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REVIEW

Homocysteine and coronary atherosclerosis: from folate fortification to the recent clinical trials

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Plasma total homocysteine (Hcy) has been associated with cardiovascular risk in multiple large-scale epidemiological studies, and it has been considered as an independent risk factor for atherosclerosis. Homocysteine lowering, achieved after the introduction of the folate food fortification programme in North America, was accompanied by an accelerated decline of cardiovascular risk and especially of stroke. Although the initial clinical trials suggested that homocysteine-lowering treatment with folates and B vitamins induces coronary plaque regression, this finding was not confirmed by more recent clinical studies. Under the light of the findings from the recent large randomized clinical trials that failed to document a benefit of Hcy lowering on clinical outcome of patients with atherosclerosis, the role of Hcy as a risk factor and the efficacy of Hcy lowering against atherosclerosis have been questioned. Therefore, better understanding of the mechanisms relating Hcy and Hcy-lowering treatment with vascular function and atherogenesis is crucial, to help us understand why clinical trials failed to show a benefit from Hcy-lowering treatment. Are these therapeutic strategies ineffective because they fail to reduce intracellular Hcy levels and vascular redox state or should Hcy stop being considered as an independent risk factor for atherosclerosis from now on? In this review article, we provide a global approach of the molecular mechanisms relating Hcy with cardiovascular risk and introduce possible mechanistic explanations regarding the inability of clinical trials to detect any clinical benefit from Hcy-lowering treatment in secondary prevention. Finally, we provide clinical recommendations regarding the therapeutic strategies targeting homocysteine in the general population.

Keywords Atherosclerosis • Homocysteine • Folate • Endothelium • Atherothrombosis

Background

It is now widely accepted that increased plasma homocysteine (Hcy) is associated with increased cardiovascular risk, independently of other atherosclerosis risk factors.[1] From a pathophysiologic point of view, homocysteinaemia is associated with increased thrombogenicity, increased oxidative stress status,[2] over activation of redox-sensitive inflammatory pathways,[3] impaired endothelial function,[4] and finally atherogenesis. However, it is still unclear whether plasma Hcy can be approached as a modifiable risk factor for atherosclerosis, since the results regarding the effect of Hcy-lowering treatment on cardiovascular risk are contradictory.[5] Despite the decline of cardiovascular risk in North America after the introduction of folate food fortification,[6] two recently published clinical trials (the NORVIT[7] and HOPE-2[8]) failed to demonstrate any benefit from pharmacological treatment targeting Hcy levels in patients with coronary atherosclerosis, putting into question the whole concept of Hcy being a risk factor for atherosclerosis. Is the Hcy hypothesis in atherosclerosis wrong or are the therapeutic approaches used in these trials problematic? Should clinicians treat their patients with Hcy-lowering regiments or should they never measure plasma Hcy again? Understanding the mechanisms by which Hcy affects atherogenesis as well as the mechanisms by which Hcy-lowering treatment modifies this process is necessary to address these questions. In this review article, we discuss these mechanisms and we provide possible explanations for the failure of Hcy-lowering treatment to modify cardiovascular risk, suggesting alternative therapeutic strategies targeting Hcy-associated atherosclerosis.

Homocysteine as a risk factor for atherosclerosis

The role of Hcy in atherosclerosis has been well elucidated.[1] All the large meta-analyses conducted during the last decade yield consistent

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results: Hcy can be considered as an independent risk factor for cardiovascular disease (CVD).[9] The first large meta-analysis published in 1995 pointed out that Hcy is strongly associated with vascular disease, arguing that an increment in total Hcy by 5 μmol/L is equivalent to the elevation in CAD risk induced by a 20 mg/dL increase in plasma cholesterol.[9] Furthermore, it was suggested that Hcy accounts for up to 10% of the population's CAD risk.[9] Although a weaker association has been observed in prospective studies between plasma Hcy and CVD, the results in conducted meta-analyses remain unchanged.[10] The 'Homocysteine Studies Collaboration' in a recent meta-analysis of 5073 patients with CAD and 1113 with stroke showed that a reduction of 3 μmol/L of plasma Hcy is accompanied by a reduction of the relative risk for CAD by 11% and that of stroke by 19%.[10] Another large meta-analysis of more than 90 genetic and prospective studies suggested that lowering Hcy concentrations by 3 μmol/L from current levels (achievable by increasing folic acid intake) would reduce the risk of ischaemic heart disease by 16%, deep vein thrombosis by 25%, and stroke by 24%.[11] Moreover, many studies have shown that increased plasma Hcy levels in patients presenting with acute coronary syndromes are independent predictors of recurrent cardiovascular events.[12,13] Indeed, increased plasma Hcy on admission strongly predicts late cardiac events such as cardiac death and/or myocardial (re)infarction in patients with acute coronary syndromes.[14] Therefore, plasma Hcy is a strong predictor of cardiovascular risk not only in the general population but also in patients with already established atherosclerosis and/or with acute coronary events.

Therefore, the accumulated data so far provide strong evidence in support of the putative (and potentially causal) role of Hcy in atherosclerosis.[15] However, it is still unclear whether homocysteinaemia is a modifiable risk factor in atherosclerosis, and its causal role in atherosclerosis will only be demonstrated when Hcy lowering proves to reduce cardiovascular risk in prospective, randomized clinical trials.

Biosynthesis of homocysteine and reference values

Hcy production is dependent on *S*-adenosyl-methionine (SAM) that is responsible for multiple intracellular methylation reactions.[16] The by-product of these reactions is *S*-adenosyl-homocysteine (SAH), which is hydrolysed to Hcy.[16] When there is an excess of methionine, Hcy is metabolized via the pathway of *trans*-sulfurylation, producing cystathionine and cysteine in turn. The responsible enzyme for the transformation of Hcy to cystathionine is cystathionine b-synthase (CBS) that requires vitamin B6 as an essential co-factor.[16] On the other hand, under conditions of methionine deficiency, Hcy is remethylated into methionine. Hcy is remethylated in the liver via betaine-homocysteine-methyltransferase; however, in most tissues, Hcy is remethylated into methionine by methionine synthase (MS), which uses vitamin B12 as a co-factor and 5-methyltetrahydrofolate (5-MTHF) as a substrate.[16] 5-MTHF synthesis is catalysed by methyltetrahydrofolate reductase (MTHFR), which uses tetrahydrofolate as a substrate.[16]

Although there is no specific cut-off point for normal plasma Hcy, it is widely accepted that plasma total Hcy levels <15 μmol/L should be considered as 'normal'.[17] Adults, who do not eat food fortified with folic acid, appear with an upper plasma total Hcy reference limit of 15–20 μmol/L, but in adults with good B vitamin and folate status or a healthy lifestyle, the upper reference limit is 12 μmol/L.[17] Homocysteinaemia is classified according to fasting plasma Hcy levels, as moderate (fasting plasma Hcy levels 15–30 μmol/L), intermediate (fasting plasma Hcy levels 30–100 μmol/L), or severe (fasting plasma Hcy levels >100 μmol/L),[17] and this classification is important to help us decide whether homocysteinemic individuals should be treated or not.

Homocysteine and oxidative stress

Increased reactive oxygen species/reactive nitrogen species production in homocysteinaemia

In animal models, homocysteinaemia has been associated with increased vascular oxidative burden.[18] It has been shown that homocysteinaemia induces NADPH oxidase and inducible nitric oxide synthase activity, contributing to increased superoxide radicals production in rat coronary vessels.[18] Moreover, in animal models of mild homocysteinaemia, the function of intracellular antioxidant enzymes such as superoxide dismutase and glutathione peroxidase is altered.[3] In addition, the self-oxidation of Hcy to homocystine and Hcy-thiolactone generates reactive oxygen species (ROS) and further contributes to the vascular toxicity of homocysteinaemia.[19] Moreover, the elevated asymmetrical dimethylarginine (ADMA) levels observed in homocysteinaemia may also be implicated in ROS production as suggested in a recent experimental study.[20] Nevertheless, uncoupled endothelial nitric oxide synthase (eNOS) is a major source of superoxide radicals in homocysteinaemia, since under specific conditions (which will be analysed later) eNOS may become a source of superoxide radicals instead of NO.[21,22] The above-mentioned mechanisms increase the production of superoxide radicals that react with NO to form peroxynitrite radicals, leading to low NO bioavailability and endothelial dysfunction. Therefore, under the light of recent data suggesting the inability of conventional antioxidant treatment to modify clinical outcome, Hcy may prove to be a rational therapeutic target for the maintenance of vascular redox balance.[23]

Homocysteinaemia and redox signalling

The pro-oxidative state in homocysteinaemia favours the activation of several inflammatory mediators, such as the nuclear factor-kappa B (NF-κB), responsible for the transcriptional regulation of many proinflammatory genes.[24] This leads to the activation of endothelial cells and induces the expression of factors such as vascular cell adhesion molecule-1,[25] monocyte chemoattractant protein-1,[26] and others, all with a well-known role in atherogenesis. Moreover, the biochemical cascade triggered by the activation of NF-κB results in raised circulating levels of proinflammatory cytokines that also take part in the activation of inflammatory processes inside the vascular wall.[27] Cell culture studies have shown that both oxidative stress and Hcy *per se* have postulated mitogenic effect on vascular smooth muscle cell.[28] On the other hand, both

experimental[29] and clinical[30] studies have also suggested that oxidative stress induces the formation of oxidized low-density lipoproteins and the expression of LOX-1 receptor on endothelial cell surface, inducing foam cells formation and further promoting atherogenesis.[29,30] Therefore, the altered vascular redox signalling has many different effects on endothelial physiology, favouring atherosclerosis in multiple ways.[31]

Homocysteine and endothelial function

Increasing evidence suggests that Hcy may increase cardiovascular risk as a result of its detrimental effect on endothelial function. In subjects with homocystinuria, a significant impairment of endothelium-dependent dilation (EDD) has been well documented,[32] while Hcy has been associated with impaired coronary microvascular dilator function in healthy individuals.[33] Nevertheless, evidence is conflicting in mild homocysteinaemia. Not all observational studies have reported a statistically significant correlation between plasma Hcy and flow-mediated dilation of the brachial artery.[34] However, in clinical models of acute methionine-induced homocysteinaemia, EDD is rapidly blunted in parallel to the elevation of plasma Hcy, although this may be due to the simultaneous elevation of the endogenous eNOS inhibitor ADMA.[22] Indeed, we have shown that the mechanisms of endothelial dysfunction may differ between chronic and acute homocysteinaemia.[22] Chronic homocysteinaemia in humans is accompanied by increased endothelin-1 (ET-1) levels, which is the strongest vasoconstrictor in human vasculature and a key molecule involved in atherogenesis,[35] although ET-1 is not involved in the development of endothelial dysfunction in acute, methionine-induced homocysteinaemia.[36] The activating protein-1-mediated deregulation of ET-1 production is possibly one of the main mechanisms by which homocysteinaemia affects endothelial dysfunction in humans.[37]

Therefore, a more in depth analysis of the biochemical background is necessary to apprehend the effects of homocysteinaemia on vascular endothelium.

Homocysteine and endothelial nitric oxide synthase regulation

The close association of Hcy with endothelial dysfunction is largely dependent on its impact on eNOS coupling.[38] The uncoupled form of eNOS is a major source of superoxide radicals instead of nitric oxide, in the vascular wall.[39] A decreased supply of eNOS substrate L-arginine, observed in homocysteinaemia, has been demonstrated to induce eNOS uncoupling in cell cultures of endothelial cells.[40] Furthermore, the conversion of methionine to Hcy is accompanied by an activation of arginine-protein-methyltranferases, the enzymes responsible for L-arginine methylation to ADMA, which is a known endogenous eNOS inhibitor.[16,22] Stuhlinger *et al.*[41] demonstrated that ADMA is rapidly elevated during methionine loading. Furthermore, it has been shown that both Hcy *per se* and oxidative stress have the ability to down-regulate dimethylarginine-dimethylaminohydrolase, an enzyme responsible for the catabolic degradation of ADMA to arginine.[42,43] Thus, both augmented production and reduced catabolic rate contribute to the increased circulating ADMA levels in homocysteinaemia. ADMA then inhibits eNOS in vascular endothelium, leading to decreased NO bioavailability and impaired endothelial function.[4] In addition, evidence suggests that ADMA may also induce eNOS uncoupling,[44] while it has been introduced as a new risk factor for atherosclerosis. In addition, the raised inflammatory and oxidative burden observed in homocysteinaemia also induces the oxidation of eNOS co-factor tetrahydrobiopterin (BH4),[45] essential for its enzymatic coupling.[21] In states of vascular disease, as in homocysteinaemia, characterized by a raised oxidative burden, BH4 bioavailability is diminished, as BH4 is sensitive to oxidative degradation.[21] As we have previously shown, the intracellular vascular levels of total biopterins and BH4 in vascular inflammation state are reduced, leading to eNOS uncoupling and ROS production.[21] However, 5-MTHF administration, the circulating form of folic acid, has the ability to prevent peroxynitrite-mediated BH4 oxidation and improve eNOS coupling by increasing endothelial BH4 bioavailability in human vessels.[21] Additionally, Moens *et al.*[46] recently showed that folic acid has also substantial direct antioxidant effects, not only in the human vascular wall as we have described previously,[21,38] but also inside myocardial tissue.[46] Indeed, it was demonstrated that folic acid prevents the oxidation of BH4 in myocardial tissue by scavenging ROS (such as peroxynitrite and superoxide) responsible for its oxidation during experimental ischaemia and reperfusion, resulting in an improvement of eNOS dimerization and coupling.[46] Therefore, decreased bioavailability of 5-MTHF may induce eNOS uncoupling in both the vascular wall and myocardial tissue.

Studies on endothelial cells have further shown that Hcy induces threonine-495-phosphorylation of eNOS in a PKC-dependent way, a reaction leading to inactivation of the enzyme.[47] In addition, Hcy down-regulates eNOS in endothelial cell cultures, by modifying the expression of caveolin-1, which is a molecule responsible for the translocation of eNOS in endothelium caveolae.[48]

Further to the functional effects of Hcy on eNOS activity and coupling, evidence suggests that it may also affect eNOS expression in a dose-dependent way,[49] possibly by interfering with intracellular redox signalling.

In addition to these mechanisms relating Hcy and NO synthesis, the increased oxidative stress accompanying homocysteinaemia is also responsible for oxidative degradation of NO, contributing to the development of endothelial dysfunction in homocysteinaemia.[2] As endothelial dysfunction is associated with increased cardiovascular risk,[50] it is likely that therapeutic strategies targeting NO bioavailability via the reduction of Hcy levels may be a rational therapeutic strategy against atherosclerosis.

Further to these clear associations between Hcy, oxidative stress, and endothelial dysfunction in experimental models, it is still unclear whether endogenous variations of plasma Hcy within normal range have any impact on these mechanisms at a clinical level. Despite the clear effect of extreme Hcy levels (observed in chronic or experimental homocysteinaemia) on oxidative stress status[45] and endothelial function,[34] it is still unknown whether variations of plasma Hcy within the normal range have any impact on vascular function, as the results of clinical association studies have been controversial.

Homocysteine and thrombotic mechanisms

The link between homocystinuria and vascular thrombosis is now well established.[51] Severe homocysteinaemia accompanying the genetic defects of homocystinuria is closely linked with recurrent vascular thrombosis.[51] Half of the patients with homocystinuria will come up with a vascular event before the age of 30.[52] However, it seems that the homocystinuria-related pro-thrombotic state mainly concerns veins rather than arteries.[53] The therapeutic lowering of Hcy in subjects with a genetic defect of CBS significantly reduces the risk of thrombotic events, despite the fact that Hcy circulates in higher levels than normal.[53]

Subjects with moderate homocysteinaemia are also characterized by a pro-thrombotic and dysfibrinolytic state, and Hcy levels are an independent predictor of thrombotic events in these individuals.[54] Several mechanisms have been suggested to explain the link between Hcy and pro-thrombotic state. The oxidative injury of endothelium in homocysteinaemia, combined with the lack of vasculo-protective effects of NO, predispose to thrombotic events.[54] Both in animal models and humans, homocysteinaemia is characterized by platelet aggregation and formation of thrombi rich in platelets at the sites of injured endothelium.[55] This endothelium denudation exposes the subendothelial matrix to the lumen surface and leads to platelet activation and thrombosis.[55] Despite the fact that the exact biochemical background is not fully explored, the increased risk of vascular thrombosis may be derived from the vascular oxidative injury and the modification of physiological anti-thrombotic mechanisms.[56] Hcy inhibits the expression of thrombomodulin and induces the expression of tissue factor in endothelial cells.[57] In addition, Hcy promotes the expression of clotting factors II, V, X, and XII[55] and reduces the activation of protein C and antithrombin III in cell cultures.[56] An additional mechanism is possibly the dysfibrinogaemia and the impaired fibrinolytic capacity accompanying homocysteinaemia,[58] while a recent study on human umbilical vein endothelial cells showed that Hcy induces platelet activation, leading to elevation of plasma soluble CD40-ligand.[59] Taken together, all evidence supports that severe homocysteinaemia is strongly linked to a marked pro-thrombotic state. However, it is still unclear whether mild or moderate homocysteinaemia induces thrombosis, and it is rather unlikely that variations of plasma Hcy within the normal range are accompanied by clinically significant variations of the overall thrombotic state.

Homocysteine-lowering treatment and vascular function

Folic acid and vitamins B12 and B6 are essential co-factors in Hcy metabolism.[17] 5-MTHF, the circulating form of folic acid, serves as the methyl-donor in the conversion of Hcy to methionine, an enzymatic reaction catalysed by MS. Vitamin B12 is also pivotally implicated in this reaction as it constitutes an essential co-factor of MS, whereas B6 is a co-factor for CBS, the enzyme responsible for the conversion of Hcy to cystathione and finally to cysteine. Therefore, conditions associated with 5-MTHF, B12, or B6 deficiency have been associated with increased circulating Hcy levels, and these are the first therapeutic targets in the treatment of homocyateinaemia.[16,17] Folate administration has been consistently shown to reduce plasma Hcy even in healthy individuals without elevated Hcy levels.[60] Oral administration of folic acid (0.5–5.0 mg/day) reduces fasting Hcy levels by 25–30%, while supplementation with vitamin B12 (0.02–1 mg/day) yields an additional 7% reduction in Hcy levels.[61] Vitamin B6 has no effect on fasting Hcy levels but significantly lowers post-methionine loading homocysteinaemia.[61] So, over the last years, many studies were focused in Hcy lowering through folic acid administration supplemented with vitamins B6 and B12 in order to improve endothelial function in patients with atherosclerosis, with most of them demonstrating a favourable effect of folates on vascular NO bioavailability.[38,62,63] The doses of folic acid used for Hcy lowering differ among the several clinical trials, varying from doses less than the recommended daily allowance (RDA, 0.4 mg/day) to high doses such as 5 mg/day or even more.[38]

Homocysteine lowering and endothelial function

Hcy lowering by folates and vitamins B6 and B12 induce a significant improvement of vascular NO bioavailability and the overall endothelial function by decreasing plasma Hcy, which is a pro-oxidant molecule in human vasculature as we have already discussed. Notably, evidence suggests that folic acid may reverse vascular dysfunction independently of Hcy lowering.[64] In a recent double-blind crossover trial, high-dose folic acid (10 mg/day) improved endothelial function in patients post-myocardial infarction, independently of any changes in plasma Hcy levels (including total, reduced, or oxidized forms).[64] These findings support our previous observations that folic acid and its circulating metabolite 5-MTHF have direct effects on vascular function in humans, independently of any effects on oxidized or reduced plasma Hcy. Indeed, 5-MTHF has structural similarities to BH4, and evidence suggests that it has the ability to reverse endothelial dysfunction in vascular disease states associated with decreased BH4 bioavailability.[21] In our studies on venous and arterial grafts from patients undergoing coronary by-pass grafting (CABG), we have demonstrated that 5-MTHF ameliorates endothelial NO bioavailability and lowers vascular superoxide production both *in vivo* and *ex vivo*.[21] These properties of 5-MTHF were independent of any changes in Hcy levels and were not due to direct scavenging of superoxide radicals by 5-MTHF (*Figure 1*). In contrast, the improvement of endothelial function was attributed to the capacity of 5-MTHF to scavenge peroxynitrite radicals (which is the main oxidant of BH4 *in vivo*), leading to a remarkable improvement of vascular BH4 bioavailability.[21] Additionally, 5-MTHF improved eNOS dimerization, activity, and coupling, leading to a global improvement of vascular function in patients with advanced atherosclerosis.[21] Given these beneficial effects of 5-MTHF, we moved further to investigate the concentrations at which folic acid exerts its actions. Oral folic acid administration (0.4 or 5 mg/day), 7 weeks before scheduled CABG, was combined with improved vasomotor responses of the grafts (saphenous veins and internal mammary arteries), reduced vascular superoxide production, and improved eNOS coupling (*Figure 1*).[38,65] In line with

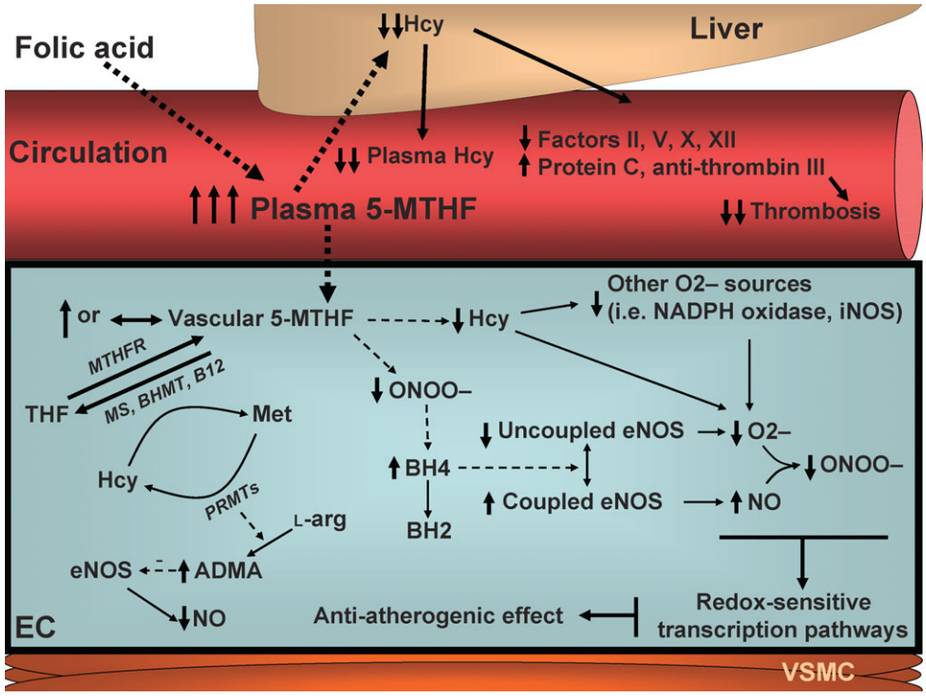

Figure 1 Homocysteine lowering with folate and atherosclerosis. Although folic acid increases plasma 5-methyltetrahydrofolate (5-MTHF), it has a modest effect on intracellular 5-MTHF levels. In vascular endothelium, 5-MTHF lowers intracellular homocysteine (Hcy) and reduces O2− radicals generation by NADPH-oxidase and inducible nitric oxide synthase. 5-MTHF directly scavenges peroxynitrite (ONOO−), protects the oxidation of tetrahydrobiopterin, and improves endothelial nitric oxide synthase (eNOS) coupling. This results in an improvement of vascular redox and endothelial function. Endogenous 5-MTHF is synthesized by methyltetrahydrofolate reductase, which uses tetrahydrofolate (THF) as a substrate. 5-MTHF is converted back to THF in parallel to the conversion of Hcy to methionine (Met) by methionine synthase (MS), which uses vitamin B12 as a co-factor, and the enzyme b-homocysteine-methyltransferase. The conversion of Met back to Hcy induces the activation of protein methyltransferases, which are responsible for the methylation of L-arginine (L-arg) to asymmetrical dimethylarginine, and inhibitor of eNOS. Homocysteine lowering in the liver decreases the synthesis of pro-atherogenic and increases the biosynthesis of anti-thrombotic factors, resulting into a net anti-thrombotic effect. EC, endothelial cell; VSMC, vascular smooth muscle cell.

our previous observations, recent evidence also suggests that folate administration prevents eNOS uncoupling not only in the vasculature but also in myocardium during experimental ischaemia and reperfusion, mainly due to the ability of 5-MTHF to prevent peroxynitrite-induced oxidation of BH4.[46] Indeed, evidence suggests that at high doses, folic acid may rescue myocardial tissue from ischaemia/reperfusion injury by improving myocardial redox state.[46] However, in human vessels, the maximum beneficial effect of folic acid has been observed at much lower doses, equal to the RDA (0.4 mg/day). A further increase in folic acid's dosage (5 mg/day) did not result in further improvement of vascular function. Despite the fact that plasma 5-MTHF increases in accordance with the dose of folic acid, vascular intracellular 5-MTHF levels do not exhibit any further increase with high doses compared with low doses of folic acid (*Figure 1*).[38]

Homocysteine lowering and plaque regression

Recent data links folate administration with a favourable effect on plaque regression. Vitamin administration (folate, B6, and B12) for 1 year has been associated with decreased intima-media thickness in patients at risk for cerebral ischaemia, an effect that was independent of plasma Hcy levels.[66] Moreover, in 2001, Schnyder *et al.*[67] published an important double-blind randomized study regarding the reduced frequency of restenosis after angioplasty with Hcy-lowering treatment. The combination of folic acid (1 mg), B12 (400 μg), and pyridoxine (10 mg) for 6 months post-angioplasty significantly reduced plasma Hcy and rates of restenosis in patients with coronary atherosclerosis. However, the same research group retracted later its previous findings, as the administration of vitamins appeared to have adverse effects on the risk of restenosis post-angioplasty excluding special subgroups as women, diabetics, and subjects with marked homocysteinaemia.[68]

Homocysteine lowering and elastic properties of large arteries

Despite the adverse effects of homocysteinaemia on vascular wall, several trials have failed to report improvement of the elastic properties of the large arteries after Hcy lowering.[69,70] In a cohort of

patients with end-stage renal disease, Hcy-lowering treatment with folic acid failed to improve common carotid artery (CCA) wall properties, such as compliance and distensibility coefficients or stiffness index.[70] In addition, a randomized, placebo-controlled trial evaluating the long-term effect of Hcy-lowering treatment with folic acid plus pyridoxine on arterial stiffness in CCA over a period of 2 years failed to demonstrate any significant effect.[69] In contrast, in another randomized trial, folic acid supplementation for 3 weeks had favourable effects on brachial pulse pressure and systemic arterial compliance.[71] However, these changes did not correlate significantly with Hcy or even folate plasma concentrations.[71] In line with these observations, we[38] have recently shown that low-dose treatment with folic acid (0.4 mg/day) leads to a significant improvement of arterial distensibility in human aorta and carotid arteries, while any further treatment with pharmacological dosage of folic acid (5 mg/day) on top of that has no additional benefit on arterial mechanics. This observation was explained by the saturation of human vascular wall with 5-MTHF after low-dose treatment, and the inability of any further increase in circulating folate levels to induce a further increase of vascular 5-MTHF.

Homocysteine lowering and biochemical markers of endothelial function and oxidative stress

Both chronic and acute, experimentally induced, homocysteinaemia are associated with increased oxidative stress and impaired endothelial function in humans.[22] Folate administration in chronic homocysteinaemia is known to reduce pro-thrombotic state, raise antioxidant defensive mechanisms, and lower systemic oxidative stress markers.[72] Concomitant administration of high doses of antioxidant vitamins in the model of experimental, methionine-induced homocysteinaemia prevented the increase of inflammatory markers such as IL-6.[22] However, despite the fact that antioxidant treatment prevented the methionine loading-induced elevation of oxidative stress, it failed to prevent the impairment of endothelial dysfunction, as a result of the increased ADMA generation observed during the conversion of methionine to Hcy, that contributes to eNOS biochemical uncoupling and diminished NO bioavailability.[22] Since ADMA is closely associated with homocysteinaemia, we anticipated that Hcy-lowering treatment would reduce ADMA levels. However, evidence suggests that folate-mediated Hcy lowering is not followed by a concomitant reduction of plasma ADMA, possibly because folate administration augments the methionine pool, resulting in enhanced protein methyltransferase-mediated conversion of L-arginine to ADMA.[73]

Homocysteine-lowering treatment and clinical outcome: its role in primary and secondary prevention

The available data for the role of homocysteinaemia in CVD reasonably posed the question of reducing cardiovascular risk through Hcy-lowering treatment by means of diet folate fortification. Remarkable results were derived from a recently published study that examined the effect of folate fortification of flour in the USA and Canada.[6] The study showed that folate fortification increased circulating plasma folate levels and reduced plasma Hcy in the general population. Importantly, since 1998, when the flour fortification programme with folate (150 μg folate per 100 g flour) became fully mandatory, an accelerated reduction in stroke mortality was observed in North America.[6] In addition, this reduction in mortality was analogous to that predicted by Hcy lowering.[10] However, the same tendency was not observed in Wales and England, where folate fortification was not introduced.[6] Although a causative relationship between Hcy, folate, and stroke cannot be deduced from these epidemiological observations, the available data are in favour of the hypothesis that folate fortification contributes to the reduction of stroke mortality, at least at the level of primary prevention.

Folic acid is a stable, inexpensive, and safe molecule as recently discussed by Moens *et al.*[61] Folic acid has no toxic effect even at extreme dosages, while the main safety concern lies in the fact that it can mask the diagnosis of pernicious anaemia, because high folic acid levels correct the anaemia but allow the neuropathy to progress undiagnosed, leading eventually to an irreversible degeneration of the spinal cord.[61] It is therefore important to measure B12 levels before the start of folate treatment. In addition, studies in the past argued that folate administration may accelerate the progression of undiagnosed malignancies, since the folate cycle is crucial in the DNA formation and subsequently to cell proliferation.[74] However, Bayston *et al.*[75] recently reported that there should be no concern to avoid folate fortification since folic acid will not enhance the risk on colorectal carcinomas.

Over the last few years, the effect of folate treatment on cardiovascular risk has been questioned.[76] The results of large randomized studies like the VISP,[77] the NORVIT,[7] and HOPE-2[8] trials, examining the effect of long-term folate, B6, and B12 vitamins administration on cardiovascular risk, provided rather disappointing results.

The VISP trial (Vitamin Intervention for Stroke Prevention)[77] was a large randomized clinical trial that started in 1996 comparing the effect of high- vs. low-dose Hcy-lowering treatment on risk for recurrent stroke, coronary events, and death. The VISP trial recruited 3680 adults with non-disabling cerebral infarction who were randomized to receive once-daily either high-dose (25 mg B6, 0.4 mg B12, and 2.5 mg folic acid) or low-dose (200 μg B6, 6 μg B12, and 20 μg folic acid) Hcy-lowering treatment. During a 2-year follow-up period, no significant effect of Hcy-lowering treatment on the pre-specified end points was reached (primary end point: recurrent cerebral infarction, secondary end points: coronary heart disease events or death).[77] Indeed, VISP results were rather disappointing, as this study was the first large clinical trial that failed to demonstrate the benefit of Hcy-lowering treatment with vitamins.[77] However, it did not include a placebo-treated group; therefore, it generated the hypothesis that low-dose treatment may have induced the maximum benefit, and any further increase in the dose had no additional effect.

The NORVIT study[7] examined the effect of folate (800 μg/day) and vitamins B6 (40 mg/day) and B12 (400 μg/day) on the clinical outcome of 3749 patients with a recent myocardial infarction, in a period of 3.5 years. The NORVIT study showed that Hcy-lowering treatment did not have any effect on the survival of

these patients. However, the study had a number of confounding factors that had to be taken into account when interpreting the results. The recorded events in the NORVIT study were mainly during the first post-infarction year, a period for which even statins cannot provide adequate protection. Furthermore, the simultaneous initiation of Hcy-lowering treatment with other drugs known to have strong pleiotropic effects (such as statins and angiotensin-converting enzyme inhibitors), possibly covered the small but possibly existing effect of Hcy-lowering treatment. On the other hand, this study displayed a non-statistically significant rise of cancer in this population, a fact that maybe should be taken into consideration.

In the second large trial, the HOPE-2 study,[8] the effect of Hcy-lowering treatment with folate (2.5 mg/day), vitamin B6 (50 mg/day), and B12 (1 mg/day) on cardiovascular risk was estimated in 5522 patients with vascular disease or diabetes in a 5-year follow-up. Although the study displayed a significant reduction of the risk for stroke, it failed to document any effect of Hcy-lowering treatment on either overall cardiovascular mortality or the combined cardiovascular risk. However, the recruitment criteria did not secure low baseline folate levels in the study population and the statistical power of the trial was significantly weak, especially for those population subgroups living in non-folate-fortified areas. Therefore, this trial also failed to address the question whether low-dose Hcy-lowering treatment (equivalent to folate fortification) could affect cardiovascular risk.

Why clinical trials did not work?

One possible explanation for the negative results of HOPE-2 comes from the study of 5-MTHF behaviour in human vascular wall. As we observed, treatment of CAD patients with folate at dose equivalent to 400 μg/day had striking beneficial effects on vascular wall, by improving endothelial function, lowering intracellular redox state and improving the elastic properties of large vessels *in vivo*.[38] A further increase in the folate dose (5 mg/day) had no additional benefit. This observation was explained by the fact that both high- and low-dose treatments lead to a similar rise in 5-MTHF levels inside the vascular endothelium, in spite of the higher 5-MTHF plasma levels achieved in the group with high folate dose. Therefore, the RDA for folic acid (400 μg/day) has the ability to induce the maximum benefit on vascular function. So, if this amount is received either by flour fortification with folate or by a folate-rich diet, then any further treatment with folate on top of that provides no additional benefit. Therefore, in populations living in folate fortified areas (i.e. North America, which corresponds to ~70% of the population in HOPE-2), it is unlikely to improve vascular function and subsequently cardiovascular risk by administering pharmacological dosage of folic acid, since high-dose treatment increases circulating but not intracellular 5-MTHF in these subjects. Despite the fact that higher folic acid doses than the RDA (400 μg/day) induce no additional vascular effects, it is still unclear whether high-dose folate treatment may have any further effect on human myocardium.[46] Recent evidence suggests that high dosage of folic acid exerts beneficial effects on myocardium in an experimental model of ischaemia and reperfusion.[46] These beneficial cardioprotective effects seem to be due to the preservation of high-energy phosphates and eNOS coupling, improvement of myocardial redox, and prevention of myocardial cell death.[46] Therefore, more clinical studies are needed to fully elucidate the possible benefits of high-dose folic acid treatment on human myocardium.

Another possible mechanism is based on the relation of Hcy to the methylation cycle: folate induces the remethylation of Hcy to methionine, which in turn lowers SAH and increases SAM levels, that regulates all methylation reactions in the cell. Therefore, in the effort to lower Hcy by administrating folate, we enhance the methylation pathway, with several consequences. First, increased methylation of arginine residues raises ADMA levels, which can adversely affect clinical outcome, by inhibiting or uncoupling eNOS.[78] In addition, the altered methylation potential of cells has an impact on gene expression. By hypermethylation of the promoter region of several pro-atherogenic genes (more specifically the methylation of CpG-rich islands that are short regions of DNA in which CG sequence is more frequent), the expression of a wide range of pro-atherogenic molecules is up-regulated.[79] Another possible mechanism is that, through its role in the synthesis of thymidine, folic acid promotes cell proliferation leading to a worsening of atherosclerosis.[79]

All the above mechanisms partly explain why clinical trials on Hcy lowering, such as the VISP, NORVIT, or HOPE-2 and possibly others to be conducted, failed to report any improvement in CVD risk and clinical outcome. Maybe more efficient ways to target Hcy metabolism, other than vitamin supplementation, should be examined. Focusing on increasing Hcy renal clearance or reducing asymmetric dimethylarginine levels would be potentially useful. In addition, other strategies able to increase intracellular 5-MTHF should be developed, to achieve the maximum regulation of intracellular Hcy metabolism.

Homocysteine lowering: when is pharmacological intervention essential?

In the general population, folic acid and B vitamins enhance Hcy metabolism and their administration consistently lowers plasma Hcy levels,[80,81] while adherence to Mediterranean diet, which is associated with high B-vitamins and folate consumption, is associated with lower plasma Hcy, as we have recently shown.[82] Folic acid in dose equal to the RDA (400 μg/day) is associated with a 25–30% reduction of plasma Hcy.[80] An additional 7% reduction can be achieved by cobalamin (B12) 0.02–1 mg/day co-administration.[81] However, current data do not support pharmacological treatment with folic acid and B vitamins in general population.[7,8] In contrast, Hcy-lowering treatment should be considered when homocysteinaemia is present. Moderate homocysteinaemia (fasting plasma total Hcy 15–30 μmol/L) is usually due to poor diet (i.e. vegetarians), mild folate/cobalamin/vitamin B6 deficiency, heterozygosity for CBS defects, hypothyroidism, impaired renal function, or use of drugs affecting Hcy, folate, or cobalamin levels.[17] When the cause of moderate homocysteinaemia is established, then the best treatment is reversal of this cause. In the case of increased Hcy levels due to the presence of MTHFR 677TT genotype, then oral 5-MTHF treatment should

be considered, since this agent does not require any conversion by MTHFR. Although there are still not enough data to support that treatment of moderate homocysteinaemia would reduce cardiovascular risk, the general consensus is that Hcy-lowering strategies targeting specific causes are at least not harmful. Intermediate homocysteinaemia (fasting plasma total Hcy 30–100 μmol/L) is usually the result of moderate/severe cobalamin or folate deficiency or renal failure.[17] Again, diagnosis and reversal of the respective cause should be the main priority, while most of the patients respond well to folate treatment alone or in combination with vitamins B12 and B6. Severe cobalamin deficiency and homocystinuria are the main causes of severe homocysteinaemia (fasting plasma total Hcy >100 μmol/L), and it should definitely be treated accordingly (cobalamin 0.02–1 mg/day), since it is associated with increased pro-thrombotic state.[83] Homocystinuria includes deficiencies in CBS, MTHFR, MS, or methionine synthase reductase, and effects in intracellular cobalamin metabolism. There are currently three recognized modalities of treatment for CBS deficiency:[83] for those who are vitamin-responsive, pyridoxine (50–250 mg/day) in combination with folic acid (0.4–5 mg/day) and/or vitamin B12 (0.02–1 mg/day). For vitamin non-responders, the treatment is with a methionine-restricted, cystine-supplemented diet.[83] Pyridoxine, folic acid, and vitamin B12 treatment may be needed in pyridoxine non-responders, as they are co-factors in methionine metabolism.[83] Betaine, a methyl donor that remethylates homocysteine to methionine, has also been used as an adjunct to treatment.[83]

Homocysteine and cardiovascular risk: future perspectives

It is now widely accepted that, at a cellular level, Hcy exerts a detrimental effect on vascular wall and especially on endothelial cells, by decreasing NO bioavailability, increasing intracellular oxidative stress, and by triggering multiple pro-atherogenic mechanisms. In this context, epidemiological studies have clearly demonstrated that plasma Hcy is an independent risk factor for atherosclerosis. The existing data clearly demonstrate that moderate/severe homocysteinaemia is associated with thromboembolic events and increased risk for atherothrombosis, and it should be treated with folic acid/B vitamins. However, it is still unclear whether Hcy-lowering treatment with folic acid/B vitamins has the potential to improve clinical outcome in subjects with mild homocysteinaemia, and the debate on whether Hcy-lowering treatment with folate has a role in primary or secondary prevention in subjects with plasma Hcy within the normal range is still unresolved. The existing data support that low-dose folate treatment (achieved by folate food fortification) may reduce cardiovascular risk, but any pharmacological treatment with folates on top of that is unlikely to achieve any additional benefit in subjects with plasma Hcy within the 'normal range'. In conclusion, more large-scale clinical trials evaluating the effect of low-dose folate treatment on cardiovascular risk in un-fortified populations are necessary to clarify whether folate food fortification has the potential to clearly decrease cardiovascular risk in the general population.

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