 0.64$). Von Willebrand factor antigen (vWFAg) was not related to plasma folate ($r_s = -0.045$, $p = 0.62$), homocysteine ($r_s = -0.019$, $p = 0.84$) or the MTHFR genotype ($p = 0.23$). Flow-mediated dilation and vWFAg did not correlate with each other ($r_s = -0.052$, $p = 0.51$).

Effects of intervention (Tables 2 and 3). Three-way ANOVA showed highly significant differences between the three interventions in plasma folate ($F = 1.66$, $p = 0.001$) and homocysteine ($F = 2.81$, $p < 0.001$), but not in FMD ($F = 0.79$, $p = 0.45$) or plasma vWFAg ($F = 0.54$, $p = 0.58$). In each case, there were highly significant differences

Table 2. Biochemical and Hemodynamic Variables at Baseline and After Each Intervention for the Study Group as a Whole

		Intervention		
	Baseline	**Placebo**	**Diet**	**Supplement**
Nutritional data				
Folate intake (μg/day)	254 ± 83	221 ± 88	468 ± 133	561 ± 98
Biochemical data				
Folate (μg/l)	7.8 ± 3.4	7.6 ± 3.2	11.4 ± 3.7	14.0 ± 7.3
Homocysteine (μmol/l)	10.2 ± 4.2	10.9 ± 6.9	8.7 ± 3.3	8.5 ± 3.1
vWFAg (U/ml)	1.15 ± 0.38	1.14 ± 0.43	1.12 ± 0.44	1.13 ± 0.40
Hemodynamic data				
Systolic BP (mm Hg)	118.4 ± 15.4	120.5 ± 15.9	120.5 ± 14.3	119.3 ± 13.6
Diastolic BP (mm Hg)	69.8 ± 11.1	67.6 ± 10.8	67.6 ± 10.4	68.0 ± 11.8
Heart rate (beats/min)	63.4 ± 8.9	64.0 ± 9.5	62.1 ± 9.1	62.7 ± 9.0
Brachial artery data				
Baseline diameter (mm)	3.60 ± 0.68	3.61 ± 0.67	3.61 ± 0.67	3.59 ± 0.69
FMD (μm)	98 ± 73	118 ± 68	110 ± 67	114 ± 59
Basal blood flow (ml/min)	32 ± 26	42 ± 39	36 ± 33	37 ± 37
Peak blood flow (ml/min)	152 ± 90	155 ± 90	157 ± 106	155 ± 106

Data are presented as the mean value ± SD.
BP = blood pressure; FMD = flow-mediated dilation, as measured by maximal change in end-diastolic diameter of the brachial artery in response to increased blood flow; vWFAg = von Willebrand Factor antigen.

between subjects ($p \leq 0.001$), but not between periods ($p \geq 0.1$).

Folate and homocysteine. The expected values for folate intake were achieved for each intervention. For the whole group and relative to baseline, the dietary folate intervention increased plasma folate by 46% ($p < 0.001$) and reduced homocysteine by 14% ($p < 0.001$). The supplement intervention increased plasma folate to a greater extent, by 79% ($p < 0.001$), but reduced homocysteine by a similar amount, 16%. After placebo, there was a trend toward reduced plasma folate and increased homocysteine, but these were not significant ($p = 0.37$ and $p = 0.071$, respectively).

For the whole group and after each intervention, plasma folate and homocysteine were inversely related ($r_s = -0.36$ to -0.52, $p < 0.001$). This inverse relationship was particularly marked for the TT-genotype subjects ($r_s = -0.53$ to -0.80, all $p < 0.002$), who had the greatest changes observed after the supplement intervention, where homocysteine fell by 24%, compared with 8% in CC-genotype subjects.

Vascular measurements. Flow-mediated dilation did not differ significantly between the three interventions for any MTHFR genotype ($p > 0.3$). The time courses of the vasodilatory response were similar after each intervention (Fig. 2). Basal blood flow was similar throughout the study, and peak flow was similar at each intervention ($p > 0.80$). Blood pressure and heart rate were similar to baseline values throughout the study.

Table 3. Biochemical and Vascular Variables at Baseline and After Each Intervention*

		Intervention		
	Baseline	**Placebo**	**Diet**	**Supplement**
Plasma folate (μg/l)				
CC	9.1 ± 3.5	8.4 ± 3.1	12.1 ± 3.6	16.2 ± 11.1
CT	7.6 ± 3.1	7.8 ± 3.0	11.0 ± 3.7	13.3 ± 3.7
TT	6.7 ± 3.3	6.5 ± 3.2	10.9 ± 3.8	12.3 ± 3.8
Homocysteine (μmol/l)				
CC	8.8 ± 2.4	8.9 ± 2.4	7.9 ± 2.6	8.1 ± 2.7
CT	9.3 ± 2.5	9.3 ± 2.0	8.2 ± 1.6	8.1 ± 1.5
TT	12.5 ± 5.7	14.5 ± 10.9	10.2 ± 4.7	9.5 ± 4.3
vWFAg (U/ml)				
CC	1.07 ± 0.30	1.14 ± 0.41	1.04 ± 0.38	1.07 ± 0.32
CT	1.16 ± 0.41	1.09 ± 0.48	1.06 ± 0.33	1.15 ± 0.42
TT	1.21 ± 0.42	1.20 ± 0.40	1.27 ± 0.56	1.17 ± 0.46
FMD (μm)				
CC	94 ± 68	113 ± 66	110 ± 69	117 ± 55
CT	93 ± 68	123 ± 81	96 ± 69	111 ± 64
TT	106 ± 85	117 ± 58	123 ± 61	115 ± 60

*The study group was split by the methylenetetrahydrofolate reductase C677T genotype (CC, CT or TT). Data are presented as the mean value ± SD.
Abbreviations as in Table 2.

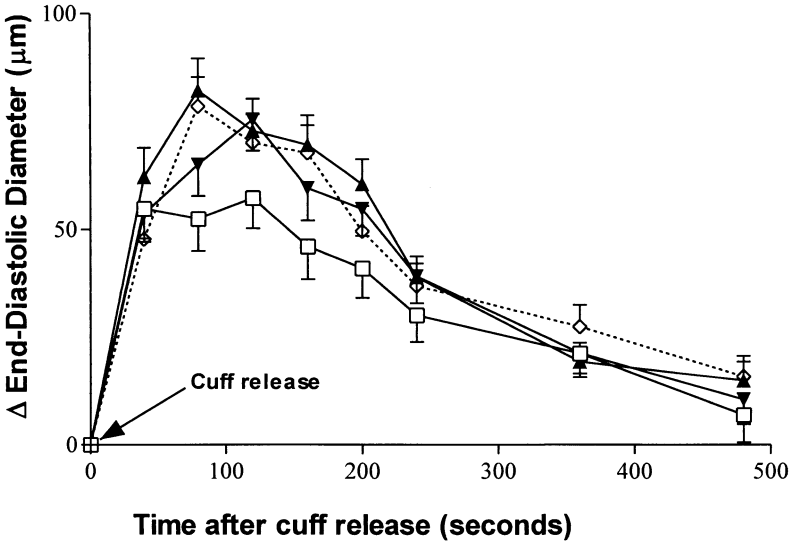

Figure 2. Time course of brachial artery response to increased blood flow at baseline and after each intervention for the group as a whole. Data are given as the change in brachial artery end-diastolic diameter (mean ± SEM). **Open squares** = baseline; **solid upward triangles** = placebo; **solid downward triangles** = diet; and **open diamonds** = supplement.

DISCUSSION

This study demonstrates that in healthy individuals, optimization of dietary folate intake or supplementation with low-dose folic acid lowers plasma homocysteine but does not enhance vascular endothelial function, as assessed by FMD or plasma vWFAg.

Folate and homocysteine responses. This study showed that subjects homozygous for the MTHFR C677T mutation had the highest homocysteine concentrations and had the greatest reductions in homocysteine with folate; however, despite this, they did not demonstrate any changes in endothelial response.

Endothelial function. The lack of any change in FMD or vWFAg is evidence against mild hyperhomocysteinemia causing endothelial dysfunction in healthy individuals, suggesting that low-dose folate may not be beneficial in preventing vascular disease in the general population.

One possible explanation of the failure to show any change in FMD is that measurement variabilities may obscure a small change. However, particular care was taken to minimize measurement errors in the vascular technique. All assessments were made in a purposely built, temperature-controlled laboratory by the same trained scientist, using the same equipment throughout.

Rapid on-line data acquisition enabled construction of a time course of FMD responses (Fig. 2). This provides a more complete picture of the vascular response, as opposed to just a single measurement of maximal artery diameter taken between 1 and 2 min after cuff release, as is usually done. The time courses of FMD are similar after each intervention.

To ensure complete objectivity, careful attention was given to the study design. The balanced cross-over study design avoids inter-subject variation, thus facilitating detection of small changes in vascular function. The study was placebo-controlled, and all vascular measurements were carried out in a blinded manner (i.e., the vascular scientist was unaware of the MTHFR genotype or intervention).

An effect of folate could have been missed if the number of study subjects was too small. However, a sample size of 126 is a large number for a study of this nature. Stratification by MTHFR genotype ensured that one-third of the subjects had the TT genotype (compared with 12% of the general population); this subgroup was expected to have the greatest response to folate. The absence of a trend for increased FMD within this subgroup suggests that the "negative" result was not caused by inadequate numbers.

Poor compliance with the interventions can be discounted because plasma folate and homocysteine responded as expected.

Thus, we are unable to identify any aspect in the study design or procedure that would explain the negative result, and we must therefore consider other physiologic interpretations.

Homocysteine and cardiovascular risk. Some studies suggest that homocysteine may only exert an effect on vascular risk in synergy with other risk factors (22,23). Subjects recruited for this study had no other cardiovascular risk factors. Our findings suggest that in the absence of other risk factors, mild hyperhomocysteinemia and low-dose folic acid supplementation may have no physiologic vascular significance.

Folate dose and mechanisms. Several studies have reported beneficial endothelial effects of high-dose folic acid (20,24). We have previously reported that folic acid, at 5,000 μg/day, improves FMD in individuals with mild hyperhomocysteinemia but with no known cardiovascular risk factors (20). In that study, plasma homocysteine was 13.6 μmol/l, which is similar to that of the TT-genotype

subjects in this study (14.5 μmol/l). However, the dose of folic acid used by Bellamy et al. (20) was pharmacologic (5,000 μg/day), which is in contrast to the physiologic dose of 400 μg/day used in our study. Daily supplementation with 500 μg to 5 mg of folic acid produces similar homocysteine-lowering effects, consistent with a plateau effect with doses of folic acid >400 μg/day (4). The homocysteine-lowering effects of these low- and high-dose folic acid interventions are approximately equivalent, so that the discrepancy in results cannot be explained by differences in the degree of homocysteine lowering. High-dose folic acid may therefore be exerting its effects by a mechanism independent of homocysteine lowering.

Other studies have reported effects of high-dose folic acid on endothelial function, independent of homocysteine lowering. Methionine loading, which rapidly elevates plasma homocysteine, has been shown to impair flow-mediated vasodilation (25,26). Concomitant administration of high-dose folic acid (5 to 20 mg orally) prevents this effect without immediately affecting plasma homocysteine (27,28). In individuals with familial hypercholesterolemia but normal homocysteine levels, endothelial dysfunction can be restored by both infusion of 5-methyltetrahydrofolate (29) and high-dose oral folic acid (30). Mechanisms whereby folic acid could act directly include modification of cellular oxidative metabolism to increase the availability of tetrahydrobiopterin, a co-factor for NO synthase. Folic acid has a twofold effect on NO synthase in vitro, both reducing superoxide production and enhancing NO synthesis (31).

It could be argued that the brachial artery does not accurately represent vascular beds in which clinically important atherosclerosis occurs. However, most systemic or circulatory cardiovascular risk factors induce generalized endothelial dysfunction, and interventions to correct these risk factors improve endothelial function. Peripheral artery NO-mediated responses appear to be a valid surrogate marker of coronary function (32), and recent studies have demonstrated that coronary endothelial dysfunction is a long-term predictor of the development of atherosclerosis and cardiac events (33,34).

This study does not support the proposal that mild elevation of plasma homocysteine in healthy individuals is a causal agent for cardiovascular disease. However, before definitive conclusions can be reached, long-term, large-scale interventional studies assessing the effects of folic acid supplementation in vascular disease are needed. There is still an important requirement for clinical trials to be carried out in high-risk populations, with monitoring of clinical events (35).

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