

Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION



Folates and Cardiovascular Disease

M.C. Verhaar, E. Stroes and T.J. Rabelink
Arterioscler Thromb Vasc Biol 2002;22;6-13
DOI: 10.1161/hq0102.102190

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association,
7272 Greenville Avenue, Dallas, TX 75214

Copyright © 2002 American Heart Association. All rights reserved. Print ISSN: 1079-5642. Online
ISSN: 1524-4636

The online version of this article, along with updated information and services, is
located on the World Wide Web at:

<http://atvb.ahajournals.org/cgi/content/full/22/1/6>

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular
Biology is online at

<http://atvb.ahajournals.org/subscriptions/>

Permissions: Permissions & Rights Desk, Lippincott Williams & Wilkins, a division of Wolters
Kluwer Health, 351 West Camden Street, Baltimore, MD 21202-2436. Phone: 410-528-4050. Fax:
410-528-8550. E-mail:

journalpermissions@lww.com

Reprints: Information about reprints can be found online at

<http://www.lww.com/reprints>

Folates and Cardiovascular Disease

M.C. Verhaar, E. Stroes, T.J. Rabelink

Abstract—It is increasingly recognized that folates may play a role in the prevention of cardiovascular disease. Over the last few years, several studies have reported beneficial effects of folates on endothelial function, a surrogate end point for cardiovascular risk. Consistently, observational studies have demonstrated an association between folate levels and cardiovascular morbidity and mortality. The exact mechanisms underlying the ameliorative effects of folates on the endothelium remain to be elucidated. Thus far, most studies have focused on the homocysteine-lowering effects of folates. However, recently, benefits of folates independent of homocysteine lowering have also been reported. Potential mechanisms include antioxidant actions, effects on cofactor availability, or direct interactions with the enzyme endothelial NO synthase. Obviously, beneficial effects of folates on cardiovascular risk would have important clinical and dietary consequences. However, for definite conclusions, the completion of ongoing randomized controlled trials will have to be awaited. (*Arterioscler Thromb Vasc Biol.* 2002;22:6-13.)

Key Words: folates ■ endothelium ■ cardiovascular disease ■ cardiovascular risk

Folates are important cofactors in the transfer and utilization of 1-carbon moieties and play a key role in the synthesis of nucleic acids and methionine regeneration. It has long been known that florid folate deficiency causes a defect in DNA synthesis, leading to megaloblastic macrocytic anemia. Now, >55 years after the discovery and synthesis of folate,¹ it is recognized that more discrete deficiency of this essential nutrient is also associated with an increased risk of morbidity unrelated to anemia.

There is irrefutable evidence that folate deficiency or abnormalities in folate metabolism during pregnancy are associated with an increased risk of developmental abnormalities, particularly neural tube defects,² whereas folate supplementation can significantly reduce the occurrence and recurrence of such disorders.³ Other potential manifestations of folic acid deficiency include neurological and neuropsychiatric disorders^{4,5} and the development of certain neoplasms and preneoplastic conditions.⁶ Furthermore, folic acid deficiency has been associated with a predisposition to atherosclerotic cardiovascular disease.

In the present article, we will review recent developments regarding the role of folates in cardiovascular disease. In addition, we will discuss potential underlying mechanisms and possible clinical and/or dietary consequences.

Folate, Vascular Function, and Cardiovascular Disease Risk

In the past 3 years, several intervention studies have shown the benefits of folate therapy on endothelial function (Table). Endothelial dysfunction, assessed as impaired vasodilator response to mechanical or pharmacological stimuli, has been

increasingly recognized as a surrogate end point for cardiovascular risk. A close correlation has been observed between the presence of cardiovascular risk factors and endothelial vasodilator dysfunction.⁷ Several investigators have found an association between endothelial dysfunction and myocardial ischemia.^{8,9} Importantly, 2 recent reports have demonstrated in long-term follow-up that endothelial dysfunction is associated with a higher incidence of cardiovascular events and increased progression of atherosclerotic disease.^{10,11} Together, available data strongly suggest that improvement of endothelial function may be associated with reduced cardiovascular risk.

Two recent studies have demonstrated that folic acid supplementation in patients with asymptomatic hyperhomocysteinemia can improve endothelial function, measured as enhanced flow-mediated vasodilatation.^{12,13} In addition, endothelial function was improved after folate therapy in hyperhomocysteinemic patients with established coronary artery disease,^{14–16} whereas folic acid supplementation also caused a reduction in the rate of progression of the ultrasound-determined extracranial carotid artery plaque area in patients with premature atherosclerosis and hyperhomocysteinemia.¹⁷ More recently, high single-dose and multiple-dose folic acid administration has been shown to prevent the temporary endothelial dysfunction induced by post-methionine-load hyperhomocysteinemia in healthy volunteers.^{18,19} These data support the hypothesis that the lowering of homocysteine in hyperhomocysteinemic patients may reduce cardiovascular risk.

Interestingly, several investigators have also reported beneficial effects of folates in nonhyperhomocysteinemic

Received September 24, 2001; revision accepted October 29, 2001.

From the Department of Vascular Medicine, University Medical Centre Utrecht, Utrecht, the Netherlands.

Correspondence to Marianne C. Verhaar, Department of Vascular Medicine, F02.226, University Medical Centre Utrecht, Heidelberglaan 100, 3584 CX Utrecht, Netherlands. E-mail m.c.verhaar@digd.azu.nl

© 2002 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol. is available at <http://www.atvbaha.org>

Intervention Studies on Effects of Folates on Cardiovascular Surrogate End Points

Author, Year	n	Patient Group	Folate, dose/d; Duration	Additional Medication (Dose)	End Point
Woo et al, ¹² 1999	17	Asymptomatic hyperhomocysteinemia	FA, 10 mg; 8 wk	...	Improved flow-mediated endothelium-dependent vasodilation
Bellamy et al, ¹³ 1999	18	Asymptomatic hyperhomocysteinemia	FA, 5 mg; 6 wk	...	Improved flow-mediated endothelium-dependent vasodilation
Title et al, ¹⁴ 2000	50	Coronary atherosclerosis and hyperhomocysteinemia	FA, 5 mg; 4 mo	–/Vit C (2 g/d)+Vit E (800 IU/d)	Improved flow-mediated endothelium-dependent vasodilation
Chambers et al, ¹⁵ 2000	89	Coronary atherosclerosis and hyperhomocysteinemia	FA, 5 mg; 8 wk	Vit B ₁₂ (1 mg/d)	Improved flow-mediated endothelium-dependent vasodilation
Doshi et al, ¹⁶ 2001	52	Coronary atherosclerosis	FA, 5 mg; 6 wk	...	Improved flow-mediated endothelium-dependent vasodilation
	10	Coronary atherosclerosis	5-MTHF, 50 μg/min; 1 dose	...	Improved flow-mediated endothelium-dependent vasodilation
Peterson and Spence, ¹⁷ 1998	38	Premature atherosclerosis and hyperhomocysteinemia	FA, 5/2.5 mg; 4.4±1.5 y	Vit B ₆ (25 mg/d) and Vit B ₁₂ (250 μg/d)	Reduced progression of carotid plaque area
Usui et al, ¹⁸ 1999	10	Acute hyperhomocysteinemia in healthy volunteers	FA, 20 mg; 1 dose	...	Prevented impairment in flow-mediated endothelium-dependent vasodilation
Chao et al, ¹⁹ 1999	16	Acute hyperhomocysteinemia in healthy volunteers	FA, 5 mg; 5 wk	Vit B ₆ (100 mg)+Vit B ₁₂ (0.5 mg)	Prevented impairment in flow-mediated endothelium-dependent vasodilation
Verhaar et al, ²¹ 1998	10	Asymptomatic familial hypercholesterolemia	5-MTHF, 0–10 μg/100 mL per min; 5 min	...	Improved serotonin-induced endothelium-dependent vasodilation
Verhaar et al, ²² 1999	20	Asymptomatic familial hypercholesterolemia	FA, 5 mg; 4 wk	...	Improved serotonin-induced endothelium-dependent vasodilation
Vermeulen et al, ²³ 2000	78	Healthy siblings of patients with premature atherothrombotic disease	FA, 5 mg; 2 y	Vit B ₆ (250 mg/d)	Decreased rate of abnormal exercise ECG tests, with no effects on a-b pressure indices or on carotid/femoral ultrasonography
Wilmink et al, ²⁴ 2000	20	Postprandial lipemia in healthy volunteers	FA, 10 mg; 2 wk	...	Prevented impairment in flow-mediated endothelium-dependent vasodilation
van Guldener et al, ⁹² 1998	35	Hemodialysis patients with hyperhomocysteinemia	FA, 5/1 or 5 mg; 12 wk/1 y	–/Betaine (4 g for 12 wk)	No effects on flow-mediated endothelium-dependent vasodilation or plasma vWF, TM, E-selectin, PAI-1, tPA, or ET
van Guldener et al, ⁹³ 1998	30	Peritoneal dialysis patients with hyperhomocysteinemia	FA, 5/1 or 5 mg; 12 wk/1 y	–/Betaine (4 g for 12 wk)	No effects on flow-mediated endothelium-dependent vasodilation or plasma vWF, TM, E-selectin, PAI-1, tPA, or ET
Thambyrajah et al, ⁹⁴ 2000	100	Patients with predialysis chronic renal failure	FA, 5 mg; 12 wk	...	No effects on flow-mediated endothelium-dependent vasodilation, plasma vWF, or serum nitrite/nitrate concentrations

FA indicates folic acid; Vit, vitamin; –/Vit C and –/Betaine, absence or presence of Vit C and betaine; a-b pressure indices, ankle-brachial indices; vWF, von Willebrand factor; TM, thrombomodulin; tPA, tissue-type plasminogen activator; PAI-1, plasminogen activator inhibitor type 1; and ET, endothelin-1.

patients. A recent study has shown that folic acid can prevent endothelial dysfunction induced by continuous nitroglycerin treatment in healthy volunteers.²⁰ We have recently demonstrated that intra-arterial administration of 5,10-methylenetetrahydrofolate and oral supplementation of folic acid can restore endothelial function in patients with familial hypercholesterolemia but with normal plasma homocysteine levels^{21,22} (Figure 1). In addition, preliminary data from our group suggest a beneficial effect of folate on endothelial dysfunction in diabetes (R. Van Etten, unpublished data, 2001). Vermeulen et al²³ have demonstrated that folic acid therapy decreases the occurrence of abnormal exercise ECG tests in healthy siblings of

patients with premature atherothrombotic disease. Furthermore, we have demonstrated that folic acid treatment can prevent endothelial dysfunction due to oral fat load-induced hyperlipidemia in healthy volunteers.²⁴ Importantly, we²¹ and others^{16,18} have shown a beneficial effect of folates on endothelial function independent of changes in plasma homocysteine levels.

The above observations suggest a role for folate supplementation in cardiovascular disease. Naturally, for definite conclusions, confirmation by large, controlled, prospective trials with hard clinical end points will be essential. Unfortunately, the results of such trials, which are currently

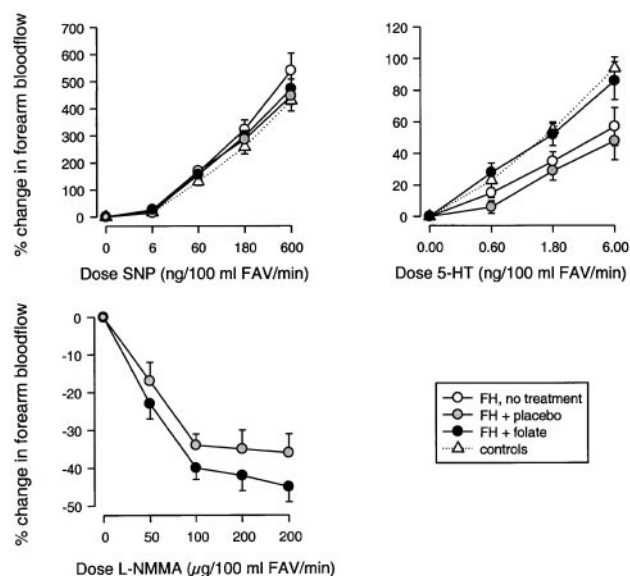


Figure 1. Percent change in forearm blood flow after stimulation of endothelium-independent and endothelium-dependent vasodilation with sodium nitroprusside (SNP) and serotonin (5-HT) and after NO synthase inhibition with *N*^G-monomethyl-L-arginine (L-NMMA) in young patients with familial hypercholesterolemia (FH) without macrovascular disease at baseline, after placebo, and after folic acid treatment (5 mg/d for 4 weeks) and in control subjects. There was no effect of folic acid supplementation in control subjects or on endothelium-independent vasodilation. Folic acid supplementation significantly enhanced endothelium-dependent vasodilation in FH patients. There was a trend toward improvement in basal NO activity.²² FAV indicates forearm value.

Various epidemiological studies seem to support a link between folate status and atherosclerotic vascular disease. In the European Concerted Action Project, a large multicenter case-control study including 750 cases and 800 controls, it was demonstrated that low serum folate levels are related to increased cardiovascular disease risk.²⁷ Similar findings have been reported in smaller cross-sectional studies,^{28–31} whereas others could not confirm such an association.^{32–34} In subsets of several prospective studies, such as the National Health and Nutrition Examination Survey,^{35–37} the Kuopio Ischemic Heart Disease Risk Factor Study,³⁸ the Framingham Heart Study,³⁹ and the Nutrition Canada Survey,⁴⁰ an inverse relation between folate status and atherosclerotic vascular disease has also been demonstrated, although this could not be confirmed by others (the Physicians' Health Study⁴¹ and Atherosclerosis Risk in Communities Study⁴²). Interestingly, in the Nurses' Health Study and in the Kuopio Ischemic Heart Disease Risk Factor Study, it was demonstrated that 20% of the individuals with the highest consumption of folate had significantly less cardiovascular disease than did those with the lowest consumption.^{43,44} On the other hand, antifolate therapy with methotrexate, an antirheumatic drug that impairs folate metabolism, has recently been suggested to promote atherosclerosis.⁴⁵

Additionally, a common mutation of 5,10-methylenetetrahydrofolate reductase (MTHFR), which causes increased thermolability and reduced activity of the enzyme catalyzing the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (5-MTHF, the main form of folate in the circulation⁴⁶), has also been reported by some to be a

risk factor for vascular disease.^{47–51} Several other large studies could not confirm such an association.^{52–56} Population differences and differences in folate status have been suggested to be responsible for these different study results.^{57,58}

Potential Mechanisms Underlying a Beneficial Effect of Folates in Cardiovascular Disease

Lowering of Homocysteine Levels

Many randomized controlled trials have demonstrated that treatment with natural dietary folate or the synthetic folic acid (pteroylmonoglutamic acid) significantly reduces plasma homocysteine levels, even when plasma levels of homocysteine and folate are already in the normal range.^{59,60} This homocysteine-lowering effect of folate can be explained by its actions as a substrate in the remethylation of homocysteine to methionine. Elevated homocysteine levels have been suggested to be a causal factor in cardiovascular disease. Thus, a beneficial effect of folates on cardiovascular risk might be explained by their effect on homocysteine plasma levels. Indeed, many cross-sectional and retrospective case-control studies have demonstrated a positive association between plasma homocysteine levels and cardiovascular risk. However, results from large prospective cohort studies are less convincing; a recent review of 7 prospective studies found no association or only a small association between plasma homocysteine levels and cardiovascular disease^{61–64} (for comprehensive review on homocysteine and cardiovascular disease, see Hankey and Eikelboom²⁶). Several pathogenetic mechanisms, based on *in vitro* experiments, have been proposed.⁶⁴ However, the clinical relevance of these *in vitro* experiments has been questioned, mainly because of the much higher homocysteine concentrations used *in vitro* than are used in patients. Definite proof that homocysteine is a causal factor for cardiovascular disease is still lacking. Alternatively, folate deficiency may be the primary cause of the increased risk of vascular disease, with elevated homocysteine levels a marker for low folate status rather than a pathogenetic factor.

Distinguishing between high homocysteine and low folate as a primary etiological factor in cardiovascular disease may not be feasible from clinical studies, considering their close metabolic relationship. For example, acute post-methionine-load hyperhomocysteinemia in healthy volunteers has been shown to induce endothelial dysfunction.⁶⁵ Although this has been viewed to be supportive of the causal relation between elevated homocysteine levels and cardiovascular disease, it may also be explained by the decrease in 5-MTHF levels that has been shown to occur after methionine loading.⁶⁶

Thus, whether the homocysteine-lowering effect of folates contributes to their beneficial effects on cardiovascular risk has not been established. However, our recent observations that the beneficial effects of folate in hypercholesterolemia occurred independent of a homocysteine-lowering effect²¹ indicate that other mechanisms must also be involved.

Antioxidant Actions

Ample evidence implicates oxidative stress in the pathogenesis of cardiovascular disease.⁶⁷ Oxidative stress is defined as a disturbance in the equilibrium between the production of reactive oxygen species (free radicals, eg, superoxide) and

antioxidant defenses. Endothelial dysfunction, a crucial early event in atherogenesis, is characterized by reduced bioavailability of endothelium-derived NO, which has been found to be due, at least in part, to enhanced oxidant degradation of NO in most cardiovascular risk factors.⁶⁸ Several antioxidants have been shown to reverse endothelial dysfunction in patients with coronary artery disease or increased risk of premature atherosclerosis.^{65,69–75}

In a series of *in vitro* experiments, we have demonstrated that folates possess antioxidant potential. Using lucigenin-enhanced chemiluminescence, we observed that 5-MTHF could reduce superoxide generation by 2 superoxide-generating systems: xanthine oxidase/hypoxanthine and endothelial NO synthase (eNOS).²¹ Others have shown that folate abolished the homocysteine-induced increases in endothelial superoxide.¹⁶ Recently, we could confirm the superoxide-scavenging capacity of 5-MTHF by electron paramagnetic resonance, with the use of 5-(Diethoxyphosphoryl)-5-methyl-L-pyrroline *N*-oxide as a spin trap for superoxide.⁷⁶ However, using this method, we also observed that the scavenging potency of 5-MTHF is ≈ 20 -fold lower than the scavenging effects of vitamin C, a well-known antioxidant vitamin. Thus, although compared with physiological vitamin C concentrations, folate clearly does exert antioxidant actions, given its lower potency and much lower plasma levels obtained *in vivo*, the relevance of such a direct antioxidant effect of folate *in vivo* is uncertain.

Interestingly, several animal and human studies do support a benefit of folates on the redox state. In rats, folate deficiency has been shown to increase lipid peroxidation and decrease cellular antioxidant defense.^{77,78} Furthermore, we observed in healthy volunteers that the beneficial effect of folates on postprandial endothelial dysfunction corresponded with a decreased urinary excretion of malondialdehyde, the radical-damage end product.²⁴ We hypothesize that an effect of folate on the NO-synthesizing enzyme eNOS may provide an explanation for the observed effects on oxidative stress and cardiovascular risk.

Interactions With eNOS

Figure 2 shows the interactions of folate with eNOS. In physiological situations, eNOS catalyzes the formation of NO by incorporating molecular oxygen into the substrate L-arginine, a reaction that requires NADPH, the allosteric activator calmodulin, and several cofactors, such as tetrahydrobiopterin (BH₄).⁷⁹ Recent data illustrate that under certain pathophysiological conditions, eNOS can “switch” from mainly NO synthesis to production of superoxide,^{80–82} a process called eNOS uncoupling (ie, uncoupling of NADPH oxidation and NO synthesis). Addition of the essential cofactor BH₄ has previously been shown to reduce superoxide production by eNOS *in vitro*^{81,83} and to improve NO availability *in vivo*,^{84,85} suggesting restoration of eNOS uncoupling by BH₄. In a recent series of *in vitro* experiments, we found that 5-MTHF can also influence the enzymatic activity of uncoupled eNOS: 5-MTHF reduces superoxide generation (more than can be explained by just a scavenging effect) and increases NO synthesis.⁷⁶ Interestingly, these effects were observed only in partially BH₄-replete, but not in BH₄-free, eNOS.

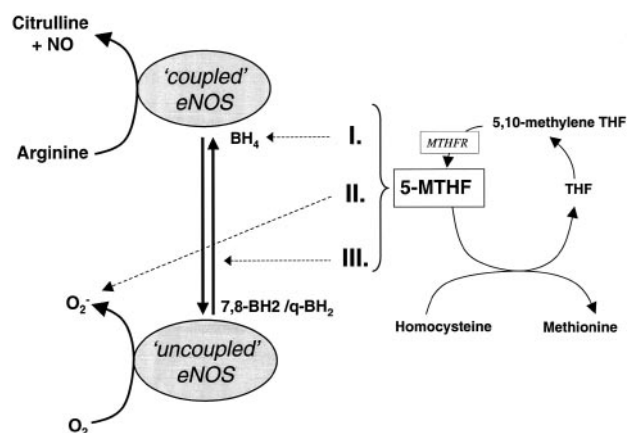


Figure 2. Potential mechanisms for the beneficial effects of folates on the heme-containing oxygenase domain of eNOS. Under certain circumstances, eNOS uncoupling can occur: a switch from the coupled (mainly NO-synthesizing) state to the uncoupled (superoxide-producing) state. The beneficial effects of folates may be explained by different mechanisms: I, BH₄ rescue or BH₄ stabilization, in which folates may stimulate endogenous BH₄ regeneration from quinoid dihydrobiopterin (q-BH₂) or lead to chemical stabilization of BH₄; II, antioxidant effects, in which folates may act as direct antioxidants; and III, direct effect on eNOS, in which folates have been shown to reduce superoxide generation and increase NO synthesis in a BH₄-dependent manner, suggesting a direct effect on eNOS. THF indicates tetrahydrofolate; 7,8-BH₂, 7,8-dihydrobiopterin.

Several mechanisms may underlie the observed folate-induced BH₄-dependent potentiation of eNOS. Recently, the presence of a pteridine-binding domain in NO synthase with similarities to the folate binding site of dihydrofolate reductase has been reported.⁸⁶ This site may act as a locus through which 5-MTHF can facilitate the electron transfer by BH₄ from the reductase domain of eNOS to heme.⁸⁷ Alternatively, 5-MTHF may enhance the binding of BH₄ to eNOS. 5-MTHF may also act by increasing BH₄ availability. This may occur through a chemical stabilization of BH₄, as has recently been described for ascorbic acid.⁸⁸ Furthermore, it has been suggested that folates may stimulate regeneration of BH₄ from the inactive oxidized quinoid dihydrobiopterin.⁸⁹ Indeed, high-dose folate supplementation has been shown to produce clinical improvement in children with BH₄ deficiency.⁹⁰

The ameliorative influence of folates on eNOS suggests a more pronounced benefit in the early phases of atherogenesis and less of a benefit on the more advanced stages, given the reduced eNOS expression observed in more advanced atherosclerosis.⁹¹ Indeed, only modest improvement of endothelial function has been observed after folate supplementation in established coronary atherosclerosis,¹⁴ whereas in dialysis patients or in patients with predialysis renal failure, no effect of folate treatment on endothelial dysfunction could be found.^{92–94}

Do We Need to Increase Our Folate Intake?

Over the years, insights on desirable folate intake have dramatically changed. Not long ago, folate sufficiency was defined as just an absence of megaloblastic anemia. In 1989, the Food and Nutrition Board in the United States even lowered the recommended dietary allowance of folate because of the low incidence of anemia due to folate deficiency.

However, during the past decade, marginal folate deficiency has been increasingly viewed as an important public health issue. Primarily, this is due to the consistent observation that folate deficiency in pregnancy is associated with birth defects. The studies reviewed above raise the question of whether the relatively low folate levels in the present-day western-style diet may also contribute to the excess cardiovascular morbidity and mortality in industrialized countries.

It is well known that dietary factors can influence the development of atherosclerosis. Recently, several dietary factors, such as an oral fat load,²⁴ a single high-fat meal,⁹⁵ an oral methionine load, or dietary animal protein,⁹⁶ have been shown to cause temporary endothelial dysfunction in healthy volunteers. Importantly, folic acid supplementation could completely prevent the observed diet-induced impairment in endothelial function.^{18,19,24} These data provide support for a protective role of folates against vascular insults. Moreover, they suggest that present-day folate intake may be too low to protect against the proatherogenic effects of our present diets, which are relatively high in fat and animal protein (methionine). Consistently, monkeys, which do not normally develop atherosclerotic disease, developed marked vascular dysfunction when fed a folate-depleted methionine-rich diet.⁹⁷ Interestingly, several human studies have shown an inverse association between fruit and vegetable (high-folate) consumption and risk of cardiovascular disease.^{98–101} Of course, fruit and vegetables may contain other vascular-protective substances, which may explain the favorable effects. In a recent large prospective study, a higher reported intake of folate was associated with reduced incidence of nonfatal myocardial infarction and fatal coronary heart disease.⁴³

Thus, increased folate intake may be desirable not only in certain subgroups but also in the general population in western countries. Improved folate status can be accomplished by increasing natural dietary folate (a diet rich in vegetables and citrus fruit) supplementation with the synthetic folic acid (pteroylmonoglutamic acid), which is heat stable and approximately twice as bioavailable, or folic acid food fortification. For all 3 methods, the potential to increase serum folate has been demonstrated.^{59,60,102} However, the actual effectiveness of the first 2 methods may be low because of poor compliance: surveys to evaluate folic acid supplementation for the prevention of birth defects uncovered that only a small minority of pregnant women had, in fact, taken advised folic acid supplements.¹⁰³ This has caused the Food and Drug Administration to introduce folic acid fortification of “enriched” cereal grains (1.4 mg/kg grain) in the United States in 1998. Similar fortification programs were introduced in Canada and several other industrialized countries. The Framingham Offspring Study has demonstrated that this policy has led to a substantial improvement in folate status in a population of middle-aged and older adults (mean folate concentrations increased from 11 to 23 nmol/L).¹⁰⁴ It will be very interesting to discover whether this level of fortification will influence cardiovascular morbidity and mortality.

Present data suggest that criteria for “folate sufficiency” may have to be redefined. Preferentially, recommendations on folate intake should be tailored to the needs of the individual patient or specific subgroups. Most studies on the effects of folates on cardiovascular end points have, thus far,

shown the benefits of relatively large quantities of folic acid (≥ 5 mg every day). No dose-response studies have been reported. It is highly likely that folate requirements will vary between groups. Increased folate demands have been suggested to occur during pregnancy.¹⁰⁵ Additionally, nutritional but also genetic factors, such as MTHFR polymorphisms,⁵⁸ may influence folate requirements. Furthermore, certain conditions may be associated with increased folate needs. For example, in vitro experiments have shown that superoxide may induce folate cleavage.¹⁰⁶ Such increased folate catabolism may lead to enhanced folate needs in patients who are exposed to increased oxidative stress, such as hypercholesterolemic and diabetic patients. Increased oxidative stress may also lead to increased consumption of folate because of its role as an antioxidant or in BH4 regeneration. Considering the amount of known and probably also unknown factors influencing folate metabolism, the assessment of folate sufficiency may be complicated. Possibly, homocysteine plasma levels may provide important information with low homocysteine levels (as low as possible), reflecting adequate folate status. Alternatively, unimpaired postprandial endothelial function may indicate folate sufficiency.

Safety Considerations

In general, folic acid supplementation is considered safe.¹⁰⁷ Some adverse effects were described in case reports and uncontrolled studies, but these could not be substantiated by further studies. However, there is some concern about folic acid therapy in people with subclinical cobalamin (vitamin B₁₂) deficiency, a relatively common disorder in the elderly¹⁰⁸ and in strict vegetarians.¹⁰⁹ Folic acid therapy in these patients may mask the hematologic manifestations of the disorder and allow progression of the neurological damage, including spinal cord injury. This induced several investigators to suggest the inclusion of cobalamin in folic acid supplements. Additionally, increased awareness of cobalamin deficiency in the population must be pursued. In our opinion, if the benefits of folic acid supplementation on hard cardiovascular end points can be confirmed in large clinical trials, the profits will, by far, outweigh the potential adverse effects. Of course, we should stay on the alert to identify potential adverse effects at an early stage.

Conclusions

Overall, available data strongly suggest a benefit of folate supplementation in lowering cardiovascular risk. Observational studies demonstrated an association between folate levels and cardiovascular morbidity and mortality, and a plausible biological mechanism that was based on in vitro experiments was presented. Additionally, an increasing number of interventional studies have confirmed the benefit of folates on surrogate end points. Of course, data from large, randomized, prospective trials are required to substantiate these findings. Such trials investigating the effects of folates on hard clinical cardiovascular end points are currently ongoing.^{25,26}

References

- Angier RB, Boothe JH, Hutchings BL. Synthesis of a compound identical with the L. casei factor isolated from liver. *Science*. 1945;102:222.

2. Daly LE, Kirke PN, Molloy A, Weir DG, Scott JM. Folate levels and neural tube defects: implications for prevention. *JAMA*. 1995;274:1698–1702.
3. MRC Vitamin Study Research Group. Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. *Lancet*. 1991;338:131–137.
4. Manzoor M, Runcie J. Folate-responsive neuropathy: report of 10 cases. *BMJ*. 1976;1:1176–1178.
5. Alpert JE, Fava M. Nutrition and depression: the role of folate. *Nutr Rev*. 1997;55:145–149.
6. Choi SW, Mason JB. Folate and carcinogenesis: an integrated scheme. *J Nutr*. 2000;130:129–132.
7. Vita JA, Treasure CB, Nabel EG, McLenachan JM, Fish D, Yeung AC, Vekshtein VI, Selwyn AP, Ganz P. Coronary vasomotor response to acetylcholine relates to risk factors for coronary artery disease. *Circulation*. 1990;81:491–497.
8. Hasdai D, Gibbons RJ, Holmes DR Jr, Higano ST, Lerman A. Coronary endothelial dysfunction in humans is associated with myocardial perfusion defects. *Circulation*. 1997;96:3390–3395.
9. Zeiher AM, Krause T, Schachinger V, Minners J, Moser E. Impaired endothelium-dependent vasodilation of coronary resistance vessels is associated with exercise-induced myocardial ischemia. *Circulation*. 1995;91:2345–2352.
10. Suwaidi JA, Hamasaki S, Higano ST, Nishimura RA, Holmes DR Jr, Lerman A. Long-term follow-up of patients with mild coronary artery disease and endothelial dysfunction. *Circulation*. 2000;101:948–954.
11. Schachinger V, Britten MB, Zeiher AM. Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. *Circulation*. 2000;101:1899–1906.
12. Woo KS, Chook P, Lolin YI, Sanderson JE, Metreweli C, Celermajer DS. Folic acid improves arterial endothelial function in adults with hyperhomocystinemia. *J Am Coll Cardiol*. 1999;34:2002–2006.
13. Bellamy MF, McDowell IFW, Ramsey MW, Brownlee M, Newcombe RG, Lewis MJ. Oral folate enhances endothelial function in hyperhomocysteinemic subjects. *Eur J Clin Invest*. 1999;29:659–662.
14. Title LM, Cummings PM, Giddens K, Genest JJ Jr, Nassar BA. Effect of folic acid and antioxidant vitamins on endothelial dysfunction in patients with coronary artery disease. *J Am Coll Cardiol*. 2000;36:758–765.
15. Chambers JC, Ueland PM, Obeid OA, Wrigley J, Refsum H, Kooner JS. Improved vascular endothelial function after oral B vitamins: an effect mediated through reduced concentrations of free plasma homocysteine. *Circulation*. 2000;102:2479–2483.
16. Doshi SN, McDowell IF, Moat SJ, Lang D, Newcombe RG, Kredan MB, Lewis MJ, Goodfellow J. Folate improves endothelial function in coronary artery disease: an effect mediated by reduction of intracellular superoxide? *Arterioscler Thromb Vasc Biol*. 2001;21:1196–1202.
17. Peterson JC, Spence JD. Vitamins and progression of atherosclerosis in hyper-homocyst(e)inaemia. *Lancet*. 1998;351:263. Letter.
18. Usui M, Matsuoka H, Miyazaki H, Ueda S, Okuda S, Imaizumi T. Endothelial dysfunction by acute hyperhomocyst(e)inaemia: restoration by folic acid. *Clin Sci (Colch)*. 1999;96:235–239.
19. Chao CL, Chien KL, Lee YT. Effect of short-term vitamin (folic acid, vitamins B6 and B12) administration on endothelial dysfunction induced by post-methionine load hyperhomocysteinemia. *Am J Cardiol*. 1999;84:1359–1361.
20. Gori T, Burstein JM, Ahmed S, Miner SE, Al Hesayen A, Kelly S, Parker JD. Folic acid prevents nitroglycerin-induced nitric oxide synthase dysfunction and nitrate tolerance: a human in vivo study. *Circulation*. 2001;104:1119–1123.
21. Verhaar MC, Wever RMF, Kastelein JJP, van Dam T, Koomans HA, Rabelink TJ. 5-Methyltetrahydrofolate, the active form of folic acid, improves endothelial function in familial hypercholesterolemia. *Circulation*. 1998;97:237–241.
22. Verhaar MC, Wever RMF, Kastelein JJP, van Loon D, Milstien S, Koomans HA, Rabelink TJ. Effects of oral folic acid supplementation on endothelial function in familial hypercholesterolemia: a randomised placebo-controlled trial. *Circulation*. 1999;100:335–338.
23. Vermeulen EG, Stehouwer CD, Twisk JW, van den BM, de Jong SC, Mackaay AJ, van Campen CM, Visser FC, Jakobs CA, Bulterjys EJ, et al. Effect of homocysteine-lowering treatment with folic acid plus vitamin B6 on progression of subclinical atherosclerosis: a randomised, placebo-controlled trial. *Lancet*. 2000;355:517–522.
24. Wilmlink HW, Stroes ES, Erkelens WD, Gerritsen WB, Wever R, Banga JD, Rabelink TJ. Influence of folic acid on postprandial endothelial dysfunction. *Arterioscler Thromb Vasc Biol*. 2000;20:1335–1338.
25. Clarke R, Collins R. Can dietary supplements with folic acid or vitamin B6 reduce cardiovascular risk?: design of clinical trials to test the homocysteine hypothesis of vascular disease. *J Cardiovasc Risk*. 1998;5:249–255.
26. Hankey GJ, Eikelboom JW. Homocysteine and vascular disease. *Lancet*. 1999;354:407–413.
27. Robinson K, Arheart K, Refsum H, Brattstrom L, Boers G, Ueland P, Rubba P, Palma-Reis R, Meleady R, Daly L, et al. Low circulating folate and vitamin B6 concentrations: risk factors for stroke, peripheral vascular disease, and coronary artery disease: European COMAC Group. *Circulation*. 1998;97:437–443.
28. Pancharuniti N, Lewis CA, Sauberlich HE, Perkins LL, Go RC, Alvarez JO, Macaluso M, Acton RT, Copeland RB, Cousins AL, et al. Plasma homocyst(e)ine, folate, and vitamin B-12 concentrations and risk for early-onset coronary artery disease. *Am J Clin Nutr*. 1994;59:940–948.
29. Schwartz SM, Siscovick DS, Malinow MR, Rosendaal FR, Beverly RK, Hess DL, Psaty BM, Longstreth WT Jr, Koepsell TD, Raghunathan TE, et al. Myocardial infarction in young women in relation to plasma total homocysteine, folate, and a common variant in the methylenetetrahydrofolate reductase gene. *Circulation*. 1997;96:412–417.
30. Verhoef P, Stampfer MJ, Buring JE, Gaziano JM, Allen RH, Stabler SP, Reynolds RD, Kok FJ, Hennekens CH, Willett WC. Homocysteine metabolism and risk of myocardial infarction: relation with vitamins B6, B12, and folate. *Am J Epidemiol*. 1996;143:845–859.
31. Bunout D, Petermann M, Hirsch S, de la Maza P, Suazo M, Barrera G, Kauffman R. Low serum folate but normal homocysteine levels in patients with atherosclerotic vascular disease and matched healthy controls. *Nutrition*. 2000;16:434–438.
32. Verhoef P, Kok FJ, Kruyssen DA, Schouten EG, Witteman JC, Grobbee DE, Ueland PM, Refsum H. Plasma total homocysteine, B vitamins, and risk of coronary atherosclerosis. *Arterioscler Thromb Vasc Biol*. 1997;17:989–995.
33. Dalery K, Lussier-Cacan S, Selhub J, Davignon J, Latour Y, Genest J Jr. Homocysteine and coronary artery disease in French Canadian subjects: relation with vitamins B12, B6, pyridoxal phosphate, and folate. *Am J Cardiol*. 1995;75:1107–1111.
34. Robinson K, Mayer EL, Miller DP, Green R, van Lente F, Gupta A, Kottke-Marchant K, Savon SR, Selhub J, Nissen SE. Hyperhomocysteinemia and low pyridoxal phosphate: common and independent reversible risk factors for coronary artery disease. *Circulation*. 1995;92:2825–2830.
35. Loria CM, Ingram DD, Feldman JJ, Wright JD, Madans JH. Serum folate and cardiovascular disease mortality among US men and women. *Arch Intern Med*. 2000;160:3258–3262.
36. Ford ES, Byers TE, Giles WH. Serum folate and chronic disease risk: findings from a cohort of United States adults. *Int J Epidemiol*. 1998;27:592–598.
37. Giles WH, Kittner SJ, Anda RF, Croft JB, Casper ML. Serum folate and risk for ischemic stroke: First National Health and Nutrition Examination Survey epidemiologic follow-up study. *Stroke*. 1995;26:1166–1170.
38. Voutilainen S, Lakka TA, Porkkala-Sarataho E, Rissanen T, Kaplan GA, Salonen JT. Low serum folate concentrations are associated with an excess incidence of acute coronary events: the Kuopio Ischaemic Heart Disease Risk Factor Study. *Eur J Clin Nutr*. 2000;54:424–428.
39. Selhub J, Jacques PF, Bostom AG, D'Agostino RB, Wilson PW, Belanger AJ, O'Leary DH, Wolf PA, Schaefer EJ, Rosenberg IH. Association between plasma homocysteine concentrations and extracranial carotid-artery stenosis. *N Engl J Med*. 1995;332:286–291.
40. Morrison HI, Schaubel D, Desmeules M, Wigle DT. Serum folate and risk of fatal coronary heart disease. *JAMA*. 1996;275:1893–1896.
41. Chasan Taber L, Selhub J, Rosenberg IH, Malinow MR, Terry P, Tishler PV, Willett W, Hennekens CH, Stampfer MJ. A prospective study of folate and vitamin B6 and risk of myocardial infarction in US physicians. *J Am Coll Nutr*. 1996;15:136–143.
42. Folsom AR, Nieto FJ, McGovern PG, Tsai MY, Malinow MR, Eckfeldt JH, Hess DL, Davis CE. Prospective study of coronary heart disease incidence in relation to fasting total homocysteine, related genetic polymorphisms, and B vitamins: the Atherosclerosis Risk in Communities (ARIC) study. *Circulation*. 1998;98:204–210.
43. Rimm EB, Willett WC, Hu FB, Sampson L, Colditz GA, Manson JE, Hennekens C, Stampfer MJ. Folate and vitamin B6 from diet and supplements in relation to risk of coronary heart disease among women. *JAMA*. 1998;279:359–364.
44. Voutilainen S, Rissanen TH, Virtanen J, Lakka TA, Salonen JT. Low serum folate and risk of coronary heart disease: association with an excess incidence of acute

- coronary events: the Kuopio Ischemic Heart Disease Risk Factor Study. *Circulation*. 2001;103:2674–2680.
45. Landewe RBM, van den Borne BEEM, Breedveld FC, Dijkman BAC. Methotrexate effects in patients with rheumatoid arthritis with cardiovascular comorbidity. *Lancet*. 2000;355:1616–1617.
 46. Loehrer FM, Angst CP, Haefeli WE, Jordan PP, Ritz R, Fowler B. Low whole-blood S-adenosylmethionine and correlation between 5-methyltetrahydrofolate and homocysteine in coronary artery disease. *Arterioscler Thromb Vasc Biol*. 1996;16:727–733.
 47. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJ, den Heijer M, Kluijtmans LA, van den Heuvel LP, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet*. 1995;10:111–113. Letter.
 48. Kang SS, Passen EL, Ruggie N, Wong PW, Sora H. Thermolabile defect of methylenetetrahydrofolate reductase in coronary artery disease. *Circulation*. 1993;88:1463–1469.
 49. Gallagher PM, Meleady R, Shields DC, Tan KS, McMaster D, Rozen R, Evans A, Graham IM, Whijtman LA, van den Heuvel LP, et al. Homocysteine and risk of premature coronary heart disease: evidence for a common gene mutation. *Circulation*. 1996;94:2154–2158.
 50. Morita H, Taguchi J, Kurihara H, Kitaoka M, Kaneda H, Kurihara Y, Maemura K, Shindo T, Minamino T, Ohno M, et al. Genetic polymorphism of 5,10-methylenetetrahydrofolate reductase (MTHFR) as a risk factor for coronary artery disease. *Circulation*. 1997;95:2032–2036.
 51. Kluijtmans LA, Kastelein JJ, Lindemans J, Boers GH, Heil SG, Brusckhe AV, Jukema JW, van den Heuvel LP, Trijbels FJ, Boerma GJ, et al. Thermolabile methylenetetrahydrofolate reductase in coronary artery disease. *Circulation*. 1997;96:2573–2577.
 52. Ma J, Stampfer MJ, Hennekens CH, Frosst P, Selhub J, Horsford J, Malinow MR, Willett WC, Rozen R. Methylenetetrahydrofolate reductase polymorphism, plasma folate, homocysteine, and risk of myocardial infarction in US physicians. *Circulation*. 1996;94:2410–2416.
 53. Schmitz C, Lindpaintner K, Verhoef P, Gaziano JM, Buring J. Genetic polymorphism of methylenetetrahydrofolate reductase and myocardial infarction: a case-control study. *Circulation*. 1996;94:1812–1814.
 54. Verhoef P, Rimm EB, Hunter DJ, Chen J, Willett WC, Kelsey K, Stampfer MJ. A common mutation in the methylenetetrahydrofolate reductase gene and risk of coronary heart disease: results among U.S. men. *J Am Coll Cardiol*. 1998;32:353–359.
 55. Anderson JL, King GJ, Thomson MJ, Todd M, Bair TL, Muhlestein JB, Carlquist JF. A mutation in the methylenetetrahydrofolate reductase gene is not associated with increased risk for coronary artery disease or myocardial infarction. *J Am Coll Cardiol*. 1997;30:1206–1211.
 56. Brattstrom L, Wilcken DE, Ohrvik J, Brudin L. Common methylenetetrahydrofolate reductase gene mutation leads to hyperhomocysteinemia but not to vascular disease: the result of a meta-analysis. *Circulation*. 1998;98:2520–2526.
 57. Jacques PF, Bostom AG, Williams RR, Ellison RC, Eckfeldt JH, Rosenberg IH, Selhub J, Rozen R. Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations. *Circulation*. 1996;93:7–9.
 58. Molloy AM, Daly S, Mills JL, Kirke PN, Whitehead AS, Ramsbottom D, Conley MR, Weir DG, Scott JM. Thermolabile variant of 5,10-methylenetetrahydrofolate reductase associated with low red-cell folates: implications for folate intake recommendations. *Lancet*. 1997;349:1591–1593.
 59. Brouwer IA, van Dusseldorp M, West CE, Meyboom S, Thomas CM, Duran M, het Hof KH, Eskes TK, Hautvast JG, Steegers-Theunissen RP. Dietary folate from vegetables and citrus fruit decreases plasma homocysteine concentrations in humans in a dietary controlled trial. *J Nutr*. 1999;129:1135–1139.
 60. Collaboration HLT. Lowering blood homocysteine with folic acid based supplements: meta-analysis of randomised trials. *BMJ*. 1998;316:894–898.
 61. Christen WG, Ajani UA, Glynn RJ, Hennekens CH. Blood levels of homocysteine and increased risks of cardiovascular disease: causal or casual? *Arch Intern Med*. 2000;160:422–434.
 62. Malinow MR, Bostom AG, Krauss RM. Homocyst(e)ine, diet, and cardiovascular diseases: a statement for healthcare professionals from the Nutrition Committee, American Heart Association. *Circulation*. 1999;99:178–182.
 63. Eikelboom JW, Lonn E, Genest J Jr, Hankey G, Yusuf S. Homocyst(e)ine and cardiovascular disease: a critical review of the epidemiologic evidence. *Ann Intern Med*. 1999;131:363–375.
 64. Welch GN, Loscalzo J. Homocysteine and atherothrombosis. *N Engl J Med*. 1998;338:1042–1050.
 65. Chambers JC, McGregor A, Jean-Marie J, Obeid OA, Kooneer JS. Demonstration of rapid onset vascular endothelial dysfunction after hyperhomocysteinemia: an effect reversible with vitamin C therapy. *Circulation*. 1999;99:1156–1160.
 66. Loehrer FM, Haefeli WE, Angst CP, Browne G, Frick G, Fowler B. Effect of methionine loading on 5-methyltetrahydrofolate, S-adenosylmethionine and S-adenosylhomocysteine in plasma of healthy humans. *Clin Sci (Colch)*. 1996;91:79–86.
 67. Griendling KK, Alexander RW. Oxidative stress and cardiovascular disease. *Circulation*. 1997;96:3264–3265.
 68. Kojda G, Harrison D. Interactions between NO and reactive oxygen species: pathophysiological importance in atherosclerosis, hypertension, diabetes and heart failure. *Cardiovasc Res*. 1999;43:562–571.
 69. Timimi FK, Ting HH, Haley EA, Roddy MA, Ganz P, Creager MA. Vitamin C improves endothelium-dependent vasodilation in patients with insulin-dependent diabetes mellitus. *J Am Coll Cardiol*. 1998;31:552–557.
 70. Ting HH, Timimi FK, Boles KS, Creager SJ, Ganz P, Creager MA. Vitamin C improves endothelium-dependent vasodilation in patients with non-insulin-dependent diabetes mellitus. *J Clin Invest*. 1996;97:22–28.
 71. Ting HH, Timimi FK, Haley EA, Roddy MA, Ganz P, Creager MA. Vitamin C improves endothelium-dependent vasodilation in forearm resistance vessels of humans with hypercholesterolaemia. *Circulation*. 1997;95:2617–2622.
 72. Solzbach U, Hornig B, Jeserich M, Just H. Vitamin C improves endothelial dysfunction of epicardial coronary arteries in hypertensive patients. *Circulation*. 1997;96:1513–1519.
 73. Kugiyama K, Motoyama T, Doi H, Kawano H, Hirai N, Soejima H, Miyao Y, Takazoe K, Moriyama Y, Mizuno Y, et al. Improvement of endothelial vasomotor dysfunction by treatment with alpha-tocopherol in patients with high remnant lipoproteins levels. *J Am Coll Cardiol*. 1999;33:1512–1518.
 74. Vita JA, Frei B, Holbrook M, Gokce N, Leaf C, Keane JF Jr. L-2-Oxothiazolidine-4-carboxylic acid reverses endothelial dysfunction in patients with coronary artery disease. *J Clin Invest*. 1998;101:1408–1414.
 75. Kugiyama K, Ohgushi M, Motoyama T, Hirashima O, Soejima H, Misumi K, Yoshimura M, Ogawa H, Sugiyama S, Yasue H. Intracoronary infusion of reduced glutathione improves endothelial vasomotor response to acetylcholine in human coronary circulation. *Circulation*. 1998;97:2299–2301.
 76. Stroes ESG, van Faassen EE, Yo M, Martasek P, Boer P, Govers R, Rabelink TJ. Folic acid reverts dysfunction of endothelial nitric oxide synthase. *Circ Res*. 2000;86:1129–1134.
 77. Durand P, Prost M, Blache D. Pro-thrombotic effects of a folic acid deficient diet in rat platelets and macrophages related to elevated homocysteine and decreased n-3 polyunsaturated fatty acids. *Atherosclerosis*. 1996;121:231–243.
 78. Henning SM, Swendseid ME, Ivandic BT, Liao F. Vitamins C, E and A and heme oxygenase in rats fed methyl/foolate-deficient diets. *Free Rad Biol Med*. 1997;23:936–942.
 79. Marletta MA. Nitric oxide synthase: aspects concerning structure and catalysis. *Cell*. 1994;78:927–930.
 80. Xia Y, Tsai AL, Berka V, Zweier JL. Superoxide generation from endothelial nitric-oxide synthase: a Ca²⁺/calmodulin-dependent and tetrahydrobiopterin regulatory process. *J Biol Chem*. 1998;273:25804–25808.
 81. Stroes E, Hijmering M, van Zandvoort M, Wever R, Rabelink TJ, van Faassen EE. Origin of superoxide production by endothelial nitric oxide synthase. *FEBS Lett*. 1998;438:161–164.
 82. Pritchard KA, Groszek L, Smalley DM, Sessa WC, Wu M, Villalon P, Wolin MS, Stemerman MB. Native low-density lipoprotein increases endothelial cell nitric oxide synthase generation of superoxide anion. *Circ Res*. 1995;77:510–518.
 83. Vasquez-Vivar J, Kalyanaram B, Martasek P, Hogg N, Masters BS, Karoui H, Tordo P, Pritchard KA. Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. *Proc Natl Acad Sci U S A*. 1998;95:9220–9225.
 84. Stroes E, Kastelein J, Cosentino F, Erkelens W, Wever R, Koomans H, Luscher T, Rabelink T. Tetrahydrobiopterin restores endothelial function in hypercholesterolemia. *J Clin Invest*. 1997;99:41–46.
 85. Tiefenbacher CP, Chilian WM, Mitchell M, DeFily DV. Restoration of endothelium-dependent vasodilation after reperfusion injury by tetrahydrobiopterin. *Circulation*. 1996;94:1423–1429.
 86. Mayer B, Pitters E, Pfeiffer S, Kukovetz WR, Schmidt K. A synthetic peptide corresponding to the putative dihydrofolate reductase domain of

- nitric oxide synthase inhibits uncoupled NADPH oxidation. *Nitric Oxide*. 1997;1:50–55.
87. Loscalzo J. Folate and nitrate-induced endothelial dysfunction: a simple treatment for a complex pathobiology. *Circulation*. 2001;104:1086–1088.
 88. Heller R, Unbehaun A, Schellenberg B, Mayer B, Werner-Felmayer G, Werner ER. L-Ascorbic acid potentiates endothelial nitric oxide synthesis via a chemical stabilization of tetrahydrobiopterin. *J Biol Chem*. 2001;276:40–47.
 89. Kaufman S. Some metabolic relationships between biopterin and folate: implications for the “methyl trap hypothesis.” *Neurochem Res*. 1991;16:1031–1036.
 90. Smith I, Hyland K, Kendall B. Clinical role of pteridine therapy in tetrahydrobiopterin deficiency. *J Inherit Metab Dis*. 1985;8(suppl 1):39–45.
 91. Oemar BS, Tschudi MR, Godoy N, Brovkovich V, Malinski T, Luscher TF. Reduced endothelial nitric oxide synthase expression and production in human atherosclerosis. *Circulation*. 1998;97:2494–2498.
 92. van Guldener C, Janssen MJ, Lambert J, ter Wee PM, Jakobs C, Donker AJM, Stehouwer CDA. No change in impaired endothelial function after long-term folic acid therapy of hyperhomocysteinaemia in haemodialysis patients. *Nephrol Dial Transplant*. 1998;13:106–112.
 93. van Guldener C, Janssen MJ, Lambert J, ter Wee PM, Donker AJ, Stehouwer CD. Folic acid treatment of hyperhomocysteinemia in peritoneal dialysis patients: no change in endothelial function after long-term therapy. *Perit Dial Int*. 1998;18:282–289.
 94. Thambyrajah J, Landray MJ, McGlynn FJ, Jones HJ, Wheeler DC, Townend JN. Does folic acid decrease plasma homocysteine and improve endothelial function in patient with predialysis renal failure? *Circulation*. 2000;102:871–875.
 95. Vogel RA, Corretti MC, Plotnick GD. Effect of a single high-fat meal on endothelial function in healthy subjects. *Am J Cardiol*. 1997;79:350–354.
 96. Chambers JC, Obeid OA, Kooner JS. Physiological increments in plasma homocysteine induce vascular endothelial dysfunction in normal human subjects. *Arterioscler Thromb Vasc Biol*. 1999;19:2922–2927.
 97. Lentz SR, Sobey CG, Piegors DJ, Bhopatkar MY, Faraci FM, Malinow MR, Heistad DD. Vascular dysfunction in monkeys with diet-induced hyperhomocyst(e)inemia. *J Clin Invest*. 1996;98:24–29.
 98. La Vecchia C, Decarli A, Pagano R. Vegetable consumption and risk of chronic disease. *Epidemiology*. 1998;9:208–210.
 99. Singh RB, Rastogi SS, Verma R, Laxmi B, Singh R, Ghosh S, Niaz MA. Randomised controlled trial of cardioprotective diet in patients with recent acute myocardial infarction: results of one year follow up. *BMJ*. 1992;304:1015–1019.
 100. Josphipura KJ, Ascherio A, Manson JE, Stampfer MJ, Rimm EB, Speizer FE, Hennekens CH, Spiegelman D, Willett WC. Fruit and vegetable intake in relation to risk of ischemic stroke. *JAMA*. 1999;282:1233–1239.
 101. Appel LJ, Moore TJ, Obarzanek E, Vollmer WM, Svetkey LP, Sacks FM, Bray GA, Vogt TM, Cutler JA, Windhauser MM, et al. A clinical trial of the effects of dietary patterns on blood pressure: DASH Collaborative Research Group. *N Engl J Med*. 1997;336:1117–1124.
 102. Lawrence JM, Petitti DB, Watkins M, Umekubo MA. Trends in serum folate after food fortification. *Lancet*. 1999;354:915–916.
 103. Wild J, Sutcliffe M, Schorah CJ, Levene MI. Prevention of neural-tube defects. *Lancet*. 1997;350:30–31. Letter.
 104. Jacques PF, Selhub J, Bostom AG, Wilson PW, Rosenberg IH. The effect of folic acid fortification on plasma folate and total homocysteine concentrations. *N Engl J Med*. 1999;340:1449–1454.
 105. Ek J, Magnus EM. Plasma and red blood cell folate during normal pregnancies. *Acta Obstet Gynecol Scand*. 1981;60:247–251.
 106. Shaw S, Jayatilake E, Herbert V, Colman N. Cleavage of folates during ethanol metabolism. *Biochem J*. 1989;257:277–280.
 107. Campbell NR. How safe are folic acid supplements? *Arch Intern Med*. 1996;156:1638–1644.
 108. Lindenbaum J, Rosenberg IH, Wilson PW, Stabler SP, Allen RH. Prevalence of cobalamin deficiency in the Framingham elderly population. *Am J Clin Nutr*. 1994;60:2–11.
 109. Chanarin I, Malkowska V, O’Hea AM, Rinsler MG, Price AB. Megaloblastic anaemia in a vegetarian Hindu community. *Lancet*. 1985;2:1168–1172.