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Folate, homocysteine, endothelial function and cardiovascular disease

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Abstract

Evidence reported from numerous clinical studies over the past decade has revealed an association between increased plasma total homocysteine (tHcy) concentrations and cardiovascular disease (CVD). In addition, epidemiological studies have identified an inverse association between blood folate concentrations, folate intake and cardiovascular endpoints, that are independent of homocysteine. Folic acid supplementation can lower plasma tHcy concentrations safely and inexpensively. Furthermore, folic acid can reverse endothelial dysfunction observed in patients with CVD. This reversal in endothelial dysfunction with folic acid has been shown to be independent of plasma tHcy lowering, suggesting that folate has pleiotropic effects on the vasculature other than homocysteine lowering. *In vitro* evidence demonstrates that 5-methyltetrahydrofolate (5MeTHF) the main circulating metabolite of folate, can increase nitric oxide production and can directly scavenge superoxide radicals. The potential beneficial role of folic acid supplements on vascular disease are currently being tested in randomized placebo controlled studies.

Keywords: Folate; Homocysteine; Endothelial function; Nitric oxide (NO); Cardiovascular disease (CVD); Flow mediated dilatation (FMD)

1. Introduction

Since the initial discovery by Lucy Wills in 1931 that yeast extracts could prevent macrocytic anemia in pregnant women in India and the subsequent identification and synthesis of folic acid as the critical factor in the 1940's, folate has been implicated in numerous disease states [1]. Initial interest in the health benefits of folic acid was confined to the prevention of neural tube defects [2]. However in the past decade folate deficiency or disturbances in folate metabolism have been associated with neurological degeneration [3], cancer [4] and CVD, which is the subject of this review.

Folate, a B-vitamin is a generic term used for compounds that have a similar structure and functions to those of folic acid. Folic acid is composed of three distinct parts. A pterin core ring structure, which is conjugated to para-aminobenzoic acid via a methylene bridge to form pteroic acid. The carboxy group of the para-aminobenzoic acid is bound via a peptide to alpha amino groups of glutamate to form folic acid. All three components must be present for biological activity. Mammals lack the necessary enzymes to synthesize folate *de novo* and therefore depend entirely on preformed folates in the diet. Folate is found in a wide variety of foods. Good sources of folate include, green leafy vegetables, mushrooms, legumes and liver. Raw foods tend to be higher in folate than cooked foods due to the hydrolysis of folates during heating. Food folates exist primarily as polyglutamates and their bioavailability is ~50% of that of the synthetic form of folic acid, which is a monoglutamate.

Folates facilitate the transfer of 1-carbon units from various donor biomolecules to numerous biosynthetic reactions such as purine and pyrimidine synthesis, methionine regeneration and amino acid metabolism. Therefore, an adequate intake of folate is vital for cell division and homeostasis, by producing DNA and regulating metabolism [5].

2. Folate and homocysteine metabolism

Folate metabolism is closely linked to that of homocysteine (Fig. 1). Homocysteine is a thiol-containing amino acid derived from the essential amino acid methionine. Methionine is converted to S-adenosylmethionine via the enzyme methionine adenosyltransferase, which is the only

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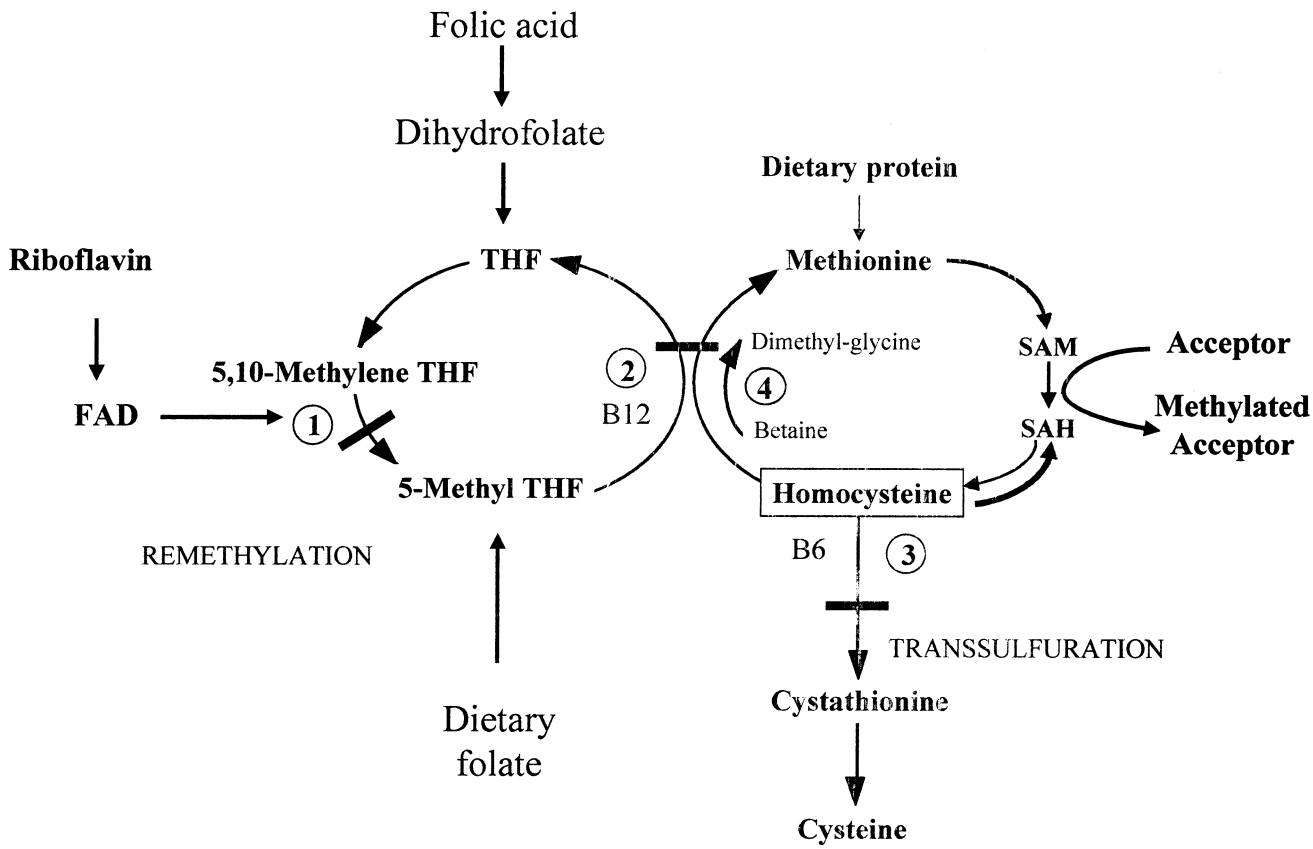

Fig. 1. Metabolism of homocysteine and folate.

methyl-donating pathway in humans. This pathway is essential in providing methyl groups to activate many biomolecules such as DNA, creatine, proteins, phospholipids and neurotransmitters. S-adenosylmethionine is demethylated to S-adenosylhomocysteine as a product of these methyl-transferase reactions. S-adenosylhomocysteine is hydrolyzed to homocysteine in a reversible reaction, in which S-adenosylhomocysteine formation is favored. Once formed, homocysteine can be metabolized via two metabolic pathways. During periods of excess methionine intake or when requirements for methyl groups are low, homocysteine enters the transulphuration pathway, where it is converted to cysteine via a two-step process involving the vitamin B_6-dependent enzyme cystathionine β-synthase (CβS) and cystathionase. Ultimately cysteine is converted to sulfate and excreted into the urine. During periods of low methionine intake and/or increased requirements of methyl groups, homocysteine is remethylated to methionine. Remethylation involves the donation of a methyl group from 5-methyltetrahydrofolate (5MeTHF) in a vitamin B_{12}-dependent reaction catalyzed by the enzyme methionine synthase (MS) (Fig. 1). 5MeTHF is formed by the conversion of 5,10-methylenetetrahydrofolate, via the enzyme 5-methyltetrahydrofolate reductase (MTHFR). Homocysteine remethylation appears to be the primary modulator of fasting and elevated plasma homocysteine concentrations [6, 7].

3. Causes of hyperhomocysteinaemia

Elevations of plasma total homocysteine (tHcy) may occur due to genetic defects and or an inadequate status of

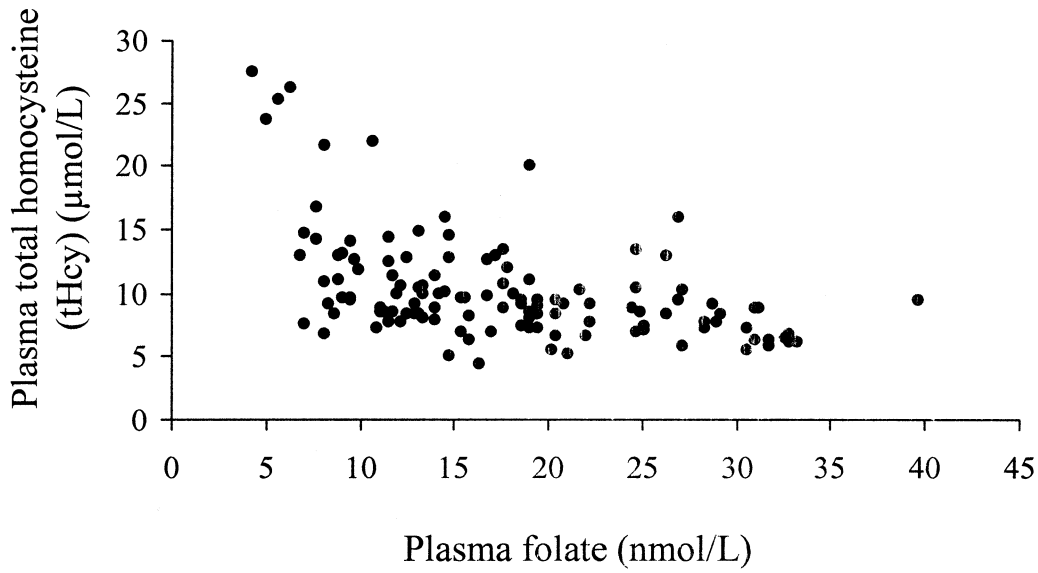

Fig. 2. Association between plasma folate and plasma total homocysteine (tHcy) in a cohort of 126 healthy men and women.

the vitamin cofactors needed for these reactions. Rare genetic disorders, which lead to the loss of enzyme activity in the pathways controlling homocysteine metabolism, result in the disorder homocystinuria, which is characterized by gross elevations of homocysteine in plasma and its excretion into the urine. Homocystinuria can be caused by three different enzymatic defects (Fig. 1). The most common is due to CβS deficiency which, has a world wide incidence of 1:300,000 live births [8] and individuals with this enzyme deficiency can have plasma tHcy concentrations as high as 300μmol/L [9]. Deficiencies in the enzymes MS and MTHFR also lead to gross elevations of homocysteine but not as high as those seen in CβS deficiency. All three enzymatic deficiencies lead to elevations of homocysteine and are associated with arteriosclerosis [10].

It was originally thought that heterozygosity for the CβS enzyme would result in mild elevations of homocysteine and lead to an increased risk for vascular disease. However, in a large study of parents and grandparents of subjects with homocystinuria, Mudd and colleagues failed to show an increased incidence of vascular events [11].

Although homozygotes and heterozygotes for severe MTHFR deficiency are very rare, a common mutation in the MTHFR gene (677C→T) has been identified, with an incidence ~12% in caucasians. Individuals homozygous (TT) for this mutation have about 50% of normal enzyme activity [12]. This reduced activity is associated with a reduced ability to catalyze the reduction of 5,10-methylenetetrahydrofolate to 5MeTHF and predisposes homozygous individuals to hyperhomocysteinaemia. It was thought that individuals with the TT genotype were more likely to have vascular disease because in combination with a low folate status they had higher concentrations of plasma tHcy than those with the CT and CC genotype. A meta-analysis including ~6000 patients with CVD has not confirmed this finding [13]. This view supported the idea that the TT genotype did not confer any risk and that tHcy may not be a risk factor. However, this meta-analysis did not have the statistical power to detect any risk related to the increase in plasma tHcy of 2.6μmol/L that was seen in subjects with the TT compared to the CC genotype. A more recent meta-analysis of 11,162 patients with CHD and 12758 controls has shown that individuals with the MTHFR TT genotype had a significantly greater risk of CHD [14].

However, there is conflicting evidence suggesting that the MTHFR TT genotype may be cardioprotective. A study assessing carotid artery geometry (internal diameter and intima-media thickness) in asymptomatic adults, showed that the TT genotype was associated with a better carotid artery geometry independent of hyperhomocysteinaemia [15]. Furthermore, recent data show that vascular function is augmented in young males with the TT genotype [16]. These data therefore suggest that the MTHFR TT allele may even protect against CVD. Further studies in subjects without overt vascular disease are needed to determine whether the MTHFR genotype is associated with CVD.

In population studies, plasma tHcy is inversely associated with plasma folate status (Fig. 2) even within the range that is considered to be normal. Folate intake is also negatively correlated with plasma homocysteine, reaching a plateau at a daily intake of ~400 μg/day. However, the associations between vitamin B_{12} and B_6 status with homocysteine are weak. Folate can lower plasma homocysteine levels safely and effectively. B_{12} and B_6 have only modest homocysteine lowering effects. There is evidence that riboflavin (vitamin B_2), as the precursor to flavin adenine dinucleotide (FAD), the cofactor for MTHFR, is also a determinant of homocysteine concentrations [17]. Plasma tHcy concentrations begin to increase at levels of folate, pyridoxal phosphate (vitamin B_6) and cobalamin (vitamin

B_{12}), that are regarded as being within the lower normal reference range. This may indicate a need to revise the references ranges for these vitamins.

4. Folate, homocysteine and cardiovascular disease: Epidemiological evidence

The interest in the effects of folic acid on vascular function and potentially the reduction in vascular and thrombotic events has stemmed from observations that an elevated plasma tHcy concentration, a risk factor for CVD, can be lowered by folate supplements or by increased dietary intake of folate. In addition, the increased levels of tHcy observed in numerous studies relating hyperhomocysteinaemia to cardiovascular risk are associated with reduced amounts of folate in plasma and red blood cells.

The importance of high circulating concentrations of plasma homocysteine was first recognized by McCully in 1969, who described similar vascular pathology in children with homocystinuria due to different inborn errors of homocysteine metabolism. The different metabolic profiles observed in these patients all resulted in similar vascular pathology suggesting that homocysteine was the causative agent [10]. These observations in individuals with homocystinuria, together with animal and laboratory studies and subsequent clinical and epidemiological studies has led to the proposal that homocysteine is a risk factor for CVD [18].

Many case-control studies have shown with consistency that mild elevations of plasma homocysteine are associated with an increased risk of CVD [19, 20]. However, the results of prospective studies have varied, with some showing associations while others have not. Prospective studies have some advantages over cross sectional studies, in that they can distinguish between cause and effect. However, they also have limitations in that during the follow-up period the association may be attenuated. This was demonstrated in some prospective studies with longer follow-up periods where no association was found [21, 22], questioning the role of homocysteine as a risk factor for CVD. However, Clarke and colleagues [23] have recently argued that failure to correct for within-person variation in plasma tHcy concentrations during the course of the study (regression-dilution bias), may underestimate any true association between plasma tHcy concentrations and CVD risk, by about one-fifth after two years and one-half after 10 years.

5. Low folate as a risk factor for cardiovascular disease?

Folate status is the most important determinant of tHcy in the general population, therefore, the association between folate status and CVD would support the concept that tHcy is a risk factor. However, an alternative hypothesis is that a low blood folate concentration is a risk factor for CVD independent of plasma homocysteine concentrations. This would indicate that a deficiency in folate rather than hyperhomocysteinaemia, may be the actual risk factor. Evidence for a primary role of low folate levels as a cause of CVD risk comes from numerous case-control and prospective epidemiological studies (Table 1 and 2). Interestingly it has been shown that a higher intake of folate in the diet is associated with a reduced risk of all cause mortality [24]. In addition, observational studies have shown that high intakes of fruit and vegetables (major sources of folates) are associated with lower rates of coronary heart disease (CHD) and stroke [25]. The association between a diet high in folate and a low CVD risk may be confounded by other dietary factors also found in these foods which may be cardioprotective.

Various studies, mostly case control in design, have demonstrated lower plasma and red cell folate concentrations in subjects with CVD compared to healthy matched control subjects. Others studies have also shown that dietary intake of folate is inversely associated with mortality from all causes of CVD. The case-control study of Verhoef and colleagues [26] of 130 patients with myocardial infarction (MI) and 118 controls, demonstrated that dietary intake of folate and plasma folate levels were significantly lower in patients than in controls and was inversely associated with the risk of MI, independently of other potential risk factors. The odds ratio (OR) of MI comparing the lowest with the highest quintile of dietary folate intake ($<$282 vs. $>$467 μg/day) was 0.3 (CI, 0.11 to 0.81, $P < 0.03$). Furthermore, the OR using the lowest and highest quintiles for dietary folate intake, including that of food and supplements ($<$310 vs. $>$682 μg/day) was 0.38 (CI, 0.15 to 0.95, $P < 0.004$). A cross-sectional study from the Framingham Heart Study [27] also demonstrated that both plasma concentrations of folate and folate intake were inversely associated with extracranial carotid stenosis after adjustment for other known risk factors. The OR for stenosis of $\geq$25% was 1.9 (CI, 1.3 to 2.7) in the lowest folate quartile ($<$2.51 μg/L). After adjustment for tHcy concentration this relationship was weakened, however, the prevalence of stenosis of $\geq$25% in subjects in the lowest plasma folate quartile remained elevated with an OR of 1.5 (CI, 1.0 to 2.3).

The COMAC European multi-center case control study [28] of 750 patients with vascular disease and 800 control subjects, demonstrated a lower red cell folate in cases compared to controls ($P < 0.005$), this association was however found only in men. In addition levels of red cell folate below the 10th centile ($<$513nmol/L) was associated with an increased risk of vascular disease ($P < 0.045$). Silberberg and colleagues [29] demonstrated significantly lower plasma folate but not red cell folate in patients with coronary artery disease (CAD) compared to controls. Quéré et al. [30], demonstrated a strong concentration-dependent association between levels of methyl folate in red cells, with the lowest folate quartile ($<$141 μg/L) being associated with a 7 fold higher risk of venous thromboembolism (CI, 3.19 to 15.75)

Table 1
Case-control studies in the association between blood folate, folate intake and cardiovascular risk

Reference & Year	Patient group	Numbers Cases/controls	Sex	Age (yrs) Cases/controls	Folate marker	Folate concentration mean (SD)		Units	P
						Cases	Controls		
Pancharuniti (1994) [31]	CAD	101/108	M	45.3/43.7*	plasma	10.0 (1.9)	12.2 (2.1)	nmol/L	<0.05
Dalery (1995) [32]	CAD	123/380	M	48.6/38.6*	plasma	4.1 (2.9)	3.8 (2.3)	ng/mL	NS
		27/204	F	50.5/36.3*	plasma	4.4 (3.3)	4.3 (2.9)		NS
Robinson (1995) [33]	CAD	304/231	M+F	62/51*	plasma	22.6 (12.4)	17.9(9.4)	nmol/L	<0.001
Loehrer (1996) [34]	CAD	70/55	M+F	52/44*	plasma 5MeTHF	19.3 (9.0)	24.2(15.0)	nmol/L	<0.05
Verhoef (1996) [26]	MI	130/118	M+F	57.7/57.7	plasma	8.76 (3.3)	9.93(4.93)	nmol/L	0.03
					dietary	340.9(107.0)	372.0(105.3)	μg/day	0.01
Graham (1997) (ECAP) [19]	VD	750/800	M+F	47.2/43.9*	red cell	811.7	840.5	nmol/L	0.1
Schwartz (1997) [35]	MI	79/386	F	N/A	plasma	12.4(13.4)	16.1(12.2)	nmol/L	0.018
Verhoef (1997) [36]	CAD	131/101	M+F	52.5/49.9	red cell	853(271)	766(263)	nmol/L	0.02
Robinson (1998) [28]	VD	514/549	M	47.2/43.9*	red cell	819	876	nmol/L	0.005
		171/226	F		red cell	727	726	nmol/L	NS
Christensen (1999) [37]	MI	107/103	M+F	62.7/62.1	whole blood	180(65)	216(112)	nmol/L	<0.01
Bunout (2000) [38]	PVD	32/24	M+F	69.6/71.8	serum	4.48(2.42)	7.14(4.04)	ng/mL	<0.02
	CAD	52/42	M+F	59.5/59.8	serum	5.15(1.9)	6.59(2.49)	ng/mL	<0.01
Silberberg (2001) [29]	CAD	255/114	M+F	N/A	plasma	18.0(7)	20.5(9)	nmol/L	<0.001
					red cell	997(340)	989(321)	nmol/L	NS
Quéré (2002) [30]	VT	243/243	M+F	54.4/55.1	plasma	6.1(2.8)	7.1(2.8)	μg/L	<0.001
					total red cell	266.0(82.4)	317.8(141.5)	μg/L	<0.001
					methyl red cell	176.8(81.8)	235.4(138.6)	μg/L	<0.001

CAD = coronary artery disease, MI = myocardial infarction, VD = vascular disease, PVD = peripheral vascular disease, VT = venous thrombosis, M = male, F = female, ECAP = Euro Concerted Action project, 5MeTHF = 5-Methyltetrahydrofolate, * age between groups significantly different, N/A = data not available. NS = not significant between cases and controls.

than the highest quartile (>249 μg/L). The finding that low folate status is associated with an increased risk of CVD has been confirmed in other cross-sectional studies. However, some cross-sectional studies have failed to demonstrate such an association (Table 1).

Inverse associations between folate intake and blood folate concentrations have also been found in a retrospective and several prospective studies (Table 2). A sub-study from the Nutrition Canada Survey [39] found that low serum folate levels were associated with an increased 15 year CHD mortality among both men and women, with a relative risk (RR) of 1.69 (CI, 1.1 to 2.61) when comparing the lowest serum folate quartile with the highest (<6.8 vs. ≥13.6nmol/L). Furthermore, increased risks were not confined to those with low serum levels, but were observed for individuals with serum levels within the normal range. However, the RR's were not adjusted for plasma tHcy levels, as the latter were not measured in this study. In the Nurses Health Study [40], 80,802 women were followed for a 14-year period. After controlling for other risk factors, the RR of CHD was 0.69 (CI, 0.55 to 0.87) when comparing those women in the highest folate intake quintile (696 μg/day) compared to those in the lowest (158 μg/day). In addition, the authors found that the strongest benefit of a folate-rich diet was in women who consumed alcohol. In the First National Health and Nutrition Examination Survey Epidemiological Follow-up study (NHANES I) the relative risk of CHD among 35 to 55 year olds was 2.4 (CI, 1.1 to 5.2) for subjects in the lowest serum folate quintile compared to those in the highest (≤9.9 vs. ≥21.8 nmol/L) [41]. The prospective study of Chasen-Taber and colleagues [42], found that men with the lowest 20% of plasma folate levels had a RR for MI of 1.4 (CI, 0.9 to 2.3) compared to those in the top 80%. Although, this association was not significant, when plasma tHcy was added to the conditional logistic regression analysis model, the RR was similar (1.3, CI 0.8 to 2.1). These results suggest that the increased risk for MI in subjects with a low folate status may be independent of plasma tHcy. In contrast to the

Table 2
Prospective studies on the association between blood folate, folate intake and cardiovascular risk

Reference & Year	n	Follow-up Years	Cases or events/ controls	Sex	Age	Folate marker	Outcome	Risk-factor adjusted RR (95%, CI)
Giles (1995) (NHANES I) [44]	2006	13	98	M+F	35–74	serum	Ischaemic stroke	1.37 (CI 0.82–2.29) folate ≤9.2 vs ≥9.2nmol/L
Chasan-Taber (1996) Physicians Health Study [42]	14,916	7.5	333/333	M	40-84	plasma	MI	1.4 (CI, 0.9–2.3) lowest quintile (<2ng/ml) vs highest
Morrison (1996) Nutrition Canada Survey [39]	5056	15	165	M+F	35–79	serum	Fatal CHD	1.69 (CI, 1.10–2.61) lowest quartile vs highest (<6.8 vs >13.6nmol/L
Folsom (1998) (ARIC) [43]	15,792	3.3	232	M+F	45–64	plasma	CHD	0.66 (CI, 0.3–1.5) highest vs lowest quintile
Rimm (1998) (Nurses Health Study) [40]	80,082	14	939	F	30–55	intake	CHD	0.69 (CI, 0.55–0.87) highest vs lowest quintile (≥545 vs <190μg/day)
Giles (1998) (NHANES I) [41]	1921	20	284	M+F	25–74	serum	CHD	2.4 (CI, 1.1–5.2) in 35–55 years olds, lowest vs highest quartile (≥9.9 vs ≤21.8nmol/L)
Ford (1998) [45]	2657 2657	19 19	215 873	M+F M+F	25–74 25–74	serum serum	Fatal CHD CHD	1.31 (CI, 0.82–2.12) lowest vs highest quintile (<9.3 vs >23.6 nmol/L) 1.04 (CI, 0.86–1.26) lowest vs highest quintile (<9.3 vs >23.6 nmol/L
Voutilainen (2002) (KIHDRFS) [46]	734	5.25	34	M	46–64	serum	ACE	0.31 (CI, 0.11–0.90) serum folate >11.3 vs <11.3nmol/L
Loria (2000) (NHANES II) [47]	689	12–16	49	M+F	30–75	serum	Fatal CVD	2.28 (CI, 0.96–5.4) lowest vs highest tertile
Voutilainen (2001) (KIHDRFS) [48]	1980	10	199	M	42–60	intake	ACE	0.45 (CI, 0.25–0.81, P=0.008) highest vs lowest quintile

MI = myocardial infarction, CHD = coronary heart disease, ACE = acute coronary events, NHANES = National Health and Nutrition Examination Survey, ARIC = Atherosclerosis Risk Communities, KIHDRFS = Kuopio Ischaemic Heart Disease Risk Study, CI = confidence interval, RR = relative risk, M = male, F = female, n = number of subjects.

other prospective studies, the Atherosclerosis Risk in Communities (ARIC) study found no such association [43].

Epidemiological studies cannot fully exclude the possibility that folate and other B-vitamins are associated with CVD risk that is independent of plasma tHcy concentrations, as the metabolism of these vitamins and homocysteine are closely interlinked. Most of these studies have been based on serum/plasma measurements and circulating folate concentrations which represent short-term body stores of folate. Therefore, prospective studies have the disadvantage that folate status was assessed once, which is not indicative of an individual's long-term folate nutriture and is likely to underestimate the role of folate in CVD. In addition, the long-term stability of folate in frozen plasma/serum is uncertain. To further assess the association between folate and CVD, studies are needed where erythrocyte folate is measured, which, is a better long-term indicator of folate status as it reflects a 3 to 4 month intake of folate.

6. Effects of folate on vascular endothelial function

The vascular endothelium is a cellular monolayer that plays an important role in cardiovascular physiology in both health and disease. The endothelium is involved in the modulation of vascular tone, initiation of coagulation and fibrinolysis and the generation of inflammatory mediators. Markers of endothelial function have been widely used in cardiovascular research since the proposal that endothelial damage was an important event in the development of CVD. Recently, two prospective studies have demonstrated that endothelial dysfunction is a long-term predictor of the development of atherosclerosis and cardiovascular events [49, 50], thereby validating the usefulness of endothelial function as a good surrogate marker for CVD.

Numerous studies have now appeared in the literature describing the effects of folic acid therapy on vascular endothelial function (Table 3). Recent studies from our

group and others have shown that high dose folic acid supplementation can ameliorate endothelial dysfunction as assessed by flow-mediated dilatation (FMD) in asymptomatic subjects with hyperhomocysteinaemia [51, 52]. Other studies have also shown that folic acid can improve vascular endothelial dysfunction in hyperhomocysteinaemic patients with vascular disease as assessed by biochemical markers. Van den Berg et al. [53], demonstrated, in young patients suffering from peripheral arterial occlusive disease and hyperhomocysteinaemia, that treatment with folic acid plus vitamin B_6 resulted in the lowering of plasma homocysteine and amelioration of endothelial damage as assessed by circulating plasma vonWillebrand factor and thrombomodulin concentrations.

Pre-treatment with folic acid can prevent endothelial dysfunction seen following an oral methionine load which is thought to be induced by experimental hyperhomocysteinaemia [54, 55]. Folic acid can also prevent nitric oxide synthase (NOS) dysfunction induced by nitroglycerin and nitrate tolerance in the arterial circulation of healthy subjects [56]. Interestingly, pre-treatment with oral folic acid can also prevent endothelial dysfunction induced by acute hyperlipidemia following an oral fat load [57]. However, folic acid therapy even at high doses appears not to ameliorate endothelial dysfunction observed in patients with renal failure [58, 59]. In addition, endothelial dysfunction was observed in patients with homocystinuria, despite these patients taking 5 to 10 mg/day of folic acid as part of their homocysteine lowering therapy [60]. In a cohort of 38 patients with premature atherothrombotic disease and homocysteine concentrations $>14\mu$mol/L, Peterson and Spence [61] reduced the size of carotid atheromatous plaques with folic acid and vitamins B_{12} and B_6. However, further studies are needed to substantiate the effect of folate and other B-vitamins in reducing plaque size/growth.

It has recently been shown by our group and others that folic acid therapy can improve endothelial function in patients with CAD with normal fasting plasma homocysteine concentrations [62–65]. Fig. 3 shows the effect of folic therapy in improving vascular endothelial function in patients with CAD as assessed by FMD in the brachial artery. Furthermore, we have recently demonstrated that improvement in endothelial function observed with 5 mg of folic acid in patients with CAD occurs acutely and is independent of changes in plasma tHcy concentrations (Fig. 4) [65]. In addition we have also shown that 5MeTHF, the main circulating metabolite of folate in plasma, also abolished this observed endothelial dysfunction in patients with CAD within 30 min of an intra-arterial infusion in the absence of a change in plasma homocysteine concentrations (Fig. 5) [62].

7. Beneficial effects of folic acid in cardiovascular disease: potential mechanisms

Initially the beneficial effects of folic acid on endothelial function were thought to be a consequence of the reduction in plasma tHcy concentrations. However, recent evidence suggests that folic acid may have several beneficial effects on vascular endothelial function other than via homocysteine lowering. Fig. 6 summarizes possible mechanisms of the beneficial action of folic acid on the cardiovasculature.

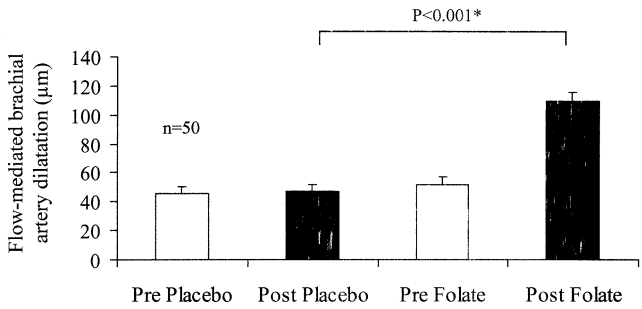

Fig. 3. Endothelial function (FMD) before and after 6 weeks of folic acid (5 mg daily) or placebo in subjects with CAD. Results shown as mean ± SEM *$P < 0.001$, comparing change with folic acid vs. placebo (reproduced with permission from Doshi SN et al., Arteriosclerosis Thrombosis and Vascular Biology 2001;21(7):1196–202).

8. Treatment of hyperhomocysteinaemia with B-vitamins?

The strongest evidence to date that an elevated plasma tHcy concentration may be a risk factor for vascular disease comes from the study of individuals with homocystinuria. If left untreated 25% of these individuals suffer a vascular event by the age of 16 years and 50% by the age of 29 years [73]. Treatment of homocystinuria is directed at lowering homocysteine concentrations by administering vitamin B_6, folate, betaine (a methyl donor) and vitamin B_{12}. This treatment has been shown to have a dramatic effect on preventing the occurrence of vascular events. Evidence from long-term follow-up of 158 patients with homocystinuria, with 2822 patient years of treatment, demonstrated that homocysteine lowering, even to concentrations several times higher than the normal reference range, significantly reduces the incidence of vascular events [74]. But patients with homocystinuria even on optimal treatment still demonstrate endothelial dysfunction [60], which is associated with the development of arteriosclerosis and an adverse cardiac prognosis.

Folate is an important substrate in the remethylation of homocysteine to methionine and numerous studies have been published on the effects of folic acid supplementation and increased dietary intake of folate, showing that homocysteine can be lowered in patients with CVD and in normal healthy subjects with or without hyperhomocysteinaemia (Table 4). However, until recently there has been uncertainty regarding the optimum vitamin dosage for lowering homocysteine concentrations. The recent meta-analysis by the homocysteine lowering trialists [75] showed that folic

Table 3
Clinical trials on the effects of folic acid on endothelial function/markers and outcome

Reference & year	Patient group	Daily dosage	Study duration	Vascular endpoint
Van den Berg (1995) [53]	HHCY & Vascular disease (n = 18)	FA(5mg) + B6 (250mg)	1 year	Decreased plasma vWF & TM
Peterson & Spence (1998) [61]	HHCY & Premature athero thrombotic patients (n = 38)	FA(5mg/2.5mg)+ B6 (25mg)+B12(250μg)	4.4±1.5 yrs	Reduced progression of carotid plaque formation
Verhaar (1998) [66]	Familial hypercholesterol-aemia (n = 10)	5-MTHF (0–10μg/ 100ml/min)	5mins	Restored serotonin-induced endothelial dependent vasodilatation
Van Guldener (1998) [58]	HHCY & Haemodialysis patients (35)	FA (5mg) or FA(5mg) + Betaine(4g)	12wks	No change in endothelial-dependent FMD or plasma vWF, TM, E-selectin PAI-I, tPA or ET
Woo (1999) [52]	Healthy HHCY subjects (n = 17)	FA(10mg)	8wks	Improved endothelial-dependent FMD
Bellamy (1999) [51]	Healthy HHCY subjects (n = 18)	FA(5mg)	6wks	Improved endothelial-dependent FMD
Verhaar (1999) [67]	Familial hypercholesterol-aemia (n = 20)	FA(5mg)	4wks	Restored serotonon-induced endothelial dependent vasodilatation
Constans (1999) [68]	HHCY & CVD patients (n = 18)	FA(5mg)+ B6(250mg)	3mo	Decreased plasma TM, no change in plasma vWF
Chao (1999) [55]	Healthy subjects with acute HHCY following a methionine load (n = 16)	FA(5mg) B6(100mg) B12(0.5mg)	5wks	Prevented impairment of endothelial-dependent FMD
Usui (1999) [54]	Healthy subjects with acute HHCY following a methionine load (n = 10)	FA(20mg)	Single dose	Prevented impairment of endothelial-dependent FMD
Title (2000) [63]	CAD & HHCY subjects (n = 75)	FA(5mg) or FA(5mg)+ VitC(2g), VitE (800IU)	4mo	Improved endothelial-dependent FMD
Chambers (2000) [64]	CHD patients (n = 89)	FA(5mg)+ B12(1mg)	8wks	Improved endothelial-dependent FMD
Vermeulen (2000) [69]	Healthy siblings of premature atherothrombotic patients (n = 78)	FA(5mg)+ B6 (250mg)	2yrs	Decreased occurrence in abnormal exercise ECG tests, no effect on ABPI or on carotid/peripheral outcome variables
Wilmink (2000) [57]	Healthy subjects with lipaemia following oral fat load (n = 20)	FA(10mg)	2wks	Prevented impairment of endothelial-dependent FMD
Thambyrajah (2000) [59]	Pre-dialysis renal failure patients (n = 100)	FA(5mg)	12wks	No change in endothelial-dependent FMD or plasma vWF
Doshi (2001) [62]	CAD patients (n = 52) CAD patients (n = 10)	FA(5mg) 5-MTHF(50μg/min)	6wks Single dose	Improved endothelial-dependent FMD Improved endothelial-dependent FMD
Pullin (2001) [70]	Healthy subjects (n = 126)	FA(400μg) or dietary folate (400μg)	4mo	No change in endothelial-dependent FMD or plasma vWF
Van Dijk (2001) [71]	Healthy siblings of premature CVD patients (n = 158)	FA(5mg)+B6(250mg)	1 and 2yrs	Decreased blood pressure, no change in endothelial-dependent FMD
Schnyder (2001) [72]	Coronary angioplasty patients (n = 205)	FA(1mg),B6(10mg)+ B12(400μg)	6mo	Decreased rate of coronary angioplasty

HHCY = hyperhomocysteinaemia, CAD = coronary artery disease, CVD = cardiovascular disease, CHD = coronary heart disease, FMD = flow-mediated dilatation, FA = folic acid, Vit C = vitamin C, ABPI = ankle-brachial pressure indicies, vWF = von Willebrand Factor, TM = thrombomdulin, PAI-1 = plasminogen activator inhibitor type 1, tPA = tissue-type plasminogen activator, ET = endothelin

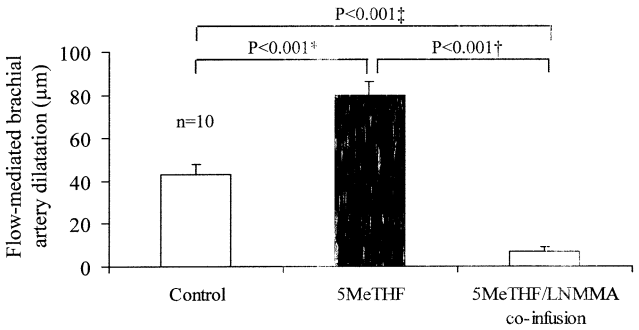

Fig. 4. Endothelial function (FMD) responses to inter-arterial saline (control) infusion, inter-arterial 5MeTHF infusion (50 μg/min) and 5MeTHF/L-NMMA co-infusion in patients with CAD. Results shown as mean ± SEM. *$P < 0.001$, comparing change following 5MeTHF infusion vs. placebo. †$P < 0.001$, comparing 5MeTHF vs. 5MeTHF/L-NMMA co-infusion. ‡$P < 0.001$, comparing control vs. 5MeTHF/L-NMMA co-infusion (reproduced with permission from Doshi SN et al., Arteriosclerosis Thrombosis and Vascular Biology 2001;21(7):1196–202).

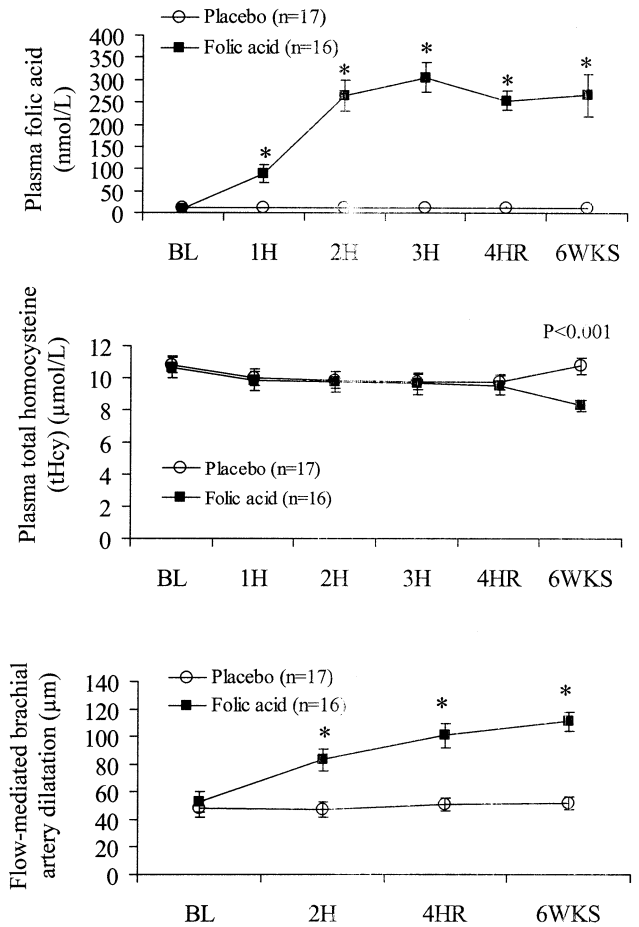

Fig. 5. Time course of endothelial function (FMD), plasma folate and plasma homocysteine (tHcy) in patients with CAD, before and after the first dose and after six weeks of folic acid (5 mg daily) or placebo. Results shown as mean ± SEM *$P < 0.001$, comparing change on folic acid with change on placebo (reproduced with permission from Doshi SN et al., Circulation 2002;105(1):22–26).

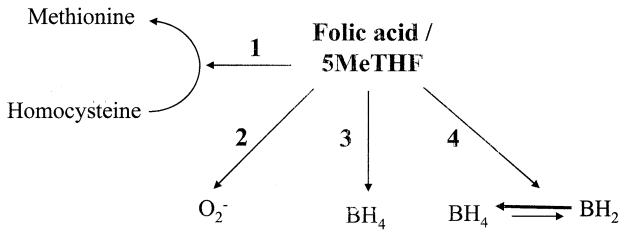

Fig. 6. Putative mechanisms for the ameliorative effects of folates on vascular endothelial function. Several beneficial effects of folates have been proposed: (1) Homocysteine lowering; 5MeTHF acts a methyl donor to convert homocysteine to methionine (2). Folic acid / folate derivatives can directly scavenge superoxide radicals (O_2^-) (3). 5MeTHF may chemically stabilize BH4 and (4) regenerate endogenous BH_4 from its oxidized and inactive form BH_2.

acid was the most effective homcysteine-lowering agent compared to vitamin B_6 and B_{12}. Folic acid reduced plasma homocysteine concentrations on average by 25%, with similar effects seen in the range of 0.5 mg to 5 mg of folic acid. The addition of vitamin B_{12} to folic acid had a small additional homocysteine lowering effect. In contrast vitamin B_6 in conjunction with folic acid did not have any additional homocysteine lowering on fasting plasma tHcy concentrations.

The study of Wald et al. [79] further assessed the lowest dose of folic acid required to produce the maximum reduction in plasma homocysteine concentration. It was shown that a dosage of 0.8 mg/day was necessary to achieve the greatest reduction in plasma homocysteine across the range of homocysteine concentrations in the general population. Therefore it would appear that the level of fortification mandated in the USA of 140 μg of folic acid per 100g of cereal grain may not achieve the maximum homocysteine lowering potential [80]. In their meta-analysis, Boushey et al. [18], using epidemiological data relating cardiovascular events and plasma homocysteine concentrations together with results from folate intervention studies, predicted that an additional daily intake of 200 μg of folate would reduce plasma tHcy concentrations by $\sim 4\mu$mol/L and that this could prevent 13,500 to 50,000 deaths per year from CAD in the USA. However, it remains to be established whether normalizing elevated plasma tHcy will influence cardiovascular mortality and morbidity. Recent evidence from our group demonstrating that folic acid can normalize endothelial dysfunction in patients with CAD independent of homocysteine lowering, as measured by total and free homocysteine suggests that folate may have a direct beneficial effect other than through homocysteine lowering [65].

9. Folate as an antioxidant?

Oxidative stress due to the increased production of reactive oxygen species both intracellularly and extracellularly has been implicated in the pathogenesis of CVD [81]. Ox-

Table 4
Folic acid supplementation and fortification trails to lower plasma total homocysteine (tHcy) levels

Reference & year	Subject Group	Dose of vitamins/day & Duration	Pre-tHcy (μmol/L)	Post-tHcy (μmol/L)	% Change
den Heijer (1998) [76]	Healthy subjects (n = 36)	Placebo, 56days	11.5	11.4	−0.9
	Healthy subjects (n = 36)	FA(0.5mg), 56days	12.2	10.0	−18.0
	Healthy subjects (n = 35)	FA(5mg), 56days	11.8	8.7	−26.3
	Healthy subjects (n = 34)	FA(5mg), B12(0.4mg), B6(50mg), 56 days	11.8	8.5	−28.0
Woo (1999) [52]	Healthy HHCY subjects (n = 17)	FA(10mg), 4wks	9.8 ± 2.8	8.1 ± 3.0	−17.3
Bellamy (1999) [51]	Healthy HHCY subjects (n = 18)	FA(5mg), 8 wks	12.1 ± 3.6	8.7 ± 2.5	−28.1
Verhaar (1999) [67]	Hypercholesterolaemia patients (n = 40)	FA(5mg), 4wks	11.5 ± 0.6	8.4 ± 0.4	−27.0
Lobo (1999) [77]	CAD patients (n = 22)	Placebo	12.0 ± 6.0	12.0 ± 3.0	0
	CAD patients (n = 27)	FA(0.4mg/d), 90days	14.0 ± 9.0	10.0 ± 2.0	−28.6
	CAD patients (n = 23)	FA(1mg/d), 90days	13.0 ± 6.0	10.0 ± 4.0	−23.1
	CAD patients (n = 23)	FA(5mg/d), 90days	15.0 ± 7.0	10.0 ± 3.0	−33.3
Constans (1999) [68]	Healthy HHCY subjects (n = 44)	FA(5mg)+B6(250mg), 3mo	11.0 ± 0.6	8.4 ± 0.4	−18.2
Title (2000) [63]	CAD & HHCY subjects (n = 25)	FA(5mg), 8wks	12.3	10.9	−11.4
Chambers (2000) [64]	CHD patients (n = 59)	FA(5mg) + B12(1mg), 8wks	13.0 ± 3.4	9.3 ± 1.9	−28.5
Vermeulen (2000) [69]	Healthy siblings of premature atherothrombotic patients (n = 78)	FA(5mg) + B6 (250mg), 2yrs	14.7 ± 8.2	7.4 ± 1.9	−49.7
Van Der Griend (2000) [78]	HHCY subjects (n = 27)	B6(200mg) 8wks	16.6 ± 7.3	17.4 ± 11.0	+4.8
	HHCY subjects (n = 30)	FA(5mg), 8wks	19.1 ± 7.5	10.7 ± 3.3	−44.0
	HHCY subjects (n = 30)	FA(0.5mg)+B6(200mg)8wks	16.5 ± 5.7	10.7 ± 4.4	−35.2
	HHCY subjects (n = 30)	FA(0.5mg), 8wks	16.8 ± 5.8	10.7 ± 3.4	−36.3
Doshi (2001) [62]	CAD patients (n = 52)	FA(5mg), 6wks	11.1 ± 2.8	9.3 ± 2.4	−16.2
Pullin (2001) [70]	Healthy subjects (n = 126)	FA(400μg), 4mo or	10.2 ± 4.2	8.5 ± 3.1	−16.7
		dietary folate (400μg), 4mo	10.2 ± 4.2	8.7 ± 3.3	−14.7
Van Dijk (2001) [71]	Healthy siblings of premature CVD patients (n = 63)	FA(5mg) + B6 (250mg), 2yrs	15.3 ± 8.9	7.5 ± 1.9	−51.0
Schynder (2001) [72]	Coronary angioplasty patients (n = 121)	FA(1mg) + B6-(10mg) B12(400μg), 6wks	11.0 ± 3.9	7.3 ± 2.4	−33.6
Doshi (2002) [65]	CAD patients (n = 16)	FA(5mg), 6wks	10.8 ± 2.1	8.3 ± 1.3	−23.1

CAD = coronary artery disease, CVD = cardiovascular disease, CHD = coronary heart disease, HHCY = hyperhomocysteinaemia, FA = folic acid, B6 = vitamin B6, B12 = vitamin B12.

idative stress can induce endothelial dysfunction by a decrease in nitric oxide (NO) bioavailability. This may be caused by a reduction in endothelial NOS (eNOS) activity or a lack of the essential substrates/cofactors required for eNOS. Antioxidants such as vitamin C can reverse endothelial dysfunction seen in patients with heart failure [82], diabetes [83], hypercholesterolaemia [84], homocystinuria [60] and following an oral methionine load [85].

Verhaar and colleagues [66] first demonstrated that 5MeTHF in relatively high concentrations reduces superoxide radicals produced by different superoxide generating systems. In addition we have shown that 5MeTHF and folic acid following conversion to 5MeTHF, abolished homocysteine-induced production of superoxide radicals in cultured porcine endothelial cells (Fig. 7) [62]. Folic acid however could not abolish this effect directly. In contrast, other *in*

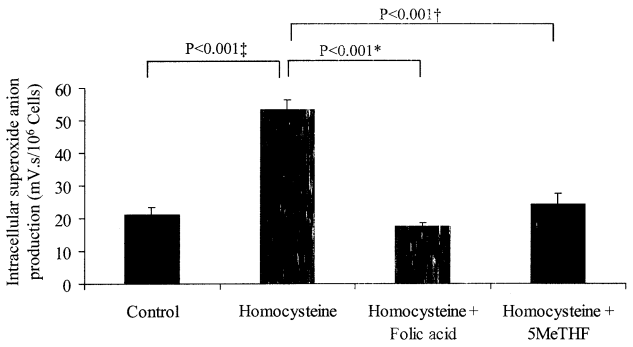

Fig. 7. Intracellular production of superoxide radicals (O_2^-) in pig aortic endothelial cells exposed to control, homocysteine alone (1mmol/L) or homocysteine (1mmol/L) with either folic acid (0.5mmol/L) or 5MeTHF (0.5mmol/L). Results shown as mean ± SEM (reproduced with permission from Doshi SN et al., Arteriosclerosis Thrombosis and Vascular Biology 2001;21(7):1196–202).

vitro experiments have shown that folic acid itself is an effective free radical scavenger and inhibitor of microsomal lipid peroxidation [86]. Endothelial dysfunction observed in healthy subjects following an oral fat load, was abolished by folic acid. This was also associated with a decreased production of malondialdehyde (MDA), an end product of lipid peroxidation [57]. In addition, 5MeTHF has been shown to reduce oxidative damage to human LDL lipid, through its free radical scavenging activity [87]. This is in contrast to other studies where, folic acid had no effect on MDA concentrations in patients with CAD, but improved vascular endothelial function [62, 63]. However, in the study of Title et al. [63], MDA concentrations were lowered in the group receiving both folic acid and the antioxidants vitamins C and E.

The relevance of 5MeTHF as an effective antioxidant *in vivo* must be questioned. Firstly, the levels of 5MeTHF achieved following a 3 mg and 5 mg oral dose of folic acid are ~150 and −200nmol/L respectively [62, 88]. Secondly, the scavenging potency of 5MeTHF was ~20 fold lower than the scavenging effects of vitamin C [89]. Therefore the high concentrations of 5MeTHF used in the these *in vitro* studies are not attainable *in vivo* following ingestion of high dose folic acid, questioning the role of folate/5MeTHF as an effective superoxide scavenger.

10. Folate interactions with nitric oxide synthase?

The majority of studies investigating the effect of folate therapy on reversing endothelial dysfunction in patients with CVD have utilized the technique of brachial artery FMD, which is a NO mediated process [90]. NO production by eNOS is dependent on optimal concentrations of the cofactor tetrahydrobiopterin (BH_4) to produce NO. Alterations in endothelial BH_4 concentrations or an increase in oxidative stress result in the oxidation of BH_4 to its inactive metabolite dihydrobiopterin (BH_2). This results in the uncoupling of the L-arginine NO pathway with subsequent production of superoxide radicals by eNOS [91]. The addition of BH_4 can reduce the production of superoxide radicals *in vitro* [62, 89]. Furthermore, supplementation with BH_4 has been shown to improve endothelium-dependent vasodilatation in patients with hypercholesterolaemia [92], CAD [93] and diabetes induced animal studies [94], indicating an increase in NO bioavailability.

The work of Stroes et al. [89], demonstrated that 5MeTHF can directly influence eNOS activity, however, this effect was not observed when eNOS was depleted of BH_4. Several theories have been proposed to account for the beneficial effects of 5MeTHF on eNOS. The ring structure of 5MeTHF is similar to that of BH_4 and Hyndman and coworkers [95], using a computer modeling system showed that 5MeTHF could bind directly into the active site of eNOS and mimic the orientation of BH_4. However, this is in contrast to the work of Stroes et al. [89]. It has been suggested that 5MeTHF may stabilize BH_4 and prevent its oxidation to its inactive metabolite BH_2. This stabilizing effect has been shown for vitamin C [96]. 5MeTHF may regenerate BH_4 from BH_2, however, folic acid therapy did not lead to any measurable increase in plasma biopterin concentrations [67]. The mechanisms(s) underlying the beneficial effects of 5MeTHF on NO bioavailability thus remain open to question.

11. Implications of these investigations

These observations indicate that folic acid or its metabolite 5MeTHF, may act directly on the vasculature by mechanisms other than through homocysteine lowering. The studies of folic acid and B-vitamin therapy on reversing endothelial dysfunction and the potential to reduce plaque size/ growth, whatever the mechanism(s), are suggestive that high dose folic acid supplements may be an important adjunct to treatment for patients with vascular and thrombotic disease.

12. What will the clinical trials with folic acid tell us?

Although there are numerous studies showing the homocysteine-lowering effects of folic acid supplementation, few data exist on the effect of folate supplementation on CVD outcome. However, there are several large scale randomized controlled clinical trials underway to assess the beneficial effects of folic acid and other B-group vitamins on CVD risk (Table 5). The hypothesis of these studies is that folic acid and other B-group vitamins can lower plasma homocysteine concentrations and therefore reduce the incidence of CVD [97]. However at the outset of these trials it was not known that folic acid may have direct effects on the vascu-

Table 5
Clinical trials underway to assess whether homocysteine lowering with folic and other B-group vitamins improves cardiovascular outcome

Study	Country	Sample size	Intervention & Study design	Disease Group	Start Date
VItamins and ThROmbosis (VITRO) trail	Netherlands	600	FA(5mg/d)+B6(50mg/d)+B12(0.4mg/d) vs placebo	Deep vein thrombosis or Pulmonary embolism	1996
Vitamin Intervention for Stroke Prevention (VISP)	USA	3600	FA(2.5mg/d)+B6(25mg/d)+B12(0.4mg/d) vs FA (0.2mg/d)+B6(0.2mg/d)+B12(0.06mg/d)	Stroke	1998
Women's Antioxidant and Cardio-vascular Disease Study (WACS)	USA	6000	FA(2.5mg/d)+B6(50mg/d)+B12(1mg/d) vs placebo	Vascular disease and high risk for vascular disease	1998
Cambridge Heart Antioxidant Study (CHAOS 2)	UK	4000	FA(5mg/d) vs placebo	Myocardial infarction or unstable angina	1998
Norwegian Study of Homocysteine Lowering with B-vitamins in Myocardial Infarction (NORVIT)	Norway	3000	FA(5mg/d) for 2 weeks, then FA (0.8mg/d)+ B12(0.4mg/d) vs placebo in a 2×2 factorial design with B6 (40mg/d) vs placebo.	Myocardial infarction	1998
Western Norway B Vitamin Trial (WENBIT)	Norway	2000	FA(5mg/d) for 2 weeks, then FA(0.8mg/d), vs placebo; B6 (40mg/d) vs placebo in a 2×2 factorial design	Coronary heart disease	1999
GOES Study	Netherlands	2000	FA(0.5mg/d) vs placebo	High risk for CVD	1998
Vitamins to Prevent Stroke Study VITATOPS	Australia	5000	FA(2mg/d)+B6(25mg/d)+B12(2mg/d)	Stroke	1999
Heart Outcomes Prevention Evaluation-2 (HOPE-2)	Canada	5000	FA(2.5mg/d)+B6(50mg/d)+B12(0.4mg/d) vs placebo	Arterial vascular disease	1999
Study of the Effectiveness of Additional Reductions in Cholesterol and Homocysteine (SEARCH)	UK	12,000	FA(2mg/d)+B12(1mg/d) vs placebo within a 2×2 factorial design with simivastatin (80mg/d vs 20mg/d)	Myocardial infarction	1999
Prevention with a Combined Inhibitor and Folate in Coronary Heart Disease (PACIFIC)	Australia	10,000	FA(0.2mg/d or 2mg/d) vs placebo in a 2×2 factorial design with Omapatrilat (ACE-inhibitor) vs placebo.	High risk vascular disease of previous history of	2000

FA = folic acid, CVD = cardiovascular disease
Data obtained in part from Clarke and Armitage [97]

lature other than homocysteine lowering. The trials underway at the moment are using daily doses of folic acid in the range of 0.2 mg – 5 mg/day. However, these studies cannot distinguish between the effects of homocysteine lowering and the direct effects of folic acid [98]. Results from the secondary intervention trial (CHAOS 2) of high dose folic acid (5 mg/day) on cardiovascular events in patients with ischemic heart disease study have recently been reported [99]. This study comprised 942 patients on folic acid and 940 patients on placebo, with a median follow-up of 1.7 years. Treatment significantly reduced plasma tHcy (11.2 ± 6.9 to 9.7 ± 5.3μmol/L) and a 2-fold reduction in non-fatal MI was observed. However, no reduction in total deaths was seen, possibly reflecting the short duration of the study. The GOES study, a secondary intervention trial comprising 593 patients with CHD (300 received folic acid, 293 controls) recently reported no clinical benefit following a 2-year intervention with low dose folic acid (0.5 mg/day) [100]. This study was underpowered and secondly the dose of folic acid used may be too low to demonstrate any beneficial effect, other than tHcy lowering. It is important to mention that recruitment for three large randomized, controlled studies; the Vitamin Intervention for Stroke Prevention (VISP) trial, the Women's Antioxidant Cardiovascular Disease Study (WACS), and the HEART Outcomes Prevention Evaluation (HOPE-2) were initiated before the before the introduction of the fortification of cereals and grains with folic acid by the US and Canadian Governments. Therefore, these trials will be substantially underpowered to test the hypothesis they were originally designed for [101]. Caution must therefore be taken when interpreting results from these studies [98]. Further studies with larger study sizes, longer duration and the appropriate doses of folic acid will be needed to clarify these results.

13. Risks with increasing folate intake

Pharmacological doses of folic acid far exceed the amount of this nutrient required to exert its normal physiological function. There is some evidence that this may produce adverse effects. Although, there is clear evidence that pharmacological doses can lower plasma tHcy concentrations and reverse endothelial dysfunction, a surrogate marker for vascular health, there is no clear evidence to date that lowering homocysteine or increasing folate reduces cardiovascular risk.

One concern of folic acid fortification or the administration of large doses of folic acid is that of masking the hematological effects of vitamin B_{12} deficiency thereby allowing the progression of neurological damage. However, adequate monitoring for B_{12} deficiency, or by supplementing with B_{12} will avoid such complications. Studies from the Framingham Heart Study involving more than 700 elderly subjects revealed that the benefit of folate fortification through projected decreases in plasma tHcy concentration and cardiovascular risk greatly outweigh this risk [102]. However, recommendations to increase folate intake on a public health perspective would be unwise until such trials have proven that folic acid is safe and can reduce cardiovascular risk.

14. Conclusions

Elevated plasma tHcy is associated with an increased risk of CVD and is regarded as a risk factor. However, it remains unclear as to whether hyperhomocysteinaemia is a direct cause of CVD. An alternative interpretation is that a low folate status is a risk factor for CVD. It is yet to be shown that folic acid, either through tHcy lowering or by its independent effects on the vasculature, has any preventative function in CVD. Several appropriate randomized folate intervention trials are underway to address this question. Results from which are eagerly awaited and will help to clarify whether increasing folate intake and thereby its plasma concentrations will reduce CVD risk.

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