Mechanisms and Potential Therapeutic Targets for Folic Acid in Cardiovascular Disease

An L. Moens¹,², Christiaan J. Vrints², Marc J. Claeys², Jean-Pierre Timmermans², Hunter C. Champion¹, David A. Kass¹

¹Johns Hopkins Medical Institutions, Div. of Cardiology, Baltimore, MD, USA
²University of Antwerp, Div. of Cardiology, Antwerp, Belgium

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Corresponding author:
An L. Moens, MD
University Hospital of Antwerp
Div. of Cardiology
Wilrijkstraat 10
2650 Antwerp-Edegem
Belgium
Tel:+32-3-821 4693
Fax: +32-3-830 2305
an.moens@uza.be
Abstract

Folic acid (FA) is a member of the B-vitamin family with cardiovascular roles in homocysteine regulation and endothelial nitric oxide (eNOS) activity. Its interaction with eNOS is thought due to enhancement of tetrahydrobiopterin (BH4) bioavailability, helping maintain eNOS in its coupled state to favour the generation of nitric oxide (NO) rather than oxygen free radicals. FA also plays a role in the prevention of several cardiac and non-cardiac malformations, has potent direct antioxidant and anti-thrombotic effects, and can interfere with the production of the endothelial-derived hyperpolarizing factor. These multiple mechanisms of action have led to studies regarding the therapeutic potential of FA in cardiovascular disease. To date, studies have demonstrated that FA ameliorates endothelial dysfunction and nitrate tolerance, and can improve pathologic features of atherosclerosis. These effects appear to be homocysteine-independent but rather related to its role in eNOS function. Given growing evidence that NOS uncoupling plays a major role in many cardiovascular disorders, the potential of exogenous FA as an inexpensive and safe oral therapy is intriguing and is stimulating ongoing investigations.
Introduction

The first therapeutic use of folic acid (FA) dates back to 1931, when Lucy Wills discovered that yeast extract was effective against tropical macrocytic anaemia (97) and that the critical factor involved was FA. The main role of FA was found to be its involvement in the production and maintenance of new cells (39) because it has an essential role in the integrity and function of DNA. DNA synthesis and cell proliferation require the transfer of carbon groups, a task principally fulfilled by folates. As a consequence, FA deficiency leads to inadequate nucleic acid synthesis and impairs cell division. During pregnancy, this can lead to neural tube defects, such as spina bifida, orofacial cleft and congenital heart defects (6; 20). The risk of these developmental abnormalities is significantly reduced by FA supplementation preceding conception and during pregnancy (77). FA deficiency is also associated with the development of neoplastic and pre-neoplastic conditions (17), neuropathy (56) and depression (4).

FA is a water-soluble B-vitamin that derives its name from the Latin word for leaf (folium) because it was first isolated from spinach leaves. Humans are unable to synthesize folate de novo and thus rely on dietary intake to derive sufficient levels of the vitamin. Rich sources include citrus fruits and juices, dark green leafy vegetables such as spinach, wheat and other whole grains, and liver. The synthetic form of FA is usually designated as folate. 5-Methyltetrahydrofolate (5-MTHF), the active metabolite has a structure very similar to that of tetrahydrobiopterin (BH₃), an essential cofactor of endothelial nitric oxide synthase (eNOS), with the exception of an extended tail attached to 5-MTHF. It is also the primary form of folate entering the human circulation from the intestinal cells. The conversion of FA to 5-MTHF has limited capacity, however, and if enough FA is consumed orally, unaltered FA appears in the circulation (42), is taken up by cells, and is then reduced by dihydrofolate reductase to tetrahydrofolate. Over the past 5 years, potential benefits of FA in the treatment
of cardiovascular pathology have been revealed and stimulated further clinical and experimental research. In this review, we discuss the potential mechanisms of action of FA, and its role in the pathogenesis and treatment of different cardiovascular pathologies.

**Mechanisms of action of Folic Acid**

FA is required for the re-methylation of homocysteine to methionine, which in turn reduces the concentration of homocysteine available to support oxidative stress(52). FA decreases plasma homocysteine levels of both normo-and hyperhomocysteinaemic subjects (61). However, FA also conveys protective effects in the absence of hyper-homocysteinemia, by multiple mechanisms (Fig1). In the presence of sufficient cofactor BH4, the enzyme eNOS principally synthesizes NO. Oxidative stress can oxidize BH4 to its inactive form BH2. Diminished bioavailability of BH4 leads to eNOS uncoupling with subsequent decreased NO formation and increased generation of reactive oxygen species (ROS) (19; 35; 45; 90). Folate can help to restore the bioavailability of BH4 by several mechanisms. MTHF can increase effectiveness of BH4 on eNOS uncoupling. In theory this can be explained by improved redox state or enhanced binding affinity of BH4 to eNOS (improved occupancy of eNOS by available BH4) or that MTHF facilitates the 1-electron oxidation of BH4 to the BH4 radical(78). In addition, folate can enhance the regeneration of BH4 from the inactive form BH2(41), and chemically stabilize BH4 (Fig 2). Hyndman et al.(38) also found that 5-MTHF is capable of binding the pterin site in eNOS and may directly interact with eNOS independent of BH4, though details of this interaction remain scant.

There is also evidence that FA exerts direct and indirect antioxidant effects, such as improvement of the cellular antioxidant defense system(24; 36; 92). FA deficiency in rats increases lipid peroxidation and decreases cellular antioxidant defenses(24; 36). In healthy
human volunteers, the beneficial effect of folates on postprandial endothelial dysfunction corresponded with decreased urinary excretion of malondialdehyde, a radical-damage end product(98). Administration of FA to smokers induced a significant reduction in plasma fibrinogen and D-dimer levels, markers of a prothrombotic state, and changes in plasma homocysteine did not correlate with these levels(54), suggesting an anti-thrombotic effect independent of homocysteine modulation. Other studies have shown antithrombotic effects of FA that are linked to a decline in homocysteine, which itself is pro-thrombotic(37; 47; 100) by its inhibition of a number of pathways, including thrombomodulin expression (50), antithrombin III-binding activity of heparan sulfate proteoglycan(34), and ecto-adenosine diphosphatase activity(34). Homocysteine also stimulates endothelial plasminogen activator inhibitor-1 expression(58), monocyte tissue factor expression, and potentiates platelet aggregation(23; 43).

There are several other mechanisms that may underlie the beneficial effect of FA, but these are presently less well-defined. First, 5-MTHF has been reported to restore the defective production of an unidentified endothelium-derived hyperpolarizing factor (EDHF) in diabetic rats(21). Second, we recently demonstrated improved myocardial high-energy phosphate metabolism from high dose FA pre-treatment in rats subjected to regional coronary occlusion (60). As a result, less superoxide was generated, eNOS was kept in his coupled state, myocardial function was preserved and reperfusion-injury was prevented.

**Folic acid and Congenital Heart Disease**

Congenital heart defects occur in approximately 3-8 of every 1000 births. In the United States alone the number of deaths attributed to congenital heart defects is estimated to be ~ 6000 annually(9). The etiology of non-syndromic congenital heart defects is complex, involving genetic, epigenetic, and environmental risk factors. However, one of the most
promising clues about the prevention of conotruncal defects (truncus arteriosus, transposition of great arteries and tetralogy of Fallot) is that women who use FA-containing vitamins in early pregnancy have reduced risks of delivering offspring with conotruncal defects (10; 75). FA is well-known for its beneficial effects on neural tube closure which depend on NOS activity (64). Indeed, blocking NOS activity by inhibiting BH4 or calcium-calmodulin binding to NOS, results in ablated closure of the neural tube. Therefore, it is recommended that all women capable of becoming pregnant take 400µg/d of FA in addition to a healthy diet (6). Women taking medications that interfere with folate metabolism (e.g. antiepileptic drugs such as carbamazepine and valproate) are advised to take higher doses of FA (1-5 mg/d) during pre-conception and throughout pregnancy. Furthermore, a recent study showed that offspring of pregnant rats on a protein-restricted diet during pregnancy had higher systolic blood pressure, impaired acetylcholine-induced vasodilation and reduced levels of eNOS mRNA in their thoracic aorta. Maternal folate supplementation during pregnancy in this model normalized blood pressure while having a modest effect on vascular function (84). These data provide a good example of how vitamin-supplementation can ameliorate the adverse effects of micronutrient imbalance during pregnancy.

**FA and Homocysteine**

The best-known beneficial action of FA is its homocysteine-lowering effect. Homocysteine is a sulfur-containing amino acid generated during the catabolism of methionine. Homocysteine is metabolised by two pathways, either re-methylation in the case of insufficient methionine, or trans-sulfuration, in case of excess methionine. In the re-methylation pathway, homocysteine is re-converted to methionine by methionine synthase, requiring vitamin B12 as a cofactor and FA as a methyl donor (26). Utilizing the trans-sulfuration pathway, homocysteine is catabolized by cystathionine beta-synthase, with
vitamin B6 as cofactor, to cystathionine and subsequently to cysteine, which is excreted in the urine or incorporated into glutathione. Hyper-homocysteinemia is therefore associated with low concentrations of methionine, FA, or vitamin B12.

As a consequence of the involvement of FA in the homocysteine-pathway, oral administration (0.5-5 mg/d) results in a 25-30% reduction in the fasting homocysteine concentration(1; 25; 33; 68; 87). No difference in homocysteine-lowering effects of supplementary FA has been found with daily intake ranging 0.4-5 mg(1; 87), except in patients with chronic renal failure who require a higher dose. Supplementation with vitamin B12 (0.02-1 mg daily) produces an additional 7% reduction in homocysteine levels and simultaneously eliminates the theoretical risk of precipitating subacute combined degeneration of the spinal cord. Vitamin B6 supplementation has no additional effect on fasting homocysteine levels but does significantly lower postmethionine load homocysteine and cystathionine concentrations(68). Hyper-homocysteinaemia, found in up to 40% of individuals with cerebrovascular, coronary or peripheral vascular diseases(18), can be considered an independent cardiovascular risk factor (11; 76). However, the strength of association of homocysteine with risk of cardiovascular disease may be weaker than previously believed. An up-dated meta-analysis of several large-scaled observational studies found that a decline in blood homocysteine of 25% (~3µmol/L) was associated with ~11% lower risk of CHD and 19% lower risk of stroke(2). However, most large prospective studies were underpowered for this level of risk reduction, and even larger studies are needed to prove or disprove risk modulation(3; 53).

**Folic acid and endothelial dysfunction**

Endothelial dysfunction is a major marker of cardiovascular risk(94) (69; 79) and is characterized by reduced production/availability of nitric oxide (NO) and/or an imbalance
between endothelium-derived relaxation (prostacyclin and endothelium-derived hyperpolarizing factor) and contracting (endothelin and angiotensin) factors and oxidants. A number of studies have assigned a pivotal role to oxygen-derived free radicals in accelerating NO degradation. These oxygen-derived free radicals, in particular superoxide anion, easily react with NO, decreasing its half-life.

Prevention or amelioration of coronary vascular endothelial dysfunction is an attractive goal for therapeutic interventions aimed at reducing symptoms or clinical events. In the past few years, studies have reported improved endothelial function after FA supplementation in patients with hyperhomocysteinemia (8; 14; 15; 83; 88; 99), and normo-homocysteinemic patients with familial hypercholesterolaemia (92; 93), diabetes (89), stable coronary artery disease (22; 59), and in smokers (55; 66). Chronic FA treatment for six weeks in subjects with an acute myocardial infarction resulted in improved endothelial function (61). Nitroglycerin and other nitrates are mainstay therapies for coronary artery disease but can be associated with oxygen-free radical induced nitrate tolerance and subsequent endothelial dysfunction (81). The development of tolerance during continuous therapy is a major factor limiting the efficacy of these drugs. Supplemental FA may be instrumented in preventing such tolerance and endothelial dysfunction (32).

**Folic acid and atherosclerosis**

FA may have beneficial effects on atherosclerosis. Carnicer et al (13). demonstrated that in apoE-deficient mice that FA led to a decline in atherosclerotic lesions associated with increased apolipoprotein AI-, AIV- and B-levels and decreased oxidative stress. This was independent of plasma homocysteine and cholesterol levels. Clinical studies have employed more mixed cocktails of B-vitamins. For example, 1-year daily B-vitamin supplementation
(2.5 mg FA, 25 mg vitamin B6 and 0.5 mg vitamin B12) reduced carotid intima-media thickness as compared to placebo administration (82). In one study, long term FA treatment (∼ 10 years) at a much higher dose than currently used (40-80 mg/day) was found to lower the incidence of myocardial infarction, angina pectoris and requirement for nitroglycerin in patients with coronary artery disease(67). This study was not placebo controlled, however, and remains an isolated observation.

Another manifestation of vascular disease where FA may be helpful is re-stenosis following balloon angioplasty. Schnyder et al.(72) examined 205 patients with stable coronary artery disease treated with a combination of FA (1mg), vitamin B12 (400 µg), and pyridoxine (10 mg), and found reduced restenosis rates (19.6% versus 37.6%). The extent of restenosis was also less severe. This group also observed that patients with plasma homocysteine levels below 9 µmol/L have a 49 percent lower rate of coronary restenosis than those with higher levels(71). However, other studies did not confirm these results(49), potentially in part because of the greater use of vascular stents in this latter study. In the Swiss Heart Study, reduction in restenosis with FA was most observed in vessels treated with angioplasty only (10.3% vs 41.9%, $P < .001$), whereas the benefit in stented lesions did not reach statistical significance (20.6% vs 29.9%, $P = 0.32$). Differences in the pathophysiology entailed with stent placement could underlie the difference. As thrombotic complications from sirolimus- and paclitaxel-eluting coronary stents have recently come into focus(77), the potential use of FA may again be revisited.

**Folic acid and hemodynamic parameters**

FA has been examined for potential effects on arterial blood pressure. Tawakol et al.(80) found that high doses of FA (30 mg) acutely reduced systolic, diastolic and mean
arterial pressure. In regions of normal coronary flow, FA did not alter myocardial blood flow or adenosine reserve, whereas in abnormal zones FA acid significantly improved flow reserve (49% increase with adenosine), despite the decline in pressure. Additionally, FA increased vasodilator reserve by 83% in abnormal segments, but had no effect in normal segments. In another study, low dose FA (5m/day) administered for 3 weeks lowered brachial pulse pressure, without altering mean arterial pressure(96), coupled to improvement in regional artery compliance.

Folic acid and cardiovascular mortality

Low serum folate levels are associated with a high risk of fatal coronary artery disease, especially when folate levels fall below 6.8 nmol/l( = 3 ng/ml)(63). This inverse relation between folate status and atherosclerotic vascular diseases has also been demonstrated in the Nutrition Examination Survey(28; 31; 51), the Kuopio Ischemic Heart Disease Risk Factor Study(95) and the Framingham Heart Study(74), although it has not been confirmed by others (the Physicians’ Health Study(16) and atherosclerosis Risk in Communities Study(27)). Antifolate therapy with methotrexate has been suggested to promote atherosclerosis(48). Beyond dietary reductions, a common mutation of 5,10-MTHF reductase (MTHFR) caused increased thermolability and reduced activity of the enzyme catalyzing reduction of 5,10-methylenetetrahydrofolate to 5-MTHF. This mutation has been reported as a risk factor for cardiovascular disease(29; 30; 40; 46; 62).

Pharmacologic considerations of FA

In general, FA supplementation is considered safe(12) and there is no evidence that high natural folate intake poses a toxicity risk(65). No adverse effects have been reported
when high doses of FA (40-80 mg/d) are administered for as long as 10 years(67). Only one study reported on the use of FA (300mg/kg, once a week for 4wks) in rats as a model for interstitial nephritis(86). However, converting this dose from a rat to a human of 75kg results in a dose of 22.5 gram. The main safety concern lies in the fact that folate can mask the diagnosis of pernicious anaemia, because high FA levels correct the anaemia but allow the neuropathy to progress undiagnosed to an irreversible degeneration of the spinal cord (73). Therefore, vitamin B12 levels should always be measured before the start of supplementation with FA. Another concern that needs special attention is the role of FA in carcinogenesis.

In established neoplasms, the inhibitory and promoting effect of folate deficiency and supplementation, respectively, has been well-described and has been the basis for cancer chemotherapy with several antifolate agents (eg,methotrexate) and 5-fluorouracil. In neoplastic cells, in which DNA replication and cell division occur at an accelerated rate, interruption of folate metabolism causes ineffective DNA synthesis, resulting in inhibition of tumor growth (32, 33). In contrast, the role of FA, and in particular of folate fortification, on de novo carcinogenesis in normal tissue has been the subject of many contradictory reports over the past decade(44). Very recently, Bayston et al.(7) reported in the Lancet that there is no ground to concern to avoid fortification with FA and that FA supplementation will not enhance the risk on colorectal carcinomas.

The synthetic form of FA(‘folate’) is used in supplements and is added to food because of its high stability and bioavailability. The metabolic active form of FA, (l)-5-methyltetrahydrofolate (5-MTHF) is also readily available. Unlike FA, 5-MTHF has to be converted to tetrahydrofolate (THF) via the vitamin B12-dependent enzyme methionine synthase. In case of vitamin B12 deficiency, 5-MTHF is not converted to THF, and thus is not able to improve megaloblastic anaemia, even when given at high doses. Furthermore, 5-MTHF does not require reduction by dihydrofolate reductase to be incorporated into the
active cellular folate pool(101). However, low-dose 5-MTHF is equally effective as FA in reducing homocysteine concentrations in healthy persons(91) and restoration of endothelial function can also be performed by an infusion of 5-MTHF(92).

Uraemic patients usually have elevated levels of homocysteine, and are relatively resistant to FA therapy. The reason for this phenomenon is unknown but may be due to impaired intestinal absorption and/or impaired metabolic transformation of FA to an active form(57). Folinic acid (5-formyltetrahydrofolate) supplementation to this population may be more efficient in reducing the high homocysteine level in uraemia(5; 57; 85). Folinic acid can be given intravenously, where it normally is readily converted (via 5,10 methylenetetrahydrofolate and 5,10 methylenetetrahydrofolate) to 5-MTHF (70). This form of FA is best known for counteracting the therapeutic and toxic effects of FA antagonists, such as methotrexate, in the treatment of tumors, rheumatoid arthritis and psoriasis.

**Conclusion**

Coronary artery disease has become the leading cause of death in Western countries. Various studies have demonstrated an association between low-serum folate levels and risk of fatal coronary artery disease. In the light of this observation, FA appears not only important for risk stratification, but also opens new therapeutic possibilities in the treatment of cardiovascular diseases. Apart from various promising results on eNOS-dependent superoxide generation in animal studies and its well-known homocysteine-lowering effect, FA can benefit on endothelial dysfunction, and recent work suggests a potential to preserve myocardial function and prevent tissue damage.

Some of these effects may require high doses of FA, much higher than those tested to date, and clearly much higher than those obtainable through the diet. Precisely when and why
higher doses might be required for some therapeutic targets remains unclear, and somewhat controversial. Clearly, additional studies are needed to further clarify the potential role of FA, not only for risk stratification but also for cardiovascular disease treatment and/or prevention.


mediated through reduced concentrations of free plasma homocysteine. 


15. **Chao CL, Chien KL and 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_ 84: 1359-61, A8, 1999.


57. **Massy ZA.** Reversal of hyperhomocyst(e)inaemia in chronic renal failure—is folic or folinic acid the answer? *Nephrol Dial Transplant* 14: 2810-2812, 1999.

58. **Midorikawa S, Sanada H, Hashimoto S and Watanabe T.** Enhancement by homocysteine of plasminogen activator inhibitor-1 gene expression and


88. Usui M, Matsuoka H, Miyazaki H, Ueda S, Okuda S and Imaizumi T.


89. van Etten RW, de Koning EJ, Verhaar MC, Gaillard CA and Rabelink TJ.

   Impaired NO-dependent vasodilation in patients with Type II (non-insulin-dependent) diabetes mellitus is restored by acute administration of folate. *Diabetologia* 45: 1004-1010, 2002.


93. Verhaar MC, Wever RM, Kastelein JJ, van Loon D, Milstien S, Koomans HA and Rabelink TJ. Effects of oral folic acid supplementation on endothelial


**Figure 1:** Different mechanisms of action and targets of folic acid in cardiovascular diseases

**Figure 2:** Interaction of folic acid with eNOS. 5-MTHF is capable of directly interacting with eNOS (i). Folic acid also restores the bioavailability of BH4 by ameliorating the binding affinity of BH4 to eNOS (ii), by chemically stabilizing BH4 (iii), and by enhancing the regeneration of BH4 from the inactive form BH2 (iv). Oxidative stress-induced BH4 depletion leads to an imbalance between NO production and the generation of free radicals.
Nitrate Tolerance
Restenosis
Cardiovascular Mortality
Endothelial Dysfunction
- diabetes
- hypercholesterolaemia
- Hyperhomocystaenemia
- Hypertension
- Stable Coronary Artery Disease
- Smoking
Nitrate Tolerance
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Restenosis
Cardiovascular Mortality
Endothelial Dysfunction
- diabetes
- hypercholesterolaemia
- Hyperhomocystaenemia
- Hypertension
- Stable Coronary Artery Disease
- Smoking
Ischemia- and Reperfusion-Injury
- Contractility
- Endothelial dysfunction
- Cell Death
- Lethal Arrhythmias
L-arginine \[\rightarrow\] L-citrulline

\[\text{BH}_4\] \[\rightarrow\] eNOS

\[\text{BH}_2\]

5-MTHF

(i)

(ii)

(iii)

(iv)

\[\text{NO} \downarrow + \text{superoxide anion} \uparrow\]