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Review Article

The folate puzzle. Part II: folate and cardiovascular disease - the heart of the matter

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Abstract

Cardiovascular disease is the major cause of death worldwide. Apart from the traditional risk factors of smoking, hypertension and diabetes, high homocysteine plasma concentrations have also been suggested as a factor causing atherosclerosis. It has been shown that this amino acid is able to activate the immune system in such a way that monocyte chemotaxis to the injured vessel wall (a major feature of atherosclerosis) is enhanced. In addition, high homocysteine levels may induce highly reactive oxygen species that inactivate nitric oxide (NO), an important vascular relaxing factor. High homocysteine levels have also been associated with altered lipid metabolism and increased uptake of modified low-density lipoprotein (LDL) by macrophages in the vessel wall. Numerous observational studies have demonstrated high homocysteine levels in patients with atherosclerotic disease, including myocardial infarction and cerebral stroke. However, over the past two decades, various highly powered, large, randomized, controlled and population-based studies have failed to show a relationship between homocysteine levels and the risk of vascular diseases, with the exception of stroke, calling into question whether high homocysteine levels represent a risk factor or merely a risk indicator. Furthermore, folic acid treatment alters the methylation potential, which may induce carcinogenesis. A number of well-designed epidemiological studies are still under way and at present it seems best to adhere to a wait-and-see policy.

Introduction

Cardiovascular disorders are the leading cause of mortality worldwide. The death rate exceeds that of cancer and infectious diseases. Total worldwide mortality is currently estimated at an unacceptable 17 million per year, and this number is expected to increase over the following decades (1, 2). Traditional risk factors include smoking, abnormal lipid concentrations, hypertension, diabetes, obesity, diet low in plant-based foods and lack of physical activity. In the search for other factors, it was discovered that individuals with homocystinuria suffer severe atherosclerosis and thromboembolic events. It was found that this disease is characterized by a rare inherited enzymatic defect in homocysteine (an amino acid similar to cysteine) metabolism caused by a homozygous deficiency of cystathionine β-synthase. In an excellent historical overview, McCully (3) mentions two other causes of homocystinuria, *i.e.,* deficiency of methionine synthase and deficiency of methylenetetrahydrofolate reductase. These disorders are associated with marked atherosclerotic plaque formation. Genetic studies have also revealed various other genetic defects resulting in elevated homocysteine levels (4). These findings fit well with the "homocysteine theory of atherosclerosis" proposed in 1969 by the same author (5).

In 1976, Wilcken and Wilcken published the first medical evidence from 25 patients on the role of methionine metabolism in the pathogenesis of coronary artery disease (CAD) (6). Their findings suggested a reduced ability to metabolize homocysteine in patients with premature CAD. Since then, numerous observational studies have shown that high plasma homocysteine levels represent a risk factor for the development of coronary atherosclerosis. In addition, epidemiological data from case studies and cohort studies have supported the relationship between elevated homocysteine levels and the risk of cerebrovascular and other vascular diseases (7). Moreover, studies in animals have suggested a causal relationship between hyperhomocysteinemia (HHcy) and accelerated atherosclerosis (8, 9). These findings are interesting from a public health perspective, as plasma homocysteine levels can be cheaply and effectively

reduced by the intake of folic acid (also known as vitamin B_9 or B_{11}) and vitamin B_{12} (cyanocobalamin) in various combinations.

However, recent clinical trials failed to show a clear relationship between plasma homocysteine concentrations and the risk of myocardial infarction or stroke (10, 11). A population-based study from Finland (12) showed no association between homocysteine levels and the risk of acute coronary events. The large, randomized, controlled Vitamin Intervention for Stroke Prevention (VISP) trial (13) did not even discover a trend between homocysteine and the risk of stroke. Likewise, the Norwegian Vitamin Trial (NORVIT) (14) and the Heart Outcomes Prevention Evaluation-2 (HOPE-2) (15) did not report a significant decrease in cardiovascular events as a result of vitamin B therapy to reduce plasma homocysteine concentrations. However, the HOPE-2 study found a significant reduction in stroke (RR: 0.75; 95% CI: 0.59-0.97; $p = 0.03$) in patients assigned to active treatment compared to those assigned to placebo.

In this review, we summarize the available preclinical and clinical evidence with regard to the role of homocysteine in vascular disease. To better understand the possible involvement of homocysteine, we first focus on the pathways of homocysteine metabolism.

Homocysteine metabolism

Homocysteine is a sulfur-containing amino acid originating from dietary methionine. Figure 1 depicts the major biochemical steps in the synthesis and conversion of homocysteine. Methionine from dietary products is converted to *S*-adenosylmethionine (SAM), which is the universal methyl group donor for biochemical reactions. The methylation process converts SAM to *S*-adenosylhomocysteine, which is the precursor of homocysteine. To save methionine for the body, homocysteine can be converted by a remethylation reaction, which requires methionine synthase and vitamin B_{12} as cofactor. This reaction also requires the methyl donor methylenetetrahydrofolate, which is generated from tetrahydrofolate in a reaction catalyzed by methylenetetrahydrofolate reductase. Here, the methionine cycle shares the reaction process with the folate cycle.

Tetrahydrofolate is the biologically active form of folate (folic acid). This homocysteine pathway demonstrates why folate intake may reduce plasma homocysteine concentrations. Alternatively, homocysteine may be catabolized to form cystathionine, followed by the formation of cysteine. In case of excess homocysteine, the oxidation of excess cysteine to taurine and eventually to inorganic sulfates protects the cell from toxic amounts of homocysteine and cysteine.

Homocysteine plasma levels

Under normal physiological conditions, the homocysteine concentration in plasma is below 15 μmol/l (16), although males and the elderly often show higher physiological values (17). Kang and co-workers (18) classified HHcy as mild at levels up to 50 μmol/l, intermediate at levels up to 100 μmol/l and severe at levels up to 500 μmol/l. Various lifestyle and dietary factors lead to higher homocysteine concentrations (19). In addition, the homocysteine level is affected by medications (20) and malignant diseases such as breast cancer (21), lymphoma (22), colorectal and cervical cancer (23).

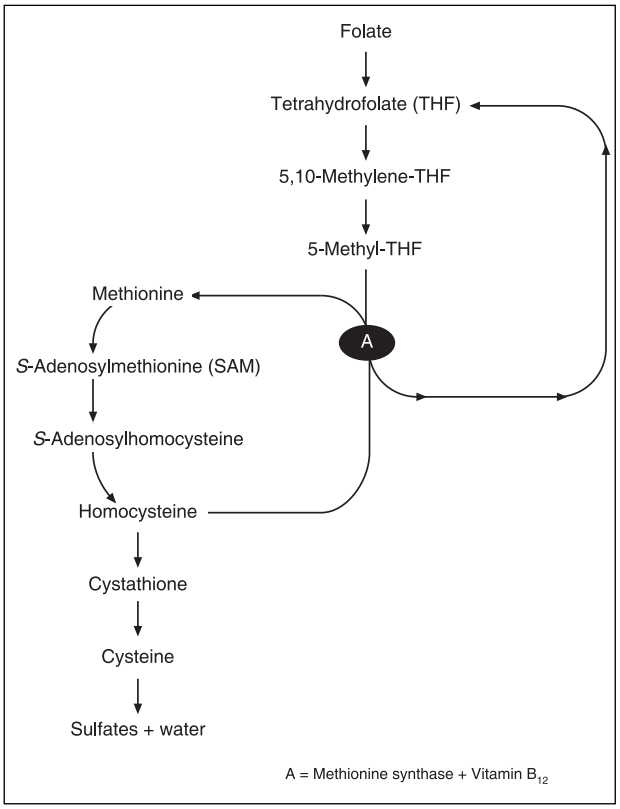

Fig. 1. Methionine and tetrahydrofolate pathways.

Interestingly, elevated homocysteine levels can also be due to genetic disorders. The catabolism of homocysteine is controlled by cystathionine β-synthase and methionine synthase (see Fig. 1). As already mentioned, homozygous mutations in the gene coding for these enzymes lead to very severe premature atherosclerosis accompanied by extremely high homocysteine levels. Fortunately, such mutations are rare (about 1:335,000) in western countries. A much more common mutation is the point mutation C677T (cytosine/thymine) at the methylenetetrahydrofolate reductase (*MTHFR*) gene, which codes for a thermolabile variant with reduced enzymatic activity in which alanine is substituted for valine. Consequently, high plasma homocysteine concentrations are found in these individuals with the TT genotype, which causes reduced formation of proper *MTHFR* to produce methyltetrahydrofolate. The heterozygous *MTHFR* mutation (affecting about 40% of the western population) is not associated with elevated homocysteine levels, whereas the homozygous mutation (affecting about 10% of the western population) is (24). Leclerc and co-workers

(25) mentioned recently that a total of 41 mutations and 60 polymorphisms have been reported for the *MTHFR* gene. However, the most common genetic cause of HHcy is the C677T variant. It is noteworthy that folate intake can decrease homocysteine concentrations, and that at high plasma folate levels the homocysteine concentration can be as low as in CT or CC genotypes (26). Thus, alteration in dietary intake or use of folic acid supplements can substantially lower plasma homocysteine levels (27).

Atherosclerosis and hyperhomocysteinemia

Atherosclerosis is a slowly progressive disease affecting the arterial blood vessels. This disorder starts as early as in childhood and may become clinically manifest after adolescence, although it usually manifests from middle age. Its clinical significance lies in the complications that may occur in medium-sized arteries, such as the coronary and carotid arteries. Atherosclerosis mainly involves the inner layers of the arterial wall, which may suffer from microinjuries due to sheer stress.

This damage to the vessel wall causes an inflammatory response. In this process, monocytes from the bloodstream adhere to the lesion and turn into macrophages. These cells ingest oxidized low-density lipoproteins (LDLs) and become large foam cells, whose cytoplasm is filled with cholesterol and cholesterol esters, leading to the formation of atheromatous plaques. Growing plaques bring about wall thickening. At some stage during the sequence of these events, plaques may rupture, causing clotting and the formation of fibrous tissue over the clot, producing stenosis. The fibrous cap over the clot may be thin and weak, and may become unstable and rupture again, and tissue fragments may enter the bloodstream. These fragments "attract" platelets, which leads to the formation of a thrombus, obstructing blood flow and causing lack of oxygen and nutrients in downstream tissue. These events may present in patients as a myocardial infarction or a stroke.

Epidemiological evidence that atherothrombotic disease often occurs in patients with HHcy has drawn the attention of biomedical researchers and led to the hypothesis that homocysteine may have a role in the development of cardiovascular disorders. The frequent finding that thrombotic disease is present in patients with autoimmune disease has suggested a relationship among immune activation, inflammation and plasma homocysteine concentrations (28). Indeed, recent investigations have demonstrated a positive relationship between homocysteine levels, biomarkers of inflammation and the risk of cardiovascular disease (29). Also, clinical signs of rheumatoid arthritis, such as the disability score, the number of swollen and painful joints and C-reactive protein (CRP) levels, are often associated with HHcy (30). Moreover, intensive steroid therapy in patients with clinically active rheumatoid arthritis brought about a significant reduction in homocysteine levels, which provides further evidence for a relationship between HHcy and inflammatory disease (31).

The mechanism by which high plasma homocysteine is associated with autoimmune disease and thrombogenesis is incompletely understood. There is ample evidence that homocysteine is able to activate the immune system and enhance the inflammatory process. For instance, it has been demonstrated that homocysteine augments cytokine-induced chemokine expression in human vascular smooth muscle cells (32). This stimulation of vascular cells and the link to atherosclerosis was already suggested more than a decade ago (33). Recently, various experiments suggest upregulation of chemokine receptor expression by homocysteine, enhancing monocyte chemotaxis (34). The attraction of monocytes is one of the major features of atherosclerotic disease, and provides further support for the idea that HHcy has a causative link to the development of atherosclerosis.

Undoubtedly, the immunostimulatory and proinflammatory consequences of HHcy produce vascular changes. Various authors have found impaired vascular relaxation that could be explained by the HHcy-induced generation of highly reactive oxygen species that consume local nitric oxide (NO), an important vascular relaxing factor, together with impairment of endothelium-dependent vasodilatation (35, 36). Other mechanisms that explain the impact of HHcy on vascular function were described by Jiang *et al.* (37) and include altered hepatic lipid metabolism and increased uptake of modified LDL by macrophages in the vessel wall.

Beneficial effects from homocysteine lowering?

Numerous studies have shown an inverse relationship between serum levels of folic acid (in combination with vitamin B_6 and B_{12}) and blood homocysteine concentrations (reviewed in Ref. 38). These findings had their influence on the design of the "polypill" containing folate, aspirin, a statin and an antihypertensive. This multicomponent pill would reduce the risk of ischemic heart disease and stroke by 88% and 80%, respectively, and presumably one-third of those taking this pill from age 55 would gain 11 healthy years as far as vascular diseases are concerned (39). This concept has created quite a stir in the medical and pharmaceutical worlds (40), as its use has been proposed as a mass prophylactic therapy. Focusing on folate alone, is there enough evidence to support the massive use of the polypill? In other words: what is the medical evidence from epidemiological studies, and more especially from randomized, controlled trials, that homocysteine-lowering treatment with folic acid has a favorable effect on the prevalence of cardiovascular diseases?

In this context, researchers carried out studies on individuals with high homocysteine levels due to *MTHFR* C677T polymorphism. A recent large meta-analysis in 26,000 cases and 31,183 controls from 80 studies in Europe, North America and Australia did not provide strong evidence that would support the association between C→T polymorphism and CAD (41). In 2007, a large prospective epidemiological study in 24,968 healthy

American women investigating genetic (*MTHFR* C677T) and dietary (folate and B vitamins) factors was published. The women were followed for 10 years. Homocysteine levels were associated with *MTHFR* genotype (1.4 μmol/l higher for TT *vs.* CC; $p < 0.001$) and inversely related to folate and vitamin B intake. The analysis showed no gene–diet or gene–homocysteine interactions in relation to cardiovascular disease (42).

The outcomes of these recent studies cast doubt on a previously performed meta-analysis that concluded that individuals with the *MTHFR* TT genotype. and particularly in the setting of a low plasma folate status, had a significantly higher risk of CAD (43). Another meta-analysis on the relationship between homocysteine levels and stroke found an increased risk for stroke among the TT homozygotes. This meta-analysis included 111 studies and a total of 15,635 people without cardiovascular disease, and consistent results were found in separate analyses by age group, ethnic background and geographical location (44). This result was in line with other more recent epidemiological studies, for example, an investigation reported by Pezzini *et al*. in a case-control study (45). These authors reported an increased risk for stroke for the TT genotype below 45 years of age. The stroke risk was associated with elevated levels of homocysteine and a causal relationship was assumed. Another case-control study (46) confirmed these results in which polymorphisms in the *MTHFR* gene represent genetic risk factors for stroke. Interestingly, the HOPE-2 study performed in 5,522 patients 55 years of age or older who had vascular disease or diabetes found a reduction in stroke in patients at risk as a result of active folic acid and vitamin B treatment. On the other hand, the HOPE-2 study did not find a significant effect on the risk of major cardiovascular events as a result of this treatment. Furthermore, a recent meta-analysis of randomized, controlled trials did not show a beneficial effect from homocysteine-lowering therapy on cardiovascular disease among participants with a prior history of cardiovascular disease (47). Another recent meta-analysis, including four large trials involving 14,000 healthy participants, demonstrated no beneficial effects for this treatment on CAD (OR: 0.99; 95% CI: 0.88-1.10) and stroke (OR: 0.98; 95% CI: 0.90-1.08). These trials are among 12 large trials involving 52,000 participants that are currently assessing the effects of vitamin B on CAD and stroke. The authors of this paper cautiously state that the results of their meta-analysis are still compatible with a homocysteine-lowering effect, demonstrated in observational studies by a 10% difference in the risk for CAD and a 20% difference in the risk for stroke associated with a 25% lower homocysteine concentration (48).

Folic acid and stroke prevention

A recent investigation on the efficacy of folic acid supplementation in stroke prevention was carried out by Wang and co-workers (49). They analyzed data from eight randomized, controlled trials that had stroke as one of the endpoints. They found an 18% reduction in the risk of stroke (RR: 0.82; 95% CI: 0.68-1.00; $p = 0.045$). An even more significant beneficial effect was observed in the stratified analysis: studies of more than 3 years' duration (RR: 0.71; 95% CI: 0.57-0.87; $p = 0.001$) and with a decrease of more than 20% in homocysteine concentrations (RR: 0.77; 95% CI: 0.63-0.94; $p = 0.012$). It is possible that this favorable effect of folic acid on the risk of stroke had not been noticed in, for example, the aforementioned VISP trial. Spence (50) conducted an efficacy analysis of a subgroup of participants where those with low and very high vitamin B_{12} levels and patients with significant renal impairment were excluded. On the basis of the data obtained, it was concluded that folate therapy is a rational approach in patients with a high risk of stroke. The author stressed that previous trials may have missed the potential benefits of HHcy lowering with regard to the risk of stroke due to the fact that the pathogenesis of stroke is different from that of myocardial infarction. The benefit of folic acid and vitamin B intake may even be enhanced in the TT genotype, as indicated from a recent study in healthy elderly subjects with lower plasma levels of vitamin B, in whom *MTHFR* TT genotype-related carotid atherosclerosis was detected.

Quo vadis?

The initial evidence for an association between elevated plasma homocysteine and the risk of occlusive vascular disease has been called into question on the basis of recent epidemiological studies. Advocates of a causal relationship emphasize that HHcy is an independent risk factor not associated with conventional risk factors such as hypertension, smoking, diabetes and hyperlipidemia (51). It has also been argued that elevated homocysteine is a strong predictor of coronary vascular disease and all-cause death in renal transplant recipients, independent of renal function (52, 53). On the other hand, it can be speculated that renal transplant patients have suffered, often silently, from long-lasting atherosclerotic disease, leading to nephrosclerosis and decline of renal function. From renal studies, it is known that reduced function results in elevated plasma homocysteine levels (54). HHcy may also be present in stroke patients secondary to the disease. Meiklejohn *et al.* (55) measured homocysteine levels immediately after an atherothrombotic stroke and in the convalescent period and found that plasma levels were only elevated during the convalescent period.

The above-mentioned findings illustrate the controversies related to the value of folic acid and vitamin B treatment in mild HHcy. Furthermore, these findings deepen the discussion of the hypothesis as to whether HHcy is a consequence or a cause of vascular disease and stroke. Therefore, the crucial question is whether HHcy is a risk indicator or a risk factor. The considerable disparities between earlier observational studies and more recent randomized, controlled studies are confusing but are probably related to the inherent limitations of observational studies. Medication is an obvious confounder in

observational studies in which participants take a variety of drugs that may interfere with folate and vitamin B_{12} metabolism or alter renal function. Pharmaceuticals that cause elevated homocysteine concentrations include lipid-lowering drugs, oral hypoglycemic drugs, insulin, antiinflammatory drugs and anticonvulsants (20).

Taken together, recent adequately powered epidemiological studies in apparently healthy participants have not demonstrated a lower risk of cardiovascular disease during folate treatment. In addition, the risk of major cardiovascular events in patients with vascular disease was not reduced. Understandably, in view of the present knowledge, leading researchers and clinicians in the field of cardiovascular disease do not support routine screening for and treatment of mildly elevated homocysteine levels (56). Certainly, the very high dose of 20-30 mg/day of folic acid, as suggested by Mangoni (57), should not be administered in view of the possible carcinogenic effects due to altered methylation potential (58). To avoid carcinogenesis due to hypermethylation, the chronic intake of folic acid should not exceed 400 μg/day.

Nevertheless, there is ample preclinical and clinical evidence supporting a relationship between HHcy and impaired endothelial function, increased carotid intima-media thickness and oxidative stress effects. This could well explain the explicit reduction in stroke risk that has been found as a result of folic acid supplementation and a decrease in homocysteine concentrations. For now, the best strategy is to await the results of ongoing trials, such as the Vitamin to Prevent Stroke (VITATOPS) trial. In the meantime, even if folic acid and vitamin B treatment does not significantly reduce the risk of CAD, the benefit may well be in the reduction of the risk of stroke. Eventually, it may appear that this is a modifiable risk.

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