Global Improvement of Vascular Function and Redox State With Low-Dose Folic Acid

Implications for Folate Therapy in Patients With Coronary Artery Disease

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Background—Although dietary folate fortification lowers plasma homocysteine and may reduce cardiovascular risk, high-dose folic acid therapy appears to not alter clinical outcome. Folic acid and its principal circulating metabolite, 5-methyltetrahydrofolate, improve vascular function, but mechanisms relating folate dose to vascular function remain unclear. We compared the effects of folic acid on human vessels using pharmacological high-dose versus low-dose treatment, equivalent to dietary folate fortification.

Methods and Results—Fifty-six non–folate-fortified patients with coronary artery disease were randomized to receive low-dose (400 µg/d) or high-dose (5 mg/d) folic acid or placebo for 7 weeks before coronary artery bypass grafting. Vascular function was quantified by magnetic resonance imaging before and after treatment. Vascular superoxide and nitric oxide bioavailability were determined in segments of saphenous vein and internal mammary artery. Low-dose folic acid increased nitric oxide–mediated endothelium-dependent vasomotor responses, reduced vascular superoxide production, and improved enzymatic coupling of endothelial nitric oxide synthase through availability of the cofactor tetrahydrobiopterin. No further improvement in these parameters occurred with high-dose compared with low-dose treatment. Whereas plasma 5-methyltetrahydrofolate increased proportionately with treatment dose of folic acid, vascular tissue 5-methyltetrahydrofolate showed no further increment with high-dose compared with low-dose folic acid.

Conclusions—Low-dose folic acid treatment, comparable to daily intake and dietary fortification, improves vascular function through effects on endothelial nitric oxide synthase and vascular oxidative stress. High-dose folic acid treatment provides no additional benefit. These direct vascular effects are related to vascular tissue levels of 5-methyltetrahydrofolate rather than plasma levels. High-dose folic acid treatment likely confers no further benefit in subjects already receiving folate supplementation. (Circulation. 2007;115:2262-2270.)

Key Words: folic acid ■ atherosclerosis ■ nitric oxide ■ magnetic resonance imaging ■ oxidative stress

Plasma total homocysteine (tHcy) is strongly associated with cardiovascular risk, but the clinical effects of lowering tHcy with folate treatment are controversial. Despite epidemiological evidence that folate fortification of the grain supply in North America has been accompanied by a reduction in cardiovascular risk, a large-scale randomized clinical trial of high-dose folic acid treatment found no beneficial effect on clinical outcomes in patients with stable coronary artery disease (CAD).
stress through regulation of endothelial nitric oxide (NO) synthase (eNOS). Specifically, intravenous administration of 5-methyl-tetrahydrofolate (5-MTHF), the principal circulating form of folic acid, improves the enzymatic “coupling” of eNOS through increasing bioavailability of the eNOS cofactor tetrahydrobiopterin (BH4) in the vascular wall. Although oral folic acid treatment rapidly elevates plasma 5-MTHF levels, it is unknown whether levels of 5-MTHF are also increased in vascular tissue and how these changes may be related to improvements in vascular function and oxidative stress. Given these mechanistic uncertainties, the dose–response relationship between oral folic acid and its vascular effects is unknown. Indeed, grain fortification with folic acid may have a major confounding effect on the results of clinical trials conducted in North America, because subjects from these populations would already receive ~380 μg of dietary folate daily, as a result of fortification, before recruitment into clinical studies.

Accordingly, we sought to evaluate the effects of oral folic acid treatment on vascular function in patients with CAD undergoing coronary artery bypass grafting (CABG) surgery, recruited from a population without dietary folate fortification. We aimed to compare the effects of low-dose folic acid treatment (400 μg daily), which is comparable to daily intake with dietary fortification, with high-dose folic acid (5 mg daily), which is typical of large-scale clinical trials. We used noninvasive magnetic resonance imaging (MRI) techniques to quantify changes in peripheral and central vascular function. Furthermore, we investigated in detail the mechanistic relationships between plasma and vascular 5-MTHF levels, endothelial function, and vascular oxidative stress in samples of blood vessels retrieved at the time of CABG.

Methods

Study Design and Subjects

Fifty-six patients with CAD undergoing elective CABG at the John Radcliffe Hospital, Oxford, United Kingdom, were randomized in a placebo-controlled, double-blind, parallel design to receive 5 mg/d (high-dose) folic acid, 400 μg/d (low-dose) folic acid, or placebo for 7 weeks before CABG. Exclusion criteria were any inflammatory, infective, liver, or renal disease or malignancy. Patients receiving nonsteroidal antiinflammatory drugs, dietary supplements of folic acid, or antioxidant vitamins were also excluded. Demographic characteristics of the patients are presented in Table 1. All patients were tested to exclude vitamin B$_2$ deficiency before randomization. The study protocol was approved by the local research ethics committee, and each patient gave written informed consent.

MRI Quantification of Vascular Function

Vascular function was quantified by high-resolution MRI at baseline and at the end of the treatment period. Images of the aorta and carotid arteries were used to determine vascular distensibility and pulse-wave velocity as indices of vascular stiffness. Flow-mediated brachial artery dilatation (FMD) was used as a measure of endothelial function. Vascular distensibility was determined in the ascending aorta, proximal descending aorta, distal descending aorta, and both common carotid arteries with a high-resolution gradient-echo pulse sequence on a 1.5-T clinical magnetic resonance scanner (Siemens Sonata, Erlangen, Germany) as we have described previously. A velocity-encoding gradient for phase-contrast MRI was applied to determine proximal pulse-wave velocity in the aortic arch. FMD and endothelium-independent dilatation of the brachial artery were quantified from cross-sectional images of the brachial artery, as described previously.

Vessel Harvesting

Paired samples of saphenous vein (SV) and internal mammary artery (IMA) were obtained from each patient at the time of CABG and transferred to the laboratory within 30 minutes in ice-cold Krebs Henseleit buffer, as described previously.

Vasomotor Studies

Endothelium-dependent and endothelium-independent dilatation were assessed with isometric tension studies in SVs obtained at the time of CABG. Four rings from each vessel were precontracted with phenylephrine (3×10$^{-8}$ mol/L), then endothelium-dependent relaxations were quantified with acetylcholine (ACH, 10$^{-6}$ to 10$^{-2}$ mol/L) and bradykinin (10$^{-6}$ to 10$^{-2}$ mol/L). Finally, relaxations to the endothelium-independent NO donor sodium nitroprusside (10$^{-6}$ to 10$^{-3}$ mol/L) were evaluated in the presence of the NO synthase inhibitor N$^\text{G}$-nitro-L-arginine methyl ester (L-NAME; 100 μmol/L).

Determination of Vascular Superoxide Production

Vascular superoxide production was measured from paired segments of intact SV and IMA by use of lucigenin-enhanced chemiluminescence, as described previously. Vessels were opened longitudinally to expose the endothelial surface and equilibrated for 20 minutes in oxygenated (95% O$_2$/5% CO$_2$) Krebs-HEPES buffer (pH 7.4) at 37°C. Lucigenin-enhanced chemiluminescence was measured with low-concentration lucigenin (5 μmol/L), because higher concentrations of lucigenin (up to 250 μmol/L) favor redox cycling. NO synthase–derived superoxide production was determined by the difference in superoxide production after 30 minutes’ preincubation with the NO synthase inhibitor L-NAME (100 μmol/L).

Oxidative Fluorescent Microphotography

In situ O$_2$ production was determined in vessel cryosections with the oxidative fluorescent dye dihydroethidium (DHE). Cryosections (30 μmol/L) were incubated with DHE (2 μmol/L) in Krebs-HEPES buffer, with or without L-NAME (100 μmol/L). Fluorescent images of the endothelium (×40, Zeiss LSM 510 META laser scanning confocal microscope, Carl Zeiss, Inc, Oberkochen, Germany) were obtained from each vessel quadrant. In each case, segments of vessel rings (with and without L-NAME) were analyzed in parallel with identical imaging parameters. DHE fluorescence was quantified by automated image analysis with Image-Pro Plus software (Media Cybernetics, Bethesda, Md); all analyses were performed in a blinded fashion by 2 independent observers.

Determination of Folate, 5-MTHF, tHcy, and Biopterins

Blood samples were collected at entry to the study and at the end of the treatment period. Plasma folate was measured by microbiological assay. Plasma tHcy was measured by high-performance liquid chromatography with fluorometric detection. For measurement of 5-MTHF, plasma was stored in vitamin C 5% and measured by high-performance liquid chromatography and fluorescence detection, as described previously. The same method was also used to measure vascular 5-MTHF. Biopterin levels in vessel tissue lysates were determined by high-performance liquid chromatography and electrochemical detection and expressed as picomoles per gram of tissue, as described previously.
Statistical Analysis
All variables were tested for normal distribution with the Kolmogorov-Smirnov test. Normally distributed variables are presented as mean ± SEM in both the tables and the figures. Because vascular superoxide was nonnormally distributed, it was log-transformed for analysis, and the results are presented as median (25th–75th percentiles). Power calculations, based on previous results from our group, showed that a total number of 15 patients per group was able to detect a 30% change of vascular MRI outcomes (within each group) and a 40% difference in the vascular vasomotor responses to ACh between groups with a power of 90% and α = 0.05. The numbers, however, were further increased to allow for a 20% dropout rate. Comparisons of baseline characteristics between the 3 groups were performed with 1-way ANOVA for continuous and χ² test for categorical variables, as appropriate. Two-way ANOVA for repeated measurements was used to assess the effect of treatment on MRI indices of vascular function and to compare the magnitude of the changes between the 3 groups. If significant time-by–treatment group interactions were detected, then multiple comparisons between the 3 groups were performed. Similarly, 2-way ANOVA for repeated measurements was also used to compare the dose-response curves for the vasomotor response to ACh. Univariate analysis was performed to assess correlations between the variables, and the Pearson r value was calculated.

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

Patient Characteristics
The patients’ demographic and clinical characteristics are presented in Table 1. No significant differences existed in clinical features or treatments at baseline between the treatment groups (Table 1).
Effect of Folic Acid on Endothelial Function
We first sought to determine the effects of low- and high-dose folic acid treatment on peripheral endothelial function, both by using noninvasive MRI of brachial artery FMD and by determining vasorelaxation responses of SVs to ACh. Pretreatment values for FMD and nitroglycerin responses were comparable between the 3 groups (P=NS between groups). Brachial artery FMD was significantly improved in both low- and high-dose folic acid groups but remained unchanged in the placebo group (Figure 1). The improvements in FMD in the 400-µg/d and 5-mg/d groups were significantly greater than with placebo (P<0.05 and P<0.001, respectively), whereas no significant difference existed in the change in FMD between the 2 active treatment groups (P=NS). Treatment with folic acid had no effect on endothelium-independent dilation in the brachial artery, as evaluated by sublingual administration of the exogenous NO donor nitroglycerin (Figure 1).

NO-mediated vasorelaxation responses in segments of SV were significantly increased in patients treated with either low- or high-dose folic acid compared with the placebo-treated patients (Figure 1), whereas endothelium-independent vasorelaxations to sodium nitroprusside were not significantly different. No significant difference existed in the vasomotor responses to ACh between the low- and high-dose groups. These findings indicate that low-dose folic acid treatment (400 µg/d) improves NO bioavailability in patients with CAD, whereas supplementation of high-dose folic acid (to 5 mg/d) does not lead to a significantly greater benefit.

Effect of Folic Acid on Vascular Distensibility
We next evaluated the effects of folic acid treatment on vascular distensibility and stiffness of the aorta and carotid arteries using noninvasive high-resolution MRI, before and after treatment with either low-dose (400 µg/d) or high-dose (5 mg/d) folic acid or placebo. Low-dose folic acid treatment significantly improved both aortic and carotid distensibility compared with placebo (Table 2). Correspondingly, low-dose folic acid significantly reduced aortic pulse-wave velocity, a measure of vascular stiffness, compared with placebo (Table 2). The changes in vascular distensibility and stiffness observed after high-dose folic acid were not significantly different from those with low-dose folic acid.

### TABLE 2. Effects of Folate Treatment on Aortic and Carotid Artery Distensibility and Pulse-Wave Velocity

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Folic Acid 400 µg Daily</th>
<th>Folic Acid 5 mg Daily</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>After Treatment</td>
<td>Δ</td>
</tr>
<tr>
<td>Distensibility, 10⁻³ mm Hg⁻¹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascending aorta</td>
<td>1.84 (0.33)</td>
<td>1.80 (0.33)</td>
<td>−0.036 (171)</td>
</tr>
<tr>
<td>Proximal descending aorta</td>
<td>2.62 (0.45)</td>
<td>2.69 (0.5)</td>
<td>0.069 (1.68)</td>
</tr>
<tr>
<td>Abdominal aorta</td>
<td>3.80 (0.63)</td>
<td>3.59 (0.53)</td>
<td>−0.328 (251)</td>
</tr>
<tr>
<td>Right carotid artery</td>
<td>3.60 (0.31)</td>
<td>3.30 (0.34)</td>
<td>−0.341 (194)</td>
</tr>
<tr>
<td>Left carotid artery</td>
<td>3.30 (0.31)</td>
<td>3.30 (0.39)</td>
<td>−0.030 (361)</td>
</tr>
<tr>
<td>Aortic pulse-wave velocity, m/s</td>
<td>7.93 (0.71)</td>
<td>8.22 (0.90)</td>
<td>0.29 (0.35)</td>
</tr>
</tbody>
</table>

Figures in parentheses represent SEM for each parameter. Δ represents the absolute change of each variable from baseline.

*P<0.05 and †P<0.01 vs placebo; ‡P<0.05 and §P<0.01 vs baseline.
Effect of Folic Acid on Vascular Superoxide Production

To further investigate the mechanisms underlying the effects of folic acid on vascular function, we next compared vascular superoxide production in samples of both SV and IMA from patients treated with low- or high-dose folic acid or placebo. Vascular superoxide production was substantially reduced in vessels from patients receiving either low- or high-dose folic acid compared with placebo (Figure 2).

Because previous studies suggest that acute administration of 5-MTHF may have direct effects on superoxide production mediated through enzymatic coupling of eNOS,\(^5\) we next examined whether oral folic acid treatment would also alter eNOS coupling. L-NAME induced a decrease in vascular superoxide production in vessels from patients who received placebo, which suggests that uncoupled eNOS was a major contributor to the overall vascular superoxide in these patients (Figure 3). However, in vessels from patients who received either low- or high-dose folic acid, this effect was reversed, which suggests that low- and high-dose folic acid induce a similar improvement in eNOS coupling (Figure 3). We further examined the effect of L-NAME on the endothelium-derived fraction of vascular superoxide in vessel cryosections by confocal imaging of DHE fluorescence (Figure 3). We observed that L-NAME decreased endothelium-derived DHE-fluorescence in vessels from placebo-treated patients, characteristic of uncoupled eNOS; however, consistent with the chemiluminescence experiments, we observed that L-NAME had the opposite effect on endothelium-derived DHE-fluorescence in patients who received low- or high-dose folic acid.
low- or high-dose folic acid compared with placebo (Figure 3). These findings suggest that low-dose folic acid treatment (400 μg/d) in patients not already receiving dietary folate supplementation improves eNOS coupling, resulting in a significant decrease in vascular superoxide production; however, high-dose folic acid (5 mg/d) did not induce a significantly greater improvement than low-dose treatment.

To investigate the mechanisms relating eNOS coupling to folate treatment, we examined the effects of low- and high-dose folic acid treatment on vascular tissue BH4 levels. We observed that vessels from patients who received either low- or high-dose folic acid treatment had significantly elevated vascular BH4 levels compared with vessels from patients who received placebo (Figure 4). Taken together, these findings suggest that oral folic acid treatment has biologically important effects on vascular oxidative stress, which are associated with improvements in vascular BH4 availability and eNOS coupling. However, oral folic acid treatment appears similarly effective at low dose as at high dose.

Effects of Folic Acid on Plasma Folate and Vascular/Plasma Levels of 5-MTHF
Baseline plasma folate levels were consistent with those of a patient group recruited from a non–folate-fortified population (Figure 5).18 Treatment with low-dose folic acid raised plasma folate to levels comparable to those observed in fortified populations,3 whereas high-dose treatment induced a further elevation of plasma circulating folate levels (P<0.001 for the change in high versus low dose; Figure 5). To explore the relationship between the dosage of folic acid and its biological effects, we next examined the effect of folic acid treatment on circulating and vascular 5-MTHF levels. Plasma levels of 5-MTHF were significantly increased after low-dose treatment with folic acid, whereas high-dose treatment induced an even higher elevation of plasma 5-MTHF (P<0.01 for the change in high versus low dose; Figure 5). Indeed, a significant correlation existed between folic acid treatment dose and plasma 5-MTHF levels (r=0.49, P<0.001); however, plasma tHcy was similarly decreased in both folic acid–treated groups compared with placebo (Figure 5), and no correlation existed between plasma tHcy and 5-MTHF levels (r=0.05 P=0.75). In contrast, plasma tHcy was similarly decreased in both active treatment groups, while remaining unchanged in the placebo group (C). Despite a significantly greater elevation of circulating 5-MTHF levels after high-dose than after low-dose folic acid supplementation, both active treatments produced significant elevations of intravascular 5-MTHF levels compared with placebo, and no significant difference in vascular 5-MTHF levels was observed between the 2 active treatment groups (D). Values are expressed as mean±SEM. *P<0.05; **P<0.01 vs baseline or placebo.

Discussion
In this randomized, double-blind, placebo-controlled trial of the effects of oral folic acid on vascular function and
oxidative stress in patients with CAD, we have made a number of novel and important observations. First, we show that folic acid improves vascular stiffness and endothelial function, assessed by both MRI studies in vivo and vasomotor studies of vessel function ex vivo. Second, we demonstrate that the salutary effects of oral folic acid treatment on endothelial function are mediated in part by an improvement in vascular BH4 bioavailability, which leads to restoration of eNOS “coupling” and a reduction of eNOS-derived superoxide production. Third, we find that these effects are observed equally with low-dose folic acid treatment as with high-dose treatment and that the biological effects of oral folic acid treatment are related to vascular tissue 5-MTHF levels rather than folic acid treatment dose. These results suggest that treatment with low-dose folic acid (equivalent to the intake of folate-rich or folate-fortified diets) increases vascular 5-MTHF levels and induces maximal improvements of vascular function. Any further increase in plasma 5-MTHF is not followed by a linear elevation of vascular 5-MTHF and offers few, if any, additional improvements in vascular function.

Both elevated plasma levels of tHcy and low folate status are associated with increased cardiovascular risk and endothelial dysfunction. Previous studies have suggested that high-dose folic acid supplementation improves endothelial function and arterial stiffness, which are both independent predictors of cardiovascular risk; however, the results from large trials examining the clinical effects of folic acid in patients with cardiovascular disease are controversial. Whereas folic acid treatment may be beneficial in patients with high tHcy levels, no clear benefit has been demonstrated in other patient groups. Two recent large clinical trials in patients with myocardial infarction or stable vascular disease found that lowering homocysteine with folic acid and B vitamins did not improve the primary clinical outcome. However, in the Heart Outcomes Prevention Evaluation-2 study, 70% of patients were recruited from countries where the grain supply is fortified with folate, in which patients may receive 380 μg of folate daily as a result of fortification. Subanalysis of nonfortified subjects, although underpowered, showed a trend toward a reduced relative risk with folic acid treatment. In addition, stroke mortality, a secondary outcome measure, was significantly reduced after folic acid treatment. Dietary folate fortification may have already had some beneficial effects on cardiovascular risk; the decline in stroke mortality has been accelerated in North America since folate fortification was introduced, compared with the nonfortified United Kingdom.

In the present study, we observed that low-dose folic acid treatment (400 μg/d, comparable to daily intake received by subjects after dietary folate fortification) significantly improved prognostic markers of cardiovascular risk, such as arterial stiffness and endothelial function, in patients who had not already received dietary folate fortification. These functional changes were accompanied by improvements in vascular oxidative stress, mediated through BH4-dependent eNOS coupling. However, treatment with a higher dose of folic acid (5 mg/d) produced no further improvements in vascular function, which suggests that the dose response of the cardiovascular benefits of folic acid treatment lies within the range of the recommended daily allowance and dietary fortification, rather than high-dose pharmacological folic acid treatment. Indeed, our observations in the present study support the recent suggestion that beyond the decrease in cardiovascular risk achieved by dietary folate fortification, additional folate treatment in fortified populations may have no additional benefit. To explain these unexpected findings, we determined both plasma folate and plasma and intracellular 5-MTHF levels in the patients in the present study. Surprisingly, despite the significantly higher plasma folate and 5-MTHF levels in patients treated with high-dose folic acid compared with those treated with low-dose folic acid, the vascular tissue levels of 5-MTHF were similar in both treatment groups, which suggests that folate plasma concentrations achieved after low-dose treatment induce a minimal elevation of vascular 5-MTHF, and any further folate administration leads to little or no further elevation of vascular 5-MTHF levels. Although we observed no statistically significant incremental effect of high- versus low-dose folic acid, the small size of the present study cannot provide sufficient power to exclude a small difference between the treatment doses.

The present study provides important insights into the mechanism of action of folic acid in patients with CAD. Increased oxidative stress is a feature of vascular disease states and in the pathogenesis of atherosclerosis, through effects of reactive oxygen species on NO bioavailability and through interactions with numerous redox-sensitive signaling pathways. Previous studies have suggested that folates may have direct effects on NO-mediated endothelial function, possibly through changes in eNOS regulation mediated by the eNOS cofactor, BH4. Indeed, we have previously shown that 5-MTHF is a potent peroxynitrite scavenger and improves eNOS coupling by preventing the ONOO⁻-induced oxidation of BH4. The targeting of BH4-dependent eNOS coupling may be a promising therapeutic target both to improve NO-mediated endothelial function and to reduce vascular oxidative stress. However, folic acid also affects methylation processes and nucleic acid synthesis, which are important in atherosclerosis. In this way, folic acid may modify cellular proliferation and affect the levels of methylarginines, which can act as eNOS inhibitors. These complex interactions between folates and vascular homeostasis may explain in part the controversial results provided after treatment with pharmacological dosages of folic acid.

In conclusion, we demonstrate that daily treatment with low-dose folic acid, within the range of the recommended daily allowance and dietary fortification, elevates vascular BH4 bioavailability, improves eNOS coupling, and globally improves vascular function in patients with atherosclerosis. However, high-dose folic acid produces little or no further improvements in vascular function. The present findings suggest that the vascular endothelium approaches its maximum capacity to take up 5-MTHF after low-dose folate supplementation (400 μg/d), and further increases in
plasma folate do not lead to a proportional rise in vascular tissue levels in patients with CAD. The results of the present study provide an explanation for the discordance between epidemiological observations that suggest a reduction of cardiovascular risk in North America after dietary folate fortification and the results of clinical trials that suggest little clinical benefit from pharmacological-dose folic acid treatment.

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Disclosures
None.

References


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**CLINICAL PERSPECTIVE**

Dietary folate fortification lowers plasma homocysteine and may reduce cardiovascular risk, whereas high-dose folic acid treatment has not been shown to alter clinical outcome. Folic acid and its principal circulating metabolite, 5-methyltetrahydrofolate, appear to improve vascular function, but the potential mechanisms relating folate dose to vascular function remain unclear. In this double-blind placebo-controlled trial, we examined the mechanistic effects of folic acid treatment in human vessels from patients undergoing coronary artery bypass grafting, using pharmacological high-dose treatment versus low-dose treatment, equivalent to dietary folate fortification. We found that a 6-week treatment with low-dose folic acid increased nitric oxide bioavailability in human vessels, reduced vascular superoxide production, and improved enzymatic coupling of endothelial nitric oxide synthase through availability of the cofactor tetrahydrobiopterin; however, high-dose folic acid treatment induced no further improvement of vascular function. Whereas plasma 5-methyltetrahydrofolate levels increased proportionately with treatment dose of folic acid, vascular tissue 5-methyltetrahydrofolate showed no further increment with high-dose compared with low-dose folic acid. These findings suggest that low-dose folic acid treatment, comparable to daily intake and dietary fortification, significantly improves vascular function through effects on endothelial nitric oxide synthase and vascular oxidative stress. Treatment with a pharmacological dosage of folic acid provides no additional benefit, as a result of the inability of high-dose treatment to induce further increases in vascular 5-methyltetrahydrofolate bioavailability. These findings provide a mechanistic explanation for the inability of high-dose treatment with folic acid to improve clinical outcome in folate-fortified populations.
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