

Homocysteine and arterial thrombosis: Challenge and opportunity

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Summary

The correlation between homocysteine and vascular disease has been assessed in several clinical studies that demonstrated that elevation of plasma total homocysteine (tHcy) was an independent risk factor for atherosclerotic disease. Major advances of homocysteine metabolism disorders have been made during the last few years, encompassing the rare homozygous enzyme deficiencies, as well as more common milder abnormalities. In experimental and clinical studies, a homocysteine-mediated oxidant stress has been shown to trigger platelet activation, in turn leading to a tendency to thrombosis, in patients with severe hyperhomocysteinaemia. Likewise, the hypomethylation hypothesis on acquired hyperhomocysteinaemia (chronic renal disease) and the interrelationship between hyperhomocysteinaemia and impaired fibrinolysis, have added further biological plausibility to the role for hyperhomocysteinaemia in vascular medicine. However, whether hyperhomocys-

teinaemia is causal or a marker of vascular disease, and whether plasma tHcy is only an indicator of the metabolic status remains to be clarified. The role of the intake of some vitamins (folic acid, vit. B₁₂, vit. B₆) on cardiovascular disease (CVD) is poorly understood: in spite of the lowering of homocysteine (Hcy) levels, vitamin supplementation failed to exert significant effects on cardiovascular risk. On the other hand, although some lipid-modifying treatments increase Hcy levels in diabetics, there is no evidence that this attenuates the beneficial effects of such treatments on the cardiovascular risk. Because of these uncertainties in the area, the data available do not provide support for routine screening and treatment for elevated Hcy to prevent CVD.

Keywords

Homocysteine, arterial thrombosis, folate

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Received: June 23, 2009

Accepted after major revision: January 15, 2010

Prepublished online: March 29, 2010

doi:10.1160/TH09-06-0393

Thromb Haemost 2010; 103: 942–961

Introduction

In the late 1960s McCully first suggested a link between homocysteine and vascular disease (1). He observed that an infant with severe hyperhomocysteinaemia leading to homocystinuria as a result of a rare condition of abnormal cobalamin metabolism, exhibited widespread severe arteriosclerosis indistinguishable from the lesions seen in cases of homocystinuria due to cystathionine β-synthase deficiency. Because hyperhomocysteinaemia (HHcy) was common to these two metabolic disorders, McCully proposed that hyperhomocysteinaemia results in arteriosclerotic disease. Seven years later, Wilcken and Wilcken showed that the concentration of homocysteine-cysteine mixed disulfide after a methionine load was slightly higher in coronary heart disease (CHD) patients than in age- and sex-matched controls (2). This pioneering work has led to important studies (3, 4) that allowed for the conclusion that elevations of total homocysteine (tHcy) in men and women are an independent graded risk factor for CHD, cerebrovascular or peripheral vascular disease. In the following years, there has been a debate on whether tHcy elevation is truly a risk factor or an epiphenomenon of atherosclerotic disease (5, 6). The debate has also been extended to those studies that failed to show any relation

between atherosclerotic vascular disease and homozygosity for the C677T mutation in the methylenetetrahydrofolate reductase (MTHFR) gene (► Fig. 1 and its legend), that is associated with mild elevations of plasma tHcy (7). A meta-analysis showed that the C677T mutation of the MTHFR gene is a weak risk factor for cardiovascular disease (8). This conclusion has been challenged, based on the different prevalence of this mutation in various ethnic groups (9). Moreover, because folate and vitamins B₆ and B₁₂ regulate tHcy, the folate status of the population should have been taken into consideration while evaluating the link between tHcy and CHD (10, 11). Finally, in an approach based on the concept of Mendelian randomisation, the observed increase in risk of stroke among individuals homozygous for the MTHFR T allele is close to that predicted from the differences in tHcy concentration conferred by this variant (12). However, the relation between elevated plasma tHcy and vascular disease is stronger in retrospective than in prospective studies (13). In the following paragraphs, we shall review and discuss this large, sometimes conflicting and complex issue, with emphasis on homocysteine metabolism and its regulation, animal models of disorders of homocysteine metabolism, thrombogenic mechanism(s) of homocysteine, and a critical review of the trials available.

Methods

We have approached the issue of homocysteine and its relevance in clinical practice with emphasis on two comprehensively reviewed relevant issues. In both cases, a series of key terms has been identified for an appropriate search strategy.

- 1) The actual strength of the evidence of the association of hyperhomocysteinaemia and arterial thrombosis. The analysis of this issue has been based on of epidemiological and intervention studies published up to the end of 2009.
- 2) The biological plausibility and the role of moderate hyperhomocysteinaemia in arterial and venous thrombosis and in atherothrombosis. This issue has been thoroughly reviewed in a series of papers concerning homocysteine in coagulation, fibrinolysis, as well as platelet and endothelial function.

Search strategy and evidence acquisition

Using the key terms terms of: Hcy and atherotrombosis; Hcy and coagulation, Hcy and fibrinolysis, Hcy and platelet activation, Hcy and endothelial function, Hcy and nitric oxide (NO), Hcy and vitamins, Hcy and lipid-lowering drugs, as to thrombogenic mechanism(s), and of: Hcy ischaemic stroke and transient ischaemic attack (TIA); Hcy and myocardial infarction (MI); Hcy and peripheral arterial disease; Hcy and unstable angina; retrospective

studies; prospective studies; intervention trials; we searched the Medline database as well as the trial register of the Cochrane group to identify studies published in the area up to the end of 2009. For an in-depth scrutiny of the information provided by the individual papers, their references were also critically reviewed. In each case and for each report, in addition to clinical relevance, emphasis has been put on the inherent potential limitations of the individual analyses. We have found the following evidence synthesis.

1. The thrombogenic mechanism of homocysteine

Abnormalities of platelet/endothelial cells

In homocystinuric patients as well as in non-human primates, platelet survival has been reported abnormally low (14, 15). In keeping with this, increased platelet stickiness has been shown in the blood of homocystinuric patients and *in vitro* after the addition of homocysteine to plasma (14). The latter observation has been challenged (15). Abnormally high platelet aggregation after homocysteine exposure has been documented. This concept has been challenged as well (14, 15). Discrepancies also exist about the effect of homocysteine thiolactone (HTL), the cyclic oxidation product of homocysteine, on platelet function. Early studies suggested that HTL had only a very small effect on platelet aggregation. However,

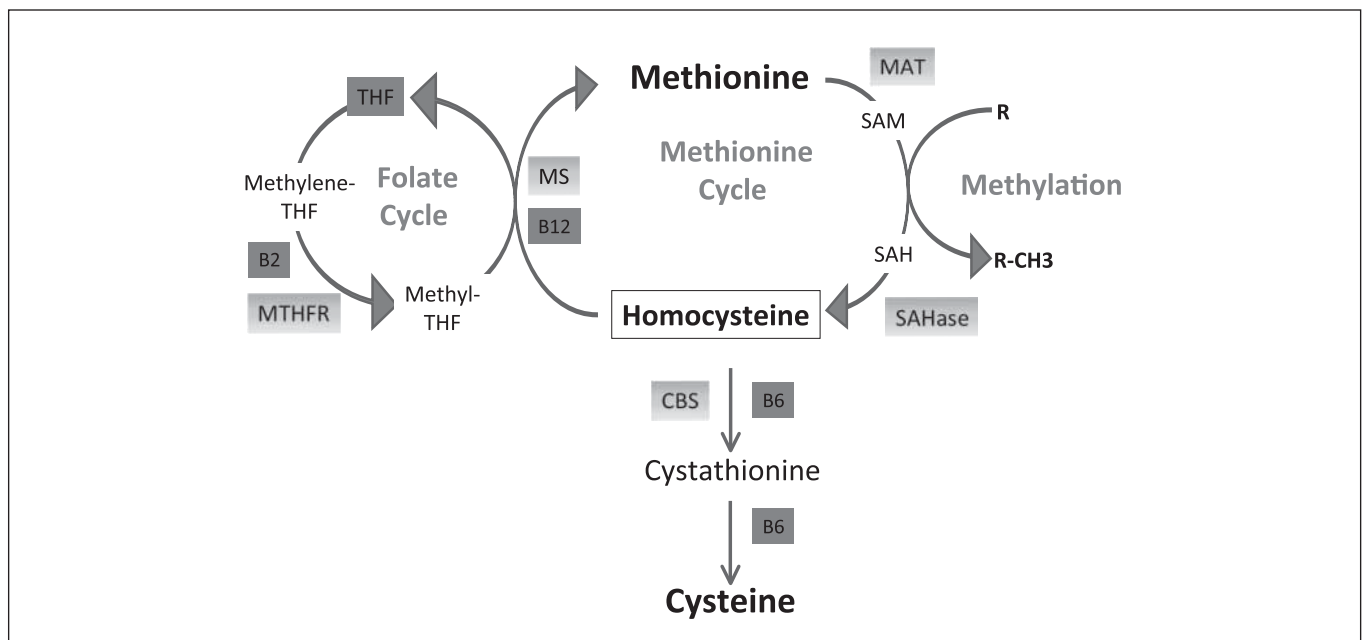


Figure 1: Genetic defects in the enzymes of Hcy metabolism that cause Hcy. Mutations in cystathione- β -synthase (CBS) leading to homocystinuria are associated with huge excess levels of Hcy (>100 μ M). While homocystinuria is rare, mild elevations of Hcy (plasma concentrations of 15–25 μ M) are common in many populations. In addition to diet and drugs (Table 1), these slight elevations are associated with common single nucleotide

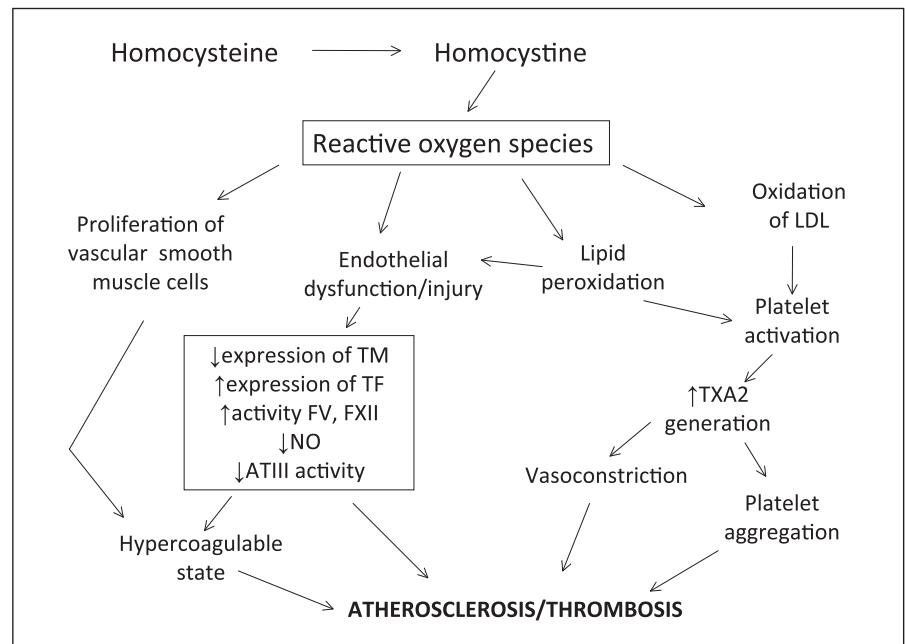
polymorphisms in enzymes in the Hcy metabolic pathway: 5,10-methylenetetrahydrofolate reductase (MTHFR) defect; thermolabile variant of MTHFR C677T (50% activity); methionine synthase (MS) defect A2756G; Cobalamine/methylcobalamine conversion defect (Cbl C, D, E, F, G). THF: tetrahydrofolate; B₂: vitamin B₂; B₆: vitamin B₆; MAT: methionine adenosyl transferase; SAM: S-adenosyl methionine; SAH: S-adenosyl homocysteine.

at variance with the inactive salt (hydrochloride form), the free base of HTL fosters platelet aggregation. On the other hand, in synergism with other methyltransferase inhibitors, HTL inhibits platelet aggregation. A study that sheds light on such discrepancies is that of Stamler et al. (16). These authors have shown that while increasing platelet adhesion to endothelial cells (EC) as a consequence of its toxic effect on the endothelium itself, homocysteine does not cause platelet aggregation *per se*. EC produces endothelium-derived relaxing factor (NO) that reacts with homocysteine to form 5 nitroso-homocysteine (SNOHO). The latter is a strong antiplatelet agent with a 5 minute half-life (for comparison, NO half-life is about 5–30 seconds). Therefore in normal conditions, the toxicity of homocysteine is prevented by the formation of SNOHO. When homocysteine levels saturate the available amounts of NO, unmodified homocysteine becomes available, thus causing endothelial injury, with a consequent reduction of the NO production, followed by reduced formation of SNOHO and in turn, of the antiplatelet potential. These data suggest a role for an oxidant stress in the risk of thrombosis related to elevated levels of homocysteine. In particular, the possibility that hydrogen peroxide is responsible for the cellular damage induced by homocysteine has been analysed in detail by Starkebaum and Harlan (17). Copper-catalysed auto-oxidation of cysteine in alkaline media leads to the reduction of oxygen and the generation of hydrogen peroxide. In view of this, Starkebaum and Harlan showed that, in a cell free system, increasing concentrations of copper (1–50 M), increases homocysteine oxidation in a dose-dependent fashion. The addition of catalase to the system reduced oxygen consumption by nearly one half, thus suggesting that H₂O₂ was formed during the reaction. However, H₂O₂ did not accumulate in the presence of homocysteine, suggesting that homocysteine itself can scavenge H₂O₂. Interestingly, concentrations of 0.05–5 fM Cu₂ increased the rate of H₂O₂ formation, whereas at concentrations above 5 pM the formation of H₂O₂ was reduced, probably due to Cu catalysed reduction of H₂O₂ to water. The relationship between copper, homocysteine and endothelial injury was further documented by the observation that a dose-dependent lysis of cultured bovine aortic endothelial cells occurred only when homocysteine (up to 5 mM) was added to endothelial cells in the presence of copper (2 pM). As for endothelial cells, the possibility has been explored that an oxidant stress may be involved in the platelet activation mediated by homocysteine. When severely increased in plasma (>100 pM/l), homocysteine can leak into the urine causing homocystinuria. In this severe form of hyperhomocysteinaemia, premature arteriosclerosis, and arterial and venous thrombosis are common findings. Biochemical measurements of urinary metabolites and clinical trials with aspirin, indicate that enhanced biosynthesis of thromboxane A₂ (TXA₂) by platelet arachidonic acid is a major contributor to the risk of thrombosis associated with several risk factors (18). We have previously reported (19) an abnormally high urinary excretion of 11-dehydro-thromboxane B₂ and of 2,3-dinor-thromboxane B₂, major enzymatic derivatives of TXA₂ in patients with homozygous homocystinuria due to cystathionine β-synthase deficiency (CBS). The abnormally high excretion of this valuable index of *in vivo*-platelet activation, was independent

of the presence of major cardiovascular risk factors. The possibility of a platelet origin for the abnormally high TXA₂, was suggested by the results of a cumulative inhibition of the excretion of TX metabolites by 50 mg/day of aspirin. On the other hand, 500 mg of the antioxidant drug probucol resulted in a 40–60% drop in the TX metabolite excretion. Interestingly, the effects of probucol were not dependent on reduction of plasma cholesterol levels. Since oxidation of lipoproteins – which can induce platelet TXA₂ formation – is facilitated by homocysteine, inhibition of TXA₂ production by probucol is consistent with the possibility that oxidised lipoproteins contribute to an increased arachidonic acid metabolism in platelets of patients with CBS. It is worth stressing that lipid peroxidation can be initiated not only by hydrogen peroxide, but also by superoxide and hydroxyl radicals, which can be generated during oxidation of thiols. Recently, an alternative metabolic route for arachidonic acid oxidation has been focused. *In vivo*, arachidonic acid derived from membrane phospholipids can undergo autooxidation, generating a mixture of hydroperoxides, epoxides, and cyclic peroxides (18). Of particular interest are the isoprostanes. They form a family of prostaglandin-related compounds acting as autacoids: presently, the urinary isoprostane index provides a reliable method to estimate *in vivo*-lipid peroxidation in various diseases. In addition, some of these isoprostanes are also able to cause activation of platelets and enhanced biosynthesis of TXA₂ linking in this manner *in vivo*-oxidative stress and the risk of thrombosis associated with several risk factors. In subjects with homozygous homocystinuria due to CBS, we (20) have reported an abnormally high *in vivo*-oxidative stress, as reflected by the excretion of major isoprostanes, leading to platelet activation. Similar results have been reported in patients with early-onset thrombosis and 677TT methylenetetrahydrofolate reductase genotype (21). In the latter subjects, the abnormally high *in vivo*-oxidative stress leading to an abnormally high TXA₂ biosynthesis, is corrected by 5-methyl-tetrahydrofolate supplementation.

Postulated atherogenic and thrombogenic mechanisms of homocysteine involving the generation of reactive oxygen species and effects on endothelial cells, vascular smooth muscle cells and platelets, as summarised in references (22–25), are shown in ►Figure 2. In this model, the observation (22) that several homocysteine effects in vascular smooth-muscle cells are due to the activation by oxygen free radicals of the transcription factor NF-κB; an essential factor for proliferation of such cells, is emphasised. The limitation of this model, however, is that it is mostly based on data from patients with homocystinuria. Data from patients with chronic renal failure and moderate HHcy (26) suggest alternative mechanisms to be followed (►Fig. 3). In red cells from uraemic patients with moderate HHcy, an abnormal adenosyl-methionine (AdoMet)/adenosyl-homocysteine (AdoHcy) ratio has been documented. Under physiological conditions, AdoHcy is rapidly hydrolysed to homocysteine and adenosine, through the action of AdoHcy-hydrolase, with a rapid removal of its products. Since thermodynamics favors AdoHcy biosynthesis rather than its hydrolysis, an increase of Hcy concentration may lead to AdoHcy accumulation within the cells, causing the reduction of AdoMet/

Figure 2: Atherogenic and thrombogenic mechanisms. Postulated atherogenic and thrombogenic mechanisms of homocysteine involving the generation of reactive oxygen species and effects on endothelial cells, vascular smooth muscle cells and platelets. A major role in this model is played by NF- κ B. See text for further details.



AdoHcy ratio, and impaired AdoMet-dependent methyltransferase reactions. Protein methylation is a key step in the repair of age-damaged proteins. These observations suggest that AdoHcy may be a mediator of *in vivo*-effects of homocysteine and that, in addition to their ability to determine cell dysfunction/lysis through the formation of free radicals, high levels of thiol-containing compounds can impair cell repair, thus providing a new mechanism leading to atherosclerosis, thrombosis and aging.

provided additional insight into the mechanism by which homocysteine impairs thrombomodulin activity in HUVEC. They found a time- and dose-dependent inhibitory effect of homocysteine on thrombomodulin cofactor activity. Thrombomodulin activity, measured as protein C activation, was reduced to 5% or 10% of the baseline values after incubation with 10 mM homocysteine when the activity was determined on the cell surface or in whole cell extracts, respectively. Thus, their data suggest that the effect of homocysteine on thrombomodulin activity is due to a reduction of the native thrombomodulin, which is followed by a compensatory

Abnormalities of coagulation/fibrinolysis

Ex vivo-data from patients with CBS indicate a variety of abnormalities of the coagulation system, which suggest a hypercoagulable state in this setting (14, 15). Reduced levels of antithrombin (AT), of factor VII, and protein C have been reported. In addition, *in vitro*-studies provide a biochemical background for a hypercoagulable syndrome in hyperhomocysteinaemia. Factor V activity and prothrombin activation have been shown to be increased by the addition of 0.5–10 mM homocysteine to cultured bovine aortic endothelial cells. While 8 hours (h) were required to detect an increase in factor V activity in the presence of 10 mM homocysteine, 24–30 h were needed at lower homocysteine concentrations (0.1 or 0.5 mM). These pro-coagulant effects may be consequent to changes in the activity of the natural anticoagulant protein C. A direct effect of homocysteine on protein C activation was subsequently shown (14). Incubation of bovine or human umbilical vein cultured endothelial cells with 7.5 to 10 mM homocysteine for 6–9 h inhibited protein C activation by 90%. This effect might be partially explained by a competitive inhibition by homocysteine of the thrombomodulin thrombin interaction. Hayashi et al. (27)

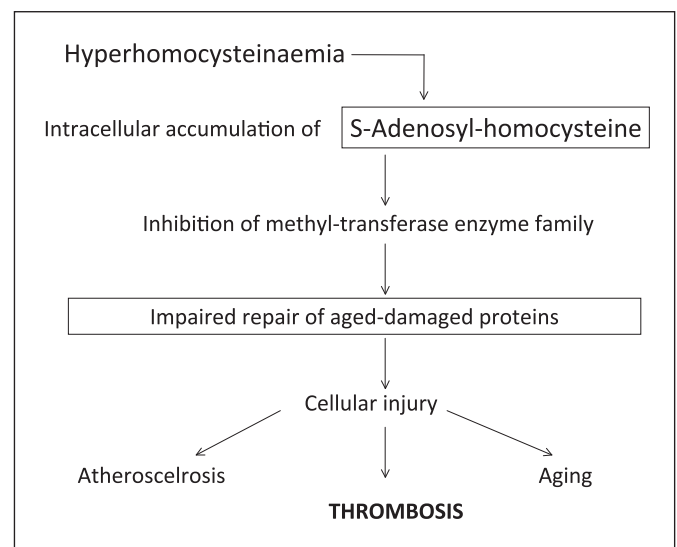


Figure 3: Adverse effects. Postulated adverse effects of homocysteine according to the hypomethylation hypothesis. See text for further details.

increase in the expression of the thrombomodulin gene and of the total thrombomodulin level. Finally, in a cell-free system, they showed that homocysteine inhibits the binding of thrombomodulin to thrombin as the result of a decreased binding capacity of the reduced thrombomodulin. Also tissue factor (TF), a central protein of the extrinsic pathway of the coagulation, has been indicated as a potential target for the thrombogenic action of homocysteine (28). Incubation of human umbilical vein endothelial cells with 10 mM homocysteine for 8 h increased TF activity by six-fold. A clear-cut concentration-dependency of this effect (0.1–10 mM homocysteine) was also shown. Homocysteine-induced TF activity was inhibited by N-ethylmaleimide, thus indicating that the sulphur group was instrumental in the observed phenomenon. Finally, the ability of homocysteine to induce TF mRNA, measured by a quantitative polymerase chain reaction technique, revealed an almost four-fold increase in the TF mRNA, when comparing HUVEC and fibroblasts after 3 h incubation with 10 mM homocysteine.

The effect of homocysteine on AT has been explored with emphasis on the interaction between AT and heparin-like glycosaminoglycans in porcine aortic endothelial cells (29). The data show that the maximal AT binding capacity to heparin sulphate is reduced to 30% of normal after a 24-h incubation with 1 mM homocysteine. This effect is dependent on sulphhydryl groups and appears to involve the generation of hydrogen peroxide, being prevented by catalase, but not by superoxide dismutase.

The interference of homocysteine with the fibrinolytic system has been first addressed by Hajjar (30). Treatment of cultured HUVEC with 1.5 to 7.5 mM homocysteine induced a 65% decrease in cellular binding sites for tissue plasminogen activator (t-PA), and this was shown to be due to a reduction of the binding sites for t-PA on the 40 kDa receptor protein. Interestingly, the receptor capacity to bind plasminogen was not altered, thus suggesting that the receptor had been altered only in the specific domain responsible for the binding of t-PA, and that the COOH-terminal domain, which binds plasminogen, remains unmodified. Along the same line, Harpel et al. (31), also focused on the potential modulation of fibrinolysis by homocysteine addressing the interaction of plasmin modified fibrin and lipoprotein (a), Lp(a). Because of its homology to kringle W of plasminogen, Lp(a) interferes with fibrinolysis by competing with plasminogen binding sites. Harpel et al. showed that homocysteine enhances the binding of Lp(a) to fibrin, especially to plasmin-treated fibrin. This binding was inhibited by ϵ -aminocaproic acid, thus indicating lysine binding site specificity, and was also increased by cysteine, glutathione and N-acetylcysteine. Using gel electrophoresis and immunoblotting, the authors observed changes in the mobility of the apo(a) moiety after exposure to homocysteine. They concluded that homocysteine alters the structure of apo(a), possibly exposing additional binding sites for the fibrin surface. As a consequence, the thrombotic potential of Lp(a) is increased by homocysteine. Together these data show that elevated Hcy levels result in increased oxidant stress, endothelial dysfunction, and a hypercoagulable state with increased thrombogenicity, all acting in combination to promote atherothrombosis (32).

As to clinical data, the interference of homocysteine with the fibrinolytic system has been addressed in a series of reports. In addition to its mitogenic potential (33, 34), Hcy has been documented to exert pro-coagulant effects by increasing TF activity (28), inducing plasminogen activator inhibitor-1 (PAI-1) (35) and altering t-PA activity (25). In individuals without evidence of coronary artery disease (CAD), an increase in Hcy levels has been associated with raised plasma levels of D-Dimer (36) as well as of t-PA and PAI-1 antigen (37, 38). In 56 patients with CAD, with hyperhomocysteinaemia and with a history of a first myocardial infarction, Speidl et al. (39) have shown a lower than normal t-PA activity that was independent of cardiovascular risk factors and medical treatment. In their study, Hcy plasma levels inversely correlated with t-PA activity ($P < 0.05$), a finding that allowed the authors to argue for homocysteine lowering therapies as being a means to increase fibrinolytic activity and help avoid newer atherothrombotic events. With one exception (40), the correlation of tHcy with PAI-1 and t-PA antigen levels has been confirmed and extended to other clinical settings (41, 42). In keeping with this, the acute increase in plasma tHcy levels after an oral methionine load has been reported to decrease the fibrinolytic activity of the euglobulin plasma fraction (43), while enhancing substance P-induced t-PA release (44). Further elucidation of the reduced fibrinolytic potential of plasma from patients with moderate hyperhomocysteinaemia has been provided by Colucci et al. (45). In 176 patients with previous venous thromboembolism (58 with moderate hyperhomocysteinaemia [HHcy], and 118 with normal tHcy levels [NHcy]), they have found that plasma levels of thrombin activatable fibrinolysis inhibitor (TAFI) and of factor VIII (FVIII) were higher in HHcy than in NHcy, whereas PAI-1, fibrinogen and the endogenous thrombin potential were similar. They have also found that fibrinolysis time was longer in HHcy than in NHcy patients, but this difference disappeared when the assay was performed in the presence of activated TAFI inhibitor, potato tuber carboxypeptidase inhibitor (PTCI). Colucci et al. have also addressed the issue whether or not homocysteine impairs fibrinolysis by altering the fibrinogen structure, thus leading to the formation of fibrin clots more tightly packed and more resistant to lysis (46–48).

In experiments in which native fibrinogen has been replaced by purified fibrinogen, the authors concluded that alterations of fibrinogen structure did not contribute to the hypofibrinolysis of HHcy plasma samples, the acute increase of tHcy either *in vivo* (after an oral methionine load) or *in vitro* (after incubation of normal plasma with 0.5 mM DL-Hcy) having no effects on fibrinolysis or TAFI levels. However, the data by Colucci et al. have been obtained in response to low (i.e. physiological) concentrations of t-PA. In contrast, tHcy concentrations inversely correlated to clot permeability and susceptibility to lysis in healthy subjects and in patients with CAD when pharmacological (i.e. > 10-fold higher than those employed by Colucci et al.) concentrations of t-PA, were employed (49, 50). Whether such high plasma levels of PAI-1 (which are poorly sensitive to the main natural regulatory mechanisms) would translate into appreciable clinical changes in the fibrinolysis rate of patients with moderate hyperhomocysteinaemia remains to be established.

2. Homocysteine and cardiovascular disease: Epidemiological evidence

Cross-sectional and case-control studies indicate an association between plasma concentrations of Hcy and the extent of carotid, coronary and peripheral vascular disease (51–53). However, the variables measured in these studies are only surrogate measures of CVD. Recently, the whole area relating Hcy to CHD has been reviewed in detail (54).

Retrospective studies (Table 1)

In a meta-analysis of 27 observational studies relating homocysteine to arteriosclerotic vascular disease and of 11 studies of folic acid effects on tHcy levels studies, Boushey et al. (4) have determined the risk of elevated tHcy levels for arteriosclerotic vascular disease, have estimated the reduction of tHcy by folic acid, and have calculated the potential reduction of CAD mortality by increasing folic acid intake. Three prospective and six high quality population-based case-control studies, five cross-sectional and 13 case-control studies, including about 4,000 subjects and all dealing with CAD, cerebrovascular disease, and peripheral arterial vascular disease, were evaluated. Elevations in tHcy were an independent graded risk factor for arteriosclerotic vascular diseases. The odds ratio (OR) for CAD of a 5 μM tHcy increment was 1.6 (95% con-

fidence interval [CI]: 1.4 to 1.7) for men and 1.8 (95% CI: 1.3 to 1.9) for women. A total of 10% of the population's CAD risk appeared attributable to tHcy. The OR for cerebrovascular disease (5 μM tHcy increment) was 1.5 (95% CI: 1.3 to 1.9). Peripheral arterial disease also showed a strong association. Increased folic acid intake (approximately 200 $\mu\text{g}/\text{day}$) reduced tHcy levels by approximately 4 μM . Assuming that lower tHcy levels decrease CAD mortality, the authors calculated the effect of (1) increased dietary folate, (2) supplementation by tablets, and (3) grain fortification. Under different assumptions, 13,500 to 50,000 CAD deaths annually could be avoided; fortification of food had the largest impact. Thus, the authors concluded that hyperhomocysteinaemia (defined as plasma Hcy levels greater than the 90th or 95th percentile of levels in controls) was associated with an increased risk of atherosclerotic disease. An increase in basal total plasma Hcy levels of 5 μM was associated with 60% and 80% increased risk of CHD in men and women, respectively, similar to the effect of raising cholesterol by 0.5 mM (20 mg/dl) (4).

Subsequent observational studies have provided consistent support for the association between hyperhomocysteinaemia and atherosclerotic vascular disease. The European Concerted Action Project, that included 750 men and women with arterial vascular disease and 800 controls, was aimed at establishing the magnitude of the vascular disease risk associated with an increased plasma homocysteine level and at examining interactions between elevated plasma homocysteine level and conventional risk factors. After a standardised methionine-loading test, the relative risk for

Table 1: Homocysteine and vascular disease: Major epidemiological evidence: retrospective studies.

Author (ref)	Study description/Results/Conclusion/Comments
Boushey CJ et al. (4)	<p>Study description: Meta-analysis of 27 observational studies relating homocysteine to arteriosclerotic vascular disease (3 prospective and 6 high quality population-based case-control studies, 5 cross-sectional and 13 case-control studies, all dealing with CAD, cerebrovascular disease, and peripheral arterial vascular disease), and of 11 studies of folic acid effects on tHcy levels that included \approx 4,000 subjects.</p> <p>Results: Elevations in tHcy were an independent graded risk factor for arteriosclerotic vascular diseases. The odds ratio for CAD of a 5 μM tHcy increment was 1.6 (95% confidence interval [CI]: 1.4–1.7) for men and 1.8 (95% CI: 1.3–1.9) for women. A total of 10% of the population's CAD risk appeared attributable to tHcy. The odds ratio for cerebrovascular disease (5 μM tHcy increment) was 1.5 (95% CI: 1.3–1.9). Peripheral arterial disease also showed a strong association. Increased folic acid intake (approximately 200 $\mu\text{g}/\text{day}$) reduced tHcy levels by approximately 4 μM. Assuming that lower tHcy levels decrease CAD mortality, the authors calculated that under different assumptions, 13,500 to 50,000 CAD deaths annually could be avoided; fortification of food had the largest impact.</p> <p>Conclusion: Hyperhomocysteinaemia (defined as plasma Hcy levels greater than the 90th or 95th percentile of levels in controls) is associated with an increased risk of atherosclerotic disease. An increase in basal total plasma Hcy levels of 5 μM is associated with 60% and 80% increased risk of CHD in men and women, respectively, similar to the effect of raising cholesterol by 0.5 mM (20 mg/dl) (4).</p>
Graham IM et al. (55)	<p>Study description: The ECAT Project included 750 men and women with arterial vascular disease and 800 controls was aimed at establishing the magnitude of the vascular disease risk associated with an increased plasma homocysteine level and at examining interactions between elevated plasma homocysteine level and conventional risk factors.</p> <p>Results: The relative risk for vascular disease in the top fifth compared with the bottom four fifths of the control fasting total homocysteine distribution was 2.2 (95% CI: 1.6–2.9). Methionine loading identified an additional 27% of at-risk cases. A dose-response effect was noted between total homocysteine level and risk. The risk was similar to and independent of that of other risk factors, but interaction effects were noted between homocysteine and these risk factors; for both sexes combined, an increased fasting homocysteine level showed a more than multiplicative effect on risk in smokers and in hypertensive subjects. Red blood cell folate, cobalamin, and pyridoxal phosphate, were inversely related to total homocysteine levels. Compared with non-users of vitamin supplements, the small number of subjects taking such vitamins appeared to have a substantially lower risk of vascular disease, a proportion of which was attributable to lower plasma homocysteine levels.</p> <p>Conclusion: An increase in plasma Hcy confers an independent risk of vascular disease similar to that of smoking or hyperlipidaemia, and powerfully increases the risk associated with smoking and hypertension.</p>

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Prospective nested case-control studies (Table 2 and Table 3)

Table 2

Among the participants in the Physicians' Health Study (PHS) (5) a nested case-control study was carried out in which blood samples, collected from a total of 14,916 male physicians aged 40 to 84 years with no prior MI or stroke who were followed up for five years, were used prospectively. Samples from 271 men who subsequently developed MI were analysed for homocyst(e)ine levels together with paired controls, matched by age and smoking. Levels of homocyst(e)ine were higher in cases than in controls (11.1 ± 4.0 [SD] vs. 10.5 ± 2.8 μM ; $P = 0.03$). The difference was attributable to an excess of high values among men who later had MIs. The relative risk for the highest 5% vs. the bottom 90% of homocyst(e)ine levels was 3.1 (95% CI: 1.4 to 6.9; $P = 0.005$). After additional adjustment for diabetes, hypertension, aspirin assignment, Quetelet's Index, and total/high-density lipoprotein cholesterol, this relative risk was 3.4 (95% CI: 1.3 to 8.8; $P = 0.01$). Thirteen controls and 31 cases (11%) had values above the 95th percentile of the controls. Thus, moderately high levels of plasma homocyst(e)ine were interpreted to be associated with subsequent risk of MI independent of other coronary risk factors. The authors also suggested that, because high levels can often be easily treated with vitamin supplements, homocyst(e)ine may be an independent, modifiable risk factor.

Based on the concept that, in the PHS, among patients with CHD, the association of homocysteine was limited to subjects above a threshold level of homocysteine. Arnesen et al. (57) conducted a nested case-control study among the 21,826 subjects, aged 12–61 years, who were surveyed in the municipality of Tromsø, Norway. Among those free from MI at the screening, 123 later developed CHD. Four controls were selected for each case. They

found that the level of homocysteine was higher in cases than in controls (12.7 ± 4.7 vs. 11.3 ± 3.7 μM (mean \pm SD); $P = 0.002$); and that the relative risk for a 4 μM increase in serum homocysteine was 1.41 (95% CI: 1.16–1.71). Adjusting for possible confounders reduced the relative risk to 1.32 (95% CI: 1.05–1.65). There was no threshold level above which serum homocysteine is associated with CHD events. Thus, they concluded that in the general population serum total homocysteine was an independent risk factor for CHD with no threshold level.

Another nested case-control study was carried out among participants to the British United Provident Association (BUPA) in which 21,520 men aged 35 to 64 years were prospectively studied. In this setting, Wald et al. (58) measured homocysteine levels in stored serum samples and analysed data from 229 men without a history of ischaemic heart disease (IHD) at study entry who subsequently died of IHD and in 1126 age-matched control subjects. They found that serum homocysteine levels were significantly higher in men who died of IHD than in men who did not (mean, 13.1 vs. 11.8 μM ; $P < 0.001$). The risk of IHD among men in the highest quartile of serum homocysteine levels was 3.7 times (or 2.9 times after adjusting for other risk factors) the risk among men in the lowest quartile (95% CI: 1.8–4.7). There was a continuous dose-response relationship, with risk increasing by 41% (95% CI: 20%–65%) for each 5 μM increase in the serum homocysteine level. After adjustment for apolipoprotein B levels and blood pressure, this estimate was 33% (95% CI: 22%–59%). They also clarified that the difference with the meta-analysis of the retrospective studies of homocysteine level and MI (4), where the age-adjusted association was stronger, was based on the fact that the participants were younger in that setting. Therefore the authors concluded that a general increase in consumption of the vitamin folic acid (which reduces serum homocysteine levels) would, therefore, be expected to reduce mortality from IHD.

In another nested case-control study, Bots et al. (59) examined the relationship of homocysteine level to incident MI and stroke among older subjects. The subjects were participants in the Rotterdam Study, a cohort study among 7,983 subjects residing in the Ommoord district of Rotterdam, the Netherlands. Baseline examinations were performed from March 1, 1990, to July 31, 1993. The analysis was restricted to MI and stroke that occurred before December 31, 1994. A total of 104 patients with a MI and 120 with a stroke were identified with complete data. Control subjects consisted of a sample of 533 subjects drawn from the study base, free of MI or stroke. Non-fasting tHcy levels were measured. When the data were adjusted for age and sex, it appeared that the risk of stroke and of MI increased directly with total homocysteine. The linear coefficient suggested a risk increase by 6% to 7% for every 1 μM increase in total homocysteine. The risk by quintiles of total homocysteine level was significantly increased only in the group with levels above 18.6 μM (upper quintile): ORs were 2.43 (95% CI: 1.11–5.35) for MI and 2.53 (95% CI: 1.19–5.35) for stroke. Associations were more pronounced among subjects with hypertension. Thus, the authors concluded that among elderly subjects, an elevated homocysteine level is associated with an increased risk of CHD.

Table 2: Homocysteine and vascular disease: Major epidemiological evidence – prospective nested case-control data. High levels of plasma homocyst(e)ine are associated with subsequent risk of MI or stroke independent of other coronary risk factors.

Author (ref)	Study description/Results/Conclusion/Comments
Stampfer MJ et al. (56)	<p>Study description: Among the participants in the PHS, samples from 271 men who subsequently developed MI were analysed for homocyst(e)ine levels together with paired controls, matched by age and smoking.</p> <p>Results: Levels of homocyst(e)ine were higher in cases than in controls ($P = 0.03$). The difference was attributable to an excess of high values among men who later had MIs. The relative risk for the highest 5% vs. the bottom 90% of homocyst(e)ine levels was 3.1 ($P = 0.005$). After adjustment for diabetes mellitus, hypertension, aspirin assignment, Quetelet's Index, and total/high-density lipoprotein cholesterol, this relative risk was 3.4 ($P = 0.01$).</p> <p>Conclusions: Moderately high levels of plasma homocyst(e)ine are associated with subsequent risk of MI independent of other coronary risk factors.</p> <p>Comments: Because high levels can often be easily treated with vitamin supplements, homocyst(e)ine may be a modifiable risk factor.</p>
Arnesen et al. (57)	<p>Study description: Among 21,826 subjects, aged 12–61 years, in the municipality of Tromsø, Norway, all free from MI at the screening, 123 later developed CHD. Four controls were selected for each case.</p> <p>Results: The level of homocysteine was higher in cases than in controls ($P = 0.002$); the RR for a 4 μM increase in serum homocysteine was 1.41 (95% CI: 1.16–1.71). Adjusting for possible confounders the RR was reduced to 1.32 (95% CI: 1.05–1.65). There was no threshold level above which serum homocysteine was associated with CHD events.</p> <p>Conclusions: In the general population serum total homocysteine is an independent risk factor for CHD with no threshold level.</p>
Wald et al. (58)	<p>Study description: Among 21,520 men aged 35 to 64 years included in the British United Provident Association (BUPA) prospective study, homocysteine levels were measured in stored serum samples from 229 men without a history of IHD at study entry who subsequently died of IHD and 1,126 age-matched control subjects.</p> <p>Results: Serum homocysteine levels were significantly higher in men who died of CHD than in men who did not ($P < 0.001$). The risk of HD among men in the highest quartile of serum homocysteine levels was 2.9 times, after adjusting for other risk factors, the risk among men in the lowest quartile. There was a continuous dose-response relationship, with risk increasing by 33%, after adjustment for apolipoprotein B levels and blood pressure, for each 5 μM increase in the serum homocysteine level.</p> <p>Conclusions: The association between serum homocysteine level and CHD is likely to be causal. A general increase in consumption of the vitamin folic acid would be expected to reduce mortality from IHD.</p> <p>Comments: The relationship between serum homocysteine level and IHD seems to be stronger in younger than in older persons.</p>
Bots et al. (59)	<p>Study description: The subjects were participants in the Rotterdam Study, a cohort study among 7,983 subjects residing in the Netherlands. The relationship of homocysteine level to incident MI and stroke among older subjects were analysed. 104 patients with a MI and 120 with a stroke were identified with complete data. Control subjects consisted of a sample of 533 subjects from the study base, free of myocardial infarction and stroke.</p> <p>Results: When the data were adjusted for age and sex, the risk of stroke and MI increased directly with total homocysteine. The linear coefficient suggested a risk increase by 6% to 7% for every 1 μM increase in total homocysteine. The risk by quintiles of tHcy level was significantly increased only in the group with levels above 18.6 μM (upper quintile). Associations were small among those with hypertension.</p> <p>Conclusion: Among elderly subjects, an elevated non-fasting tHcy level is associated with an increased risk of cardiovascular disease.</p>
Perry et al. (60)	<p>Study description: In the frame of the British Regional Heart Study cohort, between 1978 and 1980 serum was saved from 5,661 men, aged 40–59 years, randomly selected from the population of one general practice in each of 18 towns in the UK.</p> <p>Results: During 10-year follow-up, there were 141 incident cases of stroke among men with no history of stroke at screening. Serum tHcy was measured in 107 cases and 118 control men (matched for age-group and town who did not develop a stroke or MI during follow-up). tHcy concentrations were significantly higher in cases than controls ($P = 0.004$). There was a graded increase in the relative risk of stroke. Adjustment for established risk factors did not attenuate the association.</p> <p>Conclusion: tHcy is a strong and independent risk factor for stroke.</p>

The association between serum tHcy concentration and stroke was further examined by Perry et al. (60) within the British Regional Heart Study cohort. Between 1978 and 1980 serum was saved from 5,661 men, aged 40–59 years, randomly selected from the population of one general practice in each of 18 towns in the UK. During follow-up to December, 1991, there were 141 incident cases of stroke among men with no history of stroke at screening. Serum tHcy was measured in 107 cases and in 118 controls, matched for age-group and town, without a history of stroke at screening, who did not develop a stroke or MI during follow-up. They found that tHcy concentrations were significantly higher in

cases than in controls (geometric mean 13.7 [95% CI: 12.7–14.8] vs. 11.9 [11.3–12.6] μM ; $P = 0.004$). There was a graded increase in the relative risk of stroke in the second, third, and fourth quarters of the tHcy distribution (ORs 1.3, 1.9, 2.8; trend $P = 0.005$) relative to the first. Adjustment for age-group, town, social class, body mass index (BMI), hypertensive status, cigarette smoking, forced expiratory volume, packed-cell volume, alcohol intake, diabetes, high-density lipoprotein cholesterol, and serum creatinine did not attenuate the association. Thus the authors concluded that tHcy is a strong and independent risk factor for stroke (Table 3).

Table 3: Homocysteine and vascular disease: Major epidemiological evidence – prospective nested case-control data. High levels of plasma homocyst(e)ine are not associated with subsequent risk of MI or stroke independent of other coronary risk factors.

Author (ref)	Study description/Results/Conclusion/Comments
Chasan-Taber L et al. (61)	<p>Study description: Among participants in the PHS with no prior MI or stroke, followed up for a mean of 7.5 years, a nested case-control study was carried out to assess the risk of MI associated with decreased plasma levels of folate and pyridoxal phosphate (PLP) in relation to elevated levels of tHcy. The study included 333 male patients and 333 paired controls matched by age and smoking, and used prospectively blood samples collected at baseline.</p> <p>Results: Men with the lowest 20% of folate levels (<2.0 ng/ml) had a relative risk of 1.4 (95% CI: 0.9–2.3) compared with those in the top 80%. For the lowest 20% of vitamin B₆ values, the relative risk was 1.5 (95% CI: 1.0–2.2). When both folate and B₆ were included in a model with cardiovascular risk factors, the relative risk of MI for low as compared to high levels of folate was 1.3 (95% CI: 0.8–2.1) and for PLP, 1.3 (95% CI: 0.9–2.1). Adding tHcy to this model did not add significant predictive value (P >0.05). No significant association (RR, 1.7; 95% CI: 0.9–3.3) in the comparison between tHcy levels >95th vs. <95th percentile and risk of MI and of CHD death.</p> <p>Conclusion: No significant association between elevated Hcy and risk for MI, stroke and of cardio-vascular death.</p> <p>Comment: The US attitude to fortify flour with folate may have been a confounder in these studies (see below).</p>
Evans RW (62)	<p>Study description: In the frame of the Multiple Risk Factor Intervention Trial (MRFIT) cohort, a nested case-control study involving samples from 712 men, stored for up to 20 years, was undertaken. Cases involved non-fatal MIs, identified through the active phase of the study, which ended on February 28, 1982, and deaths due to CHD, monitored through 1990. The non-fatal MIs occurred within 7 years of sample collection, whereas the majority of CHD deaths occurred more than 11 years after sample collection.</p> <p>Results: Mean homocyst(e)ine concentrations were in the expected range and did not differ significantly between cases and controls: MI cases, 12.6 μM; MI controls, 13.1 μM; CHD death cases, 12.8 μM; and CHD controls, 12.7 μM. Odds ratios versus quartile 1 for CHD deaths and MIs combined were as follows: quartile 2, 1.03; quartile 3, 0.84; and quartile 4, 0.92.</p> <p>Conclusion: In this prospective study, no association of homocyst(e)ine with heart disease was detected.</p>
Folsom et al. (63)	<p>Study description: In a bi-racial sample of middle-aged men and women enrolled in the Atherosclerosis Risk in Communities (ARIC) Study cohort, a prospective case-cohort study determined whether tHcy-related factors are associated with the incidence of CHD over an average of 3.3 years of follow-up.</p> <p>Results: Age-, race-, and field center-adjusted CHD incidence was associated positively (P <0.05) with tHcy in women but not men, and CHD was associated negatively (P <0.05) with plasma folate (women only), plasma pyridoxal 5'-phosphate (both sexes), and vitamin supplementation (women only). However, after accounting for other risk factors, only plasma PLP was associated with CHD incidence; the relative risk for the highest versus lowest quintile of pyridoxal 5'-phosphate was 0.28 (95% CI: 0.1–0.7). There was no association of CHD with the C677T mutation of the methylenetetrahydrofolate reductase gene or with three mutations of the cystathionine beta-synthase gene.</p> <p>Conclusion: These findings add uncertainty to the concept that tHcy is a major, independent, causative risk factor for CHD.</p>
Alfthan G (64)	<p>Study description: The relation of serum total homocysteine with the incidence of MI or stroke was investigated in 7,424 men and women aged 40–64 years, free of the disease(s) at baseline (1977), enrolled in the North Karelia Project.</p> <p>Results: During the nine-year follow-up, 134 male and 131 female cases with either MI or stroke were identified. For each case, a control subject was selected belonging to the same sex and five-year age group. The mean serum tHcy of male cases and controls was 9.99 μM and 9.82 μM at baseline and that of female cases and controls 9.58 μM and 9.24 μM, respectively. The differences between cases and controls were not statistically significant. In logistic regression analyses, there was no significant association between homocysteine and MI or stroke, the odds ratios varying from 1.00 to 1.26.</p> <p>Conclusion: This prospective population-based study does not support the hypotheses that serum homocysteine is a risk factor for atherosclerotic disease.</p>
Verhoef et al. (65, 66)	<p>Study description: In the prolonged follow-up of the PHS, tHcy was assessed in samples from 109 subjects who subsequently developed ischaemic stroke and 427 control subjects (nested case-control design), and in 149 case control pairs, matched for age and smoking, as to risk of angina pectoris leading to coronary artery bypass surgery (66).</p> <p>Results: Mean plasma concentration of homocyst(e)ine was not statistically higher (P = 0.12) in subjects with stroke than in control subjects (65). The crude odds ratio of ischaemic stroke for subjects in the upper 20% (>12.7 μM) compared with those in the bottom 80% of homocyst(e)ine levels was 1.2 (95% CI: 0.7–2.0). In subgroup analyses, elevated homocyst(e)ine levels appeared to be more strongly predictive of ischaemic stroke in normotensive subjects and in men 60 years or younger.</p> <p>As to angina pectoris leading to coronary artery bypass surgery disease, plasma tHcy was unrelated to the overall risk as well as to the risk within the strata of major coronary risk factors.</p> <p>Conclusion: The data were compatible with a small, non-significant association between elevated plasma homocyst(e)ine and risk of ischaemic stroke but not with the risk of angina pectoris leading to coronary artery bypass surgery disease.</p> <p>Comments: As the sample size was small and the confidence intervals wide, either no association or a moderate increase in risk cannot be excluded, particularly in subgroups otherwise at low risk, e.g. younger men and those with normal blood pressure. It is possible that the US attitude to fortify flour with folate may have been a confounder in these latter studies (67).</p>

To assess prospectively the risk of MI associated with decreased plasma levels of folate and pyridoxal phosphate (PLP) in relation to elevated levels of tHcy, 14,916 male physicians participants in the PHS, aged 40–84 years, with no prior MI or stroke provided plasma samples at baseline (61). They were followed for 7.5 years. Samples from 333 men who subsequently developed MI, and their paired controls matched by age and smoking, were analysed for folate and PLP levels (nested case-control study using prospectively collected blood samples). In a model controlling for diabetes, angina, hypertension, Quetelet's index, and total/high-density lipoprotein cholesterol, men with the lowest 20% of folate levels (<2.0 ng/ml) had a relative risk of 1.4 (95% CI: 0.9–2.3) compared with those in the top 80%. For the lowest 20% of vitamin B6 values, the relative risk was 1.5 (95% CI: 1.0–2.2). When both folate and B6 were included in a model with cardiovascular risk factors, the relative risk of MI for low as compared to high levels of folate was 1.3 (95% CI: 0.8–2.1) and for PLP, 1.3 (95% CI: 0.9–2.1). Adding tHcy to this model did not add significant predictive value (chi square = 2.0, $P > 0.05$): except in the first half of the follow-up interval when men with the top 5% of tHcy values had an almost three-fold increase in risk of MI, the association between elevated Hcy and risk for MI and of CHD death was not statistically significant (relative risk 1.7, 95% CI: 0.9 to 3.3, for subjects with >95th percentile vs. <95th percentile of total Hcy levels).

No significant association between elevated Hcy levels and risk of major coronary events or stroke emerged also from the Multiple Risk Factor Intervention Trial (MRFIT) cohort (62), a nested case-control study involving samples from 712 men, stored for up to 20 years, that were analysed for homocyst(e)ine. Cases with MI, identified through the active phase of the study, which ended on February 28, 1982, and deaths due to CHD, monitored through 1990, were taken into consideration. The non-fatal MIs occurred within seven years of sample collection, whereas the majority of CHD deaths occurred more than 11 years after sample collection. Mean homocyst(e)ine concentrations were in the expected range and did not differ significantly between case patients and control subjects: MI cases, 12.6 μM ; MI controls, 13.1 μM ; CHD death cases, 12.8 μM ; and CHD controls, 12.7 μM . ORs vs. quartile 1 for CHD deaths and MIs combined were as follows: quartile 2, 1.03; quartile 3, 0.84; and quartile 4, 0.92. Thus, while weakly associated with C-reactive protein, no association of homocyst(e)ine concentration with heart disease was detected in this prospective study.

In a bi-racial sample of middle-aged men and women enrolled in the Atherosclerosis Risk in Communities (ARIC) Study cohort, a prospective case-cohort study determined whether tHcy-related factors are associated with the incidence of CHD over an average of 3.3 years of follow-up (63). Age-, race-, and field center-adjusted CHD incidence was associated positively ($P < 0.05$) with tHcy in women but not in men, and CHD was associated negatively ($P < 0.05$) with plasma folate (women only), PLP (both sexes), and vitamin supplementation (women only). However, after accounting for other risk factors, only plasma PLP was associated with CHD incidence; the relative risk for the highest vs. lowest quintile of PLP being 0.28 (95% CI: 0.1–0.7). There was no association of CHD with the C677T mutation of the MTHFR gene or with three

mutations of the CBS gene. Thus, while strongly supporting the possibility that vitamin B6 offers independent protection, these prospective findings added uncertainty to the concept that tHcy is a major, independent risk factor for CHD.

The relation of serum tHcy and Lp(a) with the incidence of atherosclerotic disease was also investigated among 7,424 men and women aged 40–64 years, free of atherosclerotic disease at baseline (1977) enrolled in the North Karelia Project (64). During the nine-year follow-up, 134 male and 131 female cases with either MI or stroke were identified. For each case, a control subject was selected belonging to the same sex and five-year age group. Serum samples collected in 1977 were stored at -20°C and analysed in 1991. The mean serum homocysteine concentration of male cases and controls was 9.99 μM and 9.82 μM at baseline; that of female cases and controls was 9.58 μM and 9.24 μM , respectively. The median serum Lp(a) concentration of male cases and controls was 73 mg/l and 108 mg/l and that of female cases and controls 113 mg/l and 91 mg/l, respectively. The differences between cases and controls were not statistically significant. In a logistic regression analysis, there was no significant association between either homocysteine or Lp(a) and atherosclerotic disease, MI or stroke, the ORs varying from 1.00 to 1.26 for homocysteine and from 0.81 to 1.06 for Lp(a).

A prolonged follow-up from the PHS confirmed a lack of association between plasma Hcy levels and risk for stroke and angina (65, 66). In the report by Verhoef et al. (65) using a nested case-control design, homocyst(e)ine was assessed in samples from 109 subjects who subsequently developed ischaemic stroke and 427 controls. Mean plasma concentration of homocyst(e)ine was slightly higher in subjects with stroke (11.1 ± 4.0 [\pm SD] μM) than in control subjects (10.6 ± 3.4 μM), but the difference was not statistically significant ($P = .12$). The crude OR of ischaemic stroke for subjects in the upper 20% (>12.7 μM) compared with those in the bottom 80% of homocyst(e)ine levels was 1.4 (95% CI: 0.8–2.2). The OR was 1.2 (95% CI: 0.7–2.0) after controlling for several risk factors and other potential confounders. In subgroup analyses, although not statistically significant, elevated homocyst(e)ine levels appeared to be more strongly predictive of ischaemic stroke in normotensive subjects and in men 60 years or younger. Thus the data were compatible with a small, non-significant association between elevated plasma homocyst(e)ine and risk of ischaemic stroke. However, since the sample size was small and the confidence intervals were wide, either no association or a moderate increase in risk could not be excluded, particularly in subgroups otherwise at low risk, e.g. younger men and those with normal blood pressure.

The same PHS setting served to prospectively investigate the relation between plasma total homocysteine levels and risk of angina pectoris leading to coronary artery bypass surgery. Among 149 case control pairs, matched for age and smoking (66), plasma total homocysteine was unrelated to risk of disease overall as well as within the strata of major coronary risk factors. It is possible, however, that the US attitude to fortify flour with folate may have been a confounder in these latter studies (67).

3. Homocysteine and vascular disease: Major epidemiological evidence – Meta-analyses (Table 4)

A meta-analysis of prospective observational studies of first events showed a weak association between hyperhomocysteinaemia and elevated risk of CHD (13). For this study, MEDLINE was searched for articles published from January 1966 to January 1999. Addi-

tional studies were identified by a hand search of references of original articles or review articles and by personal communication with investigators. Studies were included if, by January 1999, they had data on total blood homocysteine concentrations, sex, and age at event. Studies were excluded if they measured only blood concentrations of free homocysteine or of homocysteine after a methionine-loading test or if relevant clinical data were unavailable or incomplete. Data from 30 prospective or retrospective studies involving a total of 5,073 IHD events and 1,113 stroke events were in-

Table 4: Homocysteine and vascular disease: Major epidemiological evidence: meta-analyses.

Author (ref)	Study description/Results/Conclusion/Comments
Homocysteine Studies Collaboration (13)	<p>Study description: Meta-analysis of prospective observational studies of first events. MEDLINE was searched for articles published from January 1966 to January 1999. Additional studies were identified by a hand search of references of original articles or review articles and by personal communication with relevant investigators. Data from 30 prospective or retrospective studies involving a total of 5,073 IHD events and 1,113 stroke events were included.</p> <p>Results: An increase in plasma Hcy levels by 25% ($\approx 3 \mu\text{M}$) was associated with 11% and 19% excess risk for ischaemic heart disease and stroke, respectively, after correction for other cardiovascular risk factors. Stronger associations were observed in retrospective rather than in prospective studies. After adjustment for known cardiovascular risk factors and regression dilution bias in the prospective studies, a 25% lower usual (corrected for regression dilution bias) homocysteine level (about $3 \mu\text{M}$ [0.41 mg/l]) was associated with an 11% (OR, 0.89; 95% CI: 0.83–0.96) lower IHD risk and 19% (OR, 0.81; 95% CI: 0.69–0.95) lower stroke risk.</p> <p>Conclusion: Association between high Hcy and risk of CVD is weak.</p> <p>Comments, Limitations: 1. The RR associated with elevated Hcy (by $3 \mu\text{M}$) was 1.49 (95% CI: 1.41–1.61) for IHD and 1.16 (95% CI: 0.99–1.37) for cerebrovascular accident in retrospective studies, and 1.20 (95% CI: 1.12–1.30) for IHD and 1.30 (95% CI: 1.11–1.52) for prospective studies. 2. The number of strokes in these studies was relatively small.</p>
Wald et al. (68)	<p>Study description: Mendelian randomisation analyses of cohort studies. 20 prospective studies (3,820 participants) of serum homocysteine and disease risk and 72 studies in which the prevalence of a mutation in the MTHFR gene (which increases homocysteine) was determined in cases ($n = 16,849$) and controls, were analysed.</p> <p>Results: The odds ratios for a $5 \mu\text{M}$ increase in serum homocysteine were, for CHD, 1.42 (95% CI: 1.11–1.84) in the genetic studies and 1.32 (1.19–1.45) in the prospective studies; for deep vein thrombosis with or without pulmonary embolism, 1.60 (1.15–2.22) in the genetic studies (there were no prospective studies); and, for stroke, 1.65 (0.66–4.13) in the genetic studies and 1.59 (1.29–1.96) in the prospective studies.</p> <p>Conclusion: Both genetic and prospective studies yield similar highly significant strong evidence that the association between homocysteine and cardiovascular disease is causal.</p> <p>Comment: Lowering homocysteine concentrations by $3 \mu\text{M}$ from current levels (by increasing folic acid intake) would reduce the risk of CHD by 16%, deep vein thrombosis by 25%, and stroke by 24%.</p>
Brattström et al. (8)	<p>Study description: Meta-analysis of 13 studies in which there were measurements of plasma homocysteine in relation to 3 genotypes (TT, CT, and CC) and of 23 case-control studies comprising 5,869 genotyped cardiovascular disease patients (mostly CHD) and 6,644 genotyped control subjects.</p> <p>Results: Subjects with the TT genotype had plasma homocysteine concentrations $2.6 \mu\text{M}$ (25%) higher than those with the CC genotype. However, there was no difference between patients and control subjects either in the frequency of mutant alleles (T) (34.3% vs. 33.8%) or the TT genotype (11.9% vs. 11.7%). In the analysis of the 23 studies, the relative risk (OR) of vascular disease associated with the TT genotype was 1.12 (95% CI: 0.92–1.37).</p> <p>Conclusions: The TT genotype does not increase CHD. Mild hyperhomocysteinaemia found frequently in vascular disease patients is not causally related to the pathogenesis of the vascular disease.</p>
Lewis SJ et al. (69)	<p>Study description: Meta-analysis of 80 case-control and prospective studies of the association between MTHFR 677C\rightarrowT variant and MI, coronary artery occlusion, or both. Data on genotype frequency and mean homocysteine concentrations by genotype were extracted. Odds ratios were calculated for TT genotype versus CC genotype. Heterogeneity was explored, with stratification by geographical region of the study samples, and meta-regression by difference in mean serum homocysteine concentrations (CC minus TT genotypes).</p> <p>Results: 26,000 cases and 31,183 controls were included in the analysis. An overall random effects odds ratio of 1.14 (95% CI: 1.05–1.24) was found for TT versus CC genotype. There was strong evidence of heterogeneity ($P < 0.001$, I^2: 38.4%), which largely disappeared after stratification by geographical region. Odds ratios in Europe, Australia, and North America attenuated towards the null, unlike those in the Middle East and Asia.</p> <p>Conclusion: No strong evidence to support an association of the MTHFR 677C\rightarrowT polymorphism and coronary heart disease in Europe, North America, or Australia.</p>

Table 4: Continued

Author (ref)	Study description/Results/Conclusion/Comments
Casas JP et al. (12)	<p>Study description: Meta-analysis (mendelian randomisation, cohort studies) to investigate consistency between the expected odds ratio for stroke among TT homozygotes, and the observed odds ratio from a meta-analysis of genotype-disease association studies. MEDLINE and EMBASE information were used up to June, 2003, for all relevant studies on the association between homocysteine concentration and the MTHFR polymorphism, and until December, 2003, for those on the association between the polymorphism and the risk of stroke. Pooled odds ratios and 95% CI calculated by random-effects and fixed-effects models. Consistency between expected and observed odds ratios assessed by interaction test. As many as 111 studies met the selection criteria.</p> <p>Results: Among 15,635 people without cardiovascular disease, the mean difference in homocysteine concentration between TT and CC homozygotes was 1.93 μM (95% CI: 1.38 to 2.47). The expected odds ratio for stroke corresponding to this difference based was 1.20 (1.10–1.31). In the genetic meta-analysis (n = 13,928) the odds ratio for stroke was 1.26 (1.14–1.40) for TT versus CC homozygotes, similar to the expected odds ratio (P = 0.29). Consistency between the odds ratios was preserved in analyses by age-group, ethnic background, and geographical location. The TT genotype associated with a 21% (95% CI: 6–39%) higher risk of IHD and a 31% (95% CI: –20% to +215%) risk of stroke.</p> <p>Conclusion: High risk of CHD and of stroke associated with the high risk TT genotype.</p>
Ford ES et al. (70)	<p>Study description: Meta-analysis using MEDLINE (1966–1999), EMBASE (1974–1999), SciSearch (1974–1999), and Dissertation Abstracts (1999) for articles and theses about homocyst(e)ine concentration and coronary heart disease and cerebrovascular disease. 57 publications (3 cohort studies, 12 nested case-control studies, 42 case-control studies) with results on 5,518 people with CHD (11,068 control subjects) and 1,817 people with cerebrovascular disease (4,787 control subjects) were included in the analysis.</p> <p>Results: For CHD, the summary odds ratios (OR) for a 5 μM increase in homocyst(e)ine concentration were 1.06 (95% CI: 0.99–1.13) for 2 publications of cohort studies, 1.23 (95% CI: 1.07–1.41) for 10 publications of nested case-control studies, and 1.70 (95% CI: 1.50–1.93) for 26 publications of case-control studies. For cerebrovascular disease, the summary OR for a 5 μM increase in homocyst(e)ine concentration were 1.10 (95% CI: 0.94–1.28) for two publications of cohort studies, 1.58 (95% CI: 1.35–1.85) for five publications of nested case-control studies, and 2.16 (95% CI: 1.65–2.82) for 17 publications of case-control studies.</p> <p>Conclusion: Prospective studies offer weaker support than case-control studies for an association between homocyst(e)ine concentration and CHD.</p>

cluded in the meta-analysis of individual participant data, with allowance made for differences between studies, for confounding by known cardiovascular risk factors, and for regression dilution bias. An increase in plasma Hcy levels by 25% (i.e. about 3 μ M) was associated with 11% and 19% excess risk for CHD and stroke, respectively, after correction for other cardiovascular risk factors. Stronger associations were observed in retrospective than in prospective studies. After adjustment for known cardiovascular risk factors and regression dilution bias in the prospective studies, a 25% lower usual (corrected for regression dilution bias) homocysteine level (about 3 μ M [0.41 mg/l]) was associated with an 11% (OR, 0.89; 95% CI: 0.83–0.96) lower IHD risk and a 19% (OR, 0.81; 95% CI: 0.69–0.95) lower stroke risk. However, bias may exist in this analysis, as the relative risks associated with elevated Hcy (by 3 μ M) were 1.49 (95% CI: 1.41–1.61) for IHD and 1.16 (95% CI: 0.99–1.37) for cerebrovascular accident in retrospective studies, but 1.20 (95% CI: 1.12–1.30) for CHD and 1.30 (95% CI: 1.11–1.52) for cerebrovascular accident for prospective studies. Furthermore, the number of strokes in these studies was relatively small.

Similar results were obtained by Wald et al. (68), in a meta-analysis devoted to evaluate the association of serum homocysteine concentration with CHD, deep vein thrombosis and pulmonary embolism, and stroke as well as homocysteine reduction in preventing them. The philosophy behind was that, given the definite functional nature of the MTHFR single nucleotide CT polymorphism and its relationship to plasma Hcy levels (8, 69), it is possible to perform Mendelian randomisation analyses of co-

hort studies. For this purpose, 20 prospective studies (3,820 participants) of serum homocysteine and disease risk, and 72 studies in which the prevalence of the C677T mutation of the MTHFR gene (that increases homocysteine) was determined in cases (n = 16,849) and controls, were analysed. ORs of the three diseases for a 5 μ M increase in serum homocysteine concentration; ORs of the three diseases for a 5 μ M increase in serum homocysteine concentration were determined. The ORs for a 5 μ M increase in serum homocysteine were, for CHD, 1.42 (95% CI: 1.11–1.84) in the genetic studies and 1.32 (1.19–1.45) in the prospective studies; for deep vein thrombosis with or without pulmonary embolism, 1.60 (1.15–2.22) in the genetic studies (there was no prospective study); and, for stroke, 1.65 (0.66–4.13) in the genetic studies and 1.59 (1.29–1.96) in the prospective studies. Thus, although the genetic studies and the prospective studies did not share the same potential sources of error, both yield similar highly significant evidence that the association between homocysteine and cardiovascular disease is causal. On this basis, lowering homocysteine concentrations by 3 μ M from current levels (achievable by increasing folic acid intake) would reduce the risk of ischaemic heart disease by 16% (11–20%), deep-vein thrombosis by 25% (8–38%), and stroke by 24% (15–33%).

The philosophy of the previous study was the same as in the meta-analysis by Brattström et al. (8). As mutant homozygotes (TT genotype) of the MTHFR gene often have mildly elevated circulating homocysteine, it seems likely that they would also be at increased risk of vascular disease. In this respect, they identified 13 studies in which there were measurements of plasma homocys-

teine in relation to the three genotypes (TT, CT, and CC) and 23 case-control studies comprising 5,869 genotyped cardiovascular disease patients (mostly CAD) and 6,644 genotyped control subjects. Those bearing the TT genotype had plasma homocysteine concentrations 2.6 μM (25%) higher than those with the CC genotype. However, there was no difference between patients and control subjects either in the frequency of mutant alleles (T) (34.3% versus 33.8%) or in the TT genotype (11.9% versus 11.7%). In the analysis of the 23 studies, the relative risk of vascular disease associated with the TT genotype was 1.12 (95% CI: 0.92–1.37). Thus, although the C677T/MTHFR mutation is a major cause of mild hyperhomocysteinaemia, the mutation does not increase cardiovascular risk.

The association between the MTHFR 677C-->T polymorphism and coronary heart disease, assessing small study bias and heterogeneity between studies and using Medline and Embase citation searches between January 2001 and August 2004 without any language restriction, was also analysed by Lewis SJ et al. (69). 80 case-control and prospective studies of association between MTHFR 677C-->T variant and MI, CHD, or both were included. Data on genotype frequency and mean homocysteine concentrations by genotype were extracted. ORs were calculated for TT genotype versus CC genotype. Heterogeneity was explored, with stratification by geographical region of the study samples and meta-regression by difference in mean serum homocysteine concentrations (CC minus TT genotypes) was carried out. 26,000 cases and 31,183 controls were included in the analysis. An overall random effects OR of 1.14 (95% CI: 1.05–1.24) was found for TT versus CC genotype. There was strong evidence of heterogeneity ($P < 0.001$, I^2 : 38.4%), which largely disappeared after stratification by geographical region. ORs in Europe, Australia, and North America attenuated towards the null, unlike those in the Middle East and Asia. As a whole, no strong evidence was found to support an association of the MTHFR 677C-->T polymorphism and CHD in Europe, North America, or Australia, casting doubts on the conclusion drawn from previous meta-analyses that folic acid, through lowering homocysteine, has a role in prevention of cardiovascular disease. Geographical variability may be due to higher folate intake in North America and Europe or to publication bias.

Using MEDLINE and EMBASE information up to June, 2003, for all relevant studies on the association between homocysteine concentration and the MTHFR polymorphism, and until December, 2003, for those on the association between the polymorphism and the risk of stroke, Casas JP et al. (12) investigated consistency between the expected OR for stroke among TT homozygotes, extrapolated from genotype-phenotype and phenotype-disease studies, and the observed OR from a meta-analysis of genotype-disease association studies. Pooled ORs and 95% CI were calculated by random-effects and fixed-effects models. Consistency between expected and observed ORs was assessed by interaction test. As many as 111 studies met the selection criteria. Among 15,635 people without cardiovascular disease, the weighted mean difference in homocysteine concentration between TT and CC homozygotes was 1.93 μM (95% CI: 1.38–2.47). The expected OR for stroke corresponding to this difference based on previous ob-

servational studies was 1.20 (1.10–1.31). In this genetic meta-analysis ($n=13,928$) the OR for stroke was 1.26 (1.14–1.40) for TT versus CC homozygotes, similar to the expected OR ($P = 0.29$). Consistency between the ORs was preserved in analyses by age-group, ethnic background, and geographical location. Thus, the authors concluded that the observed increase in risk of stroke among homozygous for the MTHFR T allele, is close to that predicted from the differences in homocysteine concentration conferred by this variant, a concordance consistent with a causal relation between homocysteine concentration and stroke.

Finally, Ford ES et al. (70) searched MEDLINE (1966–1999), EMBASE (1974–1999), SciSearch (1974–1999), and Dissertation Abstracts (1999) for articles and theses about homocyst(e)ine concentration and coronary heart disease and cerebrovascular disease. They included in their analysis 57 publications (3 cohort studies, 12 nested case-control studies, 42 case-control studies) that reported results on 5,518 people with CHD (11,068 control subjects) and 1,817 people with cerebrovascular disease (4,787 control subjects). For CHD, the summary ORs for a 5 μM increase in homocyst(e)ine concentration were 1.06 (95% CI: 0.99–1.13) for two publications of cohort studies, 1.23 (95% CI: 1.07–1.41) for 10 publications of nested case-control studies, and 1.70 (95% CI: 1.50–1.93) for 26 publications of case-control studies. For cerebrovascular disease, the summary ORs for a 5 μM increase in homocyst(e)ine concentration were 1.10 (95% CI: 0.94–1.28) for two publications of cohort studies, 1.58 (95% CI: 1.35–1.85) for five publications of nested case-control studies, and 2.16 (95% CI: 1.65–2.82) for 17 publications of case-control studies. Thus, they concluded that prospective studies offer weaker support than case-control studies for an association between homocyst(e)ine concentration and cardiovascular disease.

4. Homocysteine and cardiovascular disease: Intervention studies with vitamins (Table 5)

Of a number of large prospective studies initiated to address this issue, involving a projected total of 52,000 subjects (71), six have recently been reported. The first of these, the Vitamin Intervention for Stroke Prevention (VISP) study (72), was a double-blind controlled trial that included 3,680 adults with a history of non-disabling cerebral infarction, randomised (in 56 university-affiliated hospitals, community hospitals, private neurology practices, and Veterans Affairs medical centers across the United States, Canada, and Scotland) to receive once-daily doses of a high-dose vitamin formulation ($n = 1,827$), containing 25 mg of pyridoxine, 0.4 mg of cobalamin, and 2.5 mg of folic acid; or a low-dose vitamin formulation ($n = 1,853$), containing 200 μg of pyridoxine, 6 μg of cobalamin and 20 μg of folate. During a two-year follow-up, mean reduction of total homocysteine was 2 μM greater in the high-dose than in the low-dose group. Such reduction of total homocysteine after non-disabling cerebral infarction had no effect on vascular outcomes: the unadjusted risk ratio for any stroke, CHD event, or death was 1.0 (95% CI: 0.8–1.1), with chances of an event within

two years of 18.0% in the high-dose group and 18.6% in the low-dose group. Likewise, the risk of ischaemic stroke within 2 years was 9.2% for the high-dose and 8.8% for the low-dose groups (ORs, 1.0; 95% CI: 0.8–1.3; $P = 0.80$ by log-rank test of the primary hypothesis of difference in ischaemic stroke between treatment groups). In spite of this, there was a persistent and graded association between baseline total homocysteine level and outcomes. A 3 μM lower total homocysteine level was associated with a 10% lower risk of stroke ($P = 0.05$), a 26% lower risk of CHD events ($P < 0.001$), and a 16% lower risk of death ($P = 0.001$) in the low-dose group and a non-significantly lower risk in the high-dose group by 2% for stroke, 7% for CHD events, and 7% for death. Thus, the authors concluded that “the consistent findings of an association of total homocysteine with vascular risk suggests that further explo-

ration of the hypothesis is warranted and longer trials in different populations with elevated total homocysteine may be necessary”. This trial was limited by its low dose-high dose design, recruitment in the US (where flour is folate-fortified), vitamin B₁₂ pre-treatment, and low rates of stroke. A post hoc-analysis that excluded those patients with low or very high vitamin B₁₂ levels or with significant renal dysfunction, showed a 21% benefit on major cardiovascular events ($P = 0.049$; adjusted for confounders $P = 0.056$) associated with vitamin B₁₂ treatment (73). The findings of two subsequent studies, the Norwegian Vitamin (NORVIT) trial (74) and the Heart Outcomes Prevention Evaluation (HOPE) 2 study (75), were consistent with those of VISP. NORVIT was a secondary prevention trial that included 3,749 men and women with prior MI, who were randomly assigned to one of four treatments adminis-

Table 5: Homocysteine and vascular disease: Major epidemiological evidence: studies with vitamins.

Author (ref)	Study description/Results/Conclusion/Comments
Toole JF et al. (72)	<p>Study description: the Vitamin Intervention for Stroke Prevention (VISP); randomised controlled double-blind trial; 3,680 adults with a history of recent non-disabling cerebral infarction, randomised to receive once-daily doses of the high-dose formulation ($n = 1,827$), or the low-dose formulation ($n = 1,853$) vitamins.</p> <p>Results: Mean reduction of total homocysteine greater in the high-dose than in the low-dose group; no treatment effect on recurrent cerebral infarction (primary outcome); coronary heart disease (CHD) events and death.</p> <p>Conclusion: During a two-year follow-up, moderate reduction of total homocysteine after non-disabling cerebral infarction had no effect on vascular outcomes.</p> <p>Comment: There was a persistent and graded association between baseline total homocysteine level and outcomes. Thus, the authors concluded that “the consistent findings of an association of total homocysteine with vascular risk suggests that further exploration of the hypothesis is warranted and longer trials in different populations with elevated total homocysteine may be necessary”.</p> <p>Limitations: Low dose-high dose design; recruitment in the US (folate fortification); vitamin B₁₂ pre-treatment; low rates of stroke.</p>
Bonaa KH et al. (74)	<p>Study description: NORVIT: secondary prevention trial; 3,749 men and women with prior MI randomised to one of four treatments, administered once daily on top of optimal cardiovascular drug care: folic acid, vit. B₆ and vit. B₁₂ (group A); folic acid and vit. B₁₂ (group B); vit. B₆ alone (group C); or placebo (group D).</p> <p>Results: After a median follow-up of 40 months, combination vitamin treatment lowered mean total Hcy levels by 27% and increased folate levels by 600–700% in patients receiving folic acid plus vit. B₁₂. No significant effect on the primary end point (composite endpoint of recurrent MI, stroke and sudden death due to CHD).</p> <p>Conclusion: No significant difference in the rates of a composite endpoint of recurrent MI, stroke and sudden death due to CHD associated with Hcy reduction.</p> <p>Comment: Event rates for the primary end point were 18% in groups B to D. In the triple therapy group (group A) the event rate was increased to 22% and for non-fatal MI by 30%, countered by a non-significant 17% decrease in stroke. Overall, the event rate for the primary end point with triple therapy (group A) compared with the other groups was increased by 20% (95% CI: 2–41%).</p>
HOPE 2 study (75)	<p>Study description: The HOPE 2 study involved 5,522 patients with vascular disease or diabetes treated daily with a combination of 2.5 mg folic acid, 1 mg vit B₁₂ and 50 mg vit B₆ or placebo for an average of five years, recruited mostly (70%) in the US.</p> <p>Results: Despite a 3.2 μM reduction in plasma Hcy levels in the combination vitamin group, there was no significant reduction in the risk of the primary end point (a composite of death from cardiovascular causes, MI and stroke), and a marginally significant 25% (95% CI: 3–41%, $P = 0.03$) reduction in stroke in patients receiving vitamins compared with those on placebo (79). A small increase in unstable angina admissions was noted with vitamin therapy.</p> <p>Conclusion: No significant reduction in the risk of a composite end point of MI, stroke and cardiovascular death despite a substantial reduction in plasma Hcy levels in diabetics receiving vitamins.</p> <p>Comments: A Bayesian analysis using data from the NORVIT and HOPE 2 studies shows a little effect of vitamins on the mortality and MI/CV events, and beneficial effect on stroke.</p>
Bazzano LA et al. (80)	<p>Study description: Meta-analysis of 12 randomised controlled studies of folic acid supplementation; data from 16,958 subjects with pre-existing vascular disease.</p> <p>Results: The RR for subjects treated with folic acid supplementation compared with controls were: 0.95 (95% CI: 0.88–1.03) for CVD; 1.04 (95% CI: 0.92–1.17) for CHD; 0.86 (95% CI: 0.71–1.04) for stroke, and 0.96 (95% CI: 0.88–1.04) for all-cause mortality.</p> <p>Conclusion: Folic acid supplementation does not significantly reduce cardiovascular risk or all-cause mortality.</p> <p>Comments: The HOPE 2 Investigators concluded that Hcy is a marker, rather than a cause, of vascular disease; epidemiological data may thus be the result of residual confounders.</p>

Table 5: Continued

Author (ref)	Study description/Results/Conclusion/Comments
Jamison RL et al. (84)	<p>Study description: Randomised controlled trial devoted to the effect of homocysteine lowering by a placebo or by the combination of folic acid, pyridoxine hydrochloride, and cyanocobalamin on mortality and vascular disease in 2,056 individuals with high homocysteine levels and advanced chronic kidney disease.</p> <p>Results: Mean homocysteine levels were lowered by 25.8% ($P < 0.001$) in the vitamin group and by 1.7% ($P = 0.14$) in the placebo group at three months. There was no significant effect on mortality, MIs, strokes, and amputations. Nor did change the composite of MI, stroke, and amputations plus mortality, time to dialysis, and time to thrombosis in haemodialysis patients.</p> <p>Conclusion: In spite of their metabolic effects, high doses of folic acid and B vitamins did not improve survival or reduce the incidence of vascular disease in advanced chronic renal disease.</p>
Albert CM et al. (85)	<p>Study description: Women (5,442 US health professionals, 42 years or older), at high risk for CV disease (with either a history of CVD or 3 or more coronary risk factors), randomised to receive a daily intake of a folic acid, vitamin B₆, and vitamin B₁₂ or a matching placebo for 7.3 years.</p> <p>Results: Mean plasma homocysteine level was decreased by 18.5% (95% CI: 12.5–24.1%; $P < 0.001$) in the active group over that observed in the placebo group (95% CI: 1.54–2.96 μM). In spite of this, women receiving active vitamin treatment had similar risk for the composite CVD primary end point, as well as for the secondary outcomes including myocardial infarction, stroke, and CVD mortality.</p> <p>Conclusion: After 7.3 years of treatment and follow-up, a combination of folic acid, vit. B₆, and vit. B₁₂ did not reduce a combined end point of total cardiovascular events among high-risk women, despite significant homocysteine lowering.</p> <p>Comment: In spite of a 18.5% decrease of plasma homocysteine levels, women receiving vitamin treatment had similar risk for the composite CVD primary end points.</p>
Ebbing M et al. (86)	<p>Study description: A total of 3,096 adult participants undergoing coronary angiography (20.5% female; mean age: 61.7 years) were randomised to a daily oral treatment with folic acid, plus vitamin B₁₂, plus vitamin B₆; folic acid plus vitamin B₁₂; vitamin B₆ alone; or placebo (2 x 2 factorial design).</p> <p>Results: After one year of treatment, mean plasma total homocysteine concentration was reduced by 30% in the groups receiving folic acid and vitamin B₁₂. During a median 38 months of follow-up, the primary end point (a composite of all-cause death, non-fatal acute MI, acute hospitalisation for unstable angina pectoris, and non-fatal thromboembolic stroke) was experienced by 219 participants receiving folic acid/vitamin B₁₂ vs. 203 not receiving such treatment and by 200 participants receiving vit. B₆ vs. 222 not receiving vit. B₆.</p> <p>Conclusion: The trial did not find an effect of treatment with folic acid/vitamin B₁₂ or vitamin B₆ on total mortality or cardiovascular events in patients treated with homocysteine-lowering B vitamins after coronary angiography.</p> <p>Comments: The trial did not find an effect of treatment with folic acid/vitamin B₁₂ or vitamin B₆ on total mortality or cardiovascular events.</p>
Martí-Carvajal et al. (87)	<p>Study description: Systematic review on the clinical effectiveness of homocysteine-lowering interventions in people with or without pre-existing cardiovascular disease. Eight randomised clinical trials (CHAOS 2002; FOLARDA 2004; GOES 2003; HOPE-2 2006; NORVIT 2006; WAFACS 2008; WENBIT 2008), involving 24,210 participants with a low risk of bias were included in the evaluation.</p> <p>Results: In subjects at risk or with established cardiovascular disease, supplements of vitamins B₆, folate or B₁₂, alone or in combination, at any dosage, did not reduce the risk of non-fatal or fatal myocardial infarction, stroke, or death.</p> <p>Conclusion: There is no evidence to support the use of homocysteine-lowering interventions to prevent cardiovascular events.</p>

tered once daily: folic acid, vitamin B₆ and vitamin B₁₂ (group A); folic acid and vitamin B₁₂ (group B); vitamin B₆ alone (group C); or placebo on top of optimal cardiovascular drug care (group D). After a median follow-up of 40 months, combination vitamin treatment lowered mean total Hcy levels by 27% and increased folate levels by 600–700% in patients receiving folic acid plus vitamin B₁₂, but had no significant effect on the primary end point (a composite of recurrent MI, stroke and sudden death due to CHD). Event rates for the primary end point were 18% in groups B to D. In the triple therapy group (group A) the event rate was increased to 22% (95% CI: 0–50%, $P = 0.05$) and for non-fatal MI by 30% ($P = 0.05$), countered by a non-significant 17% decrease ($P = 0.52$) in stroke. Overall, the event rate for the primary end point with triple therapy (group A) compared with the other groups was increased by 20% (95% CI: 2–41%). In NORVIT a 14% increase in events

($P = 0.09$) was seen in the vitamin B₆ group (29% in a subgroup of smokers; $P = 0.05$), comprising increased rates of MI (19%; $P = 0.05$) and death (19%; $P = 0.11$). The HOPE 2 study involved 5,522 patients with vascular disease or diabetes treated daily with a combination of 2.5 mg folic acid, 1 mg vitamin B₁₂ and 50 mg vitamin B₆ or placebo for an average of five years, recruited again mostly (70%) in the US. Despite a substantial reduction in plasma Hcy levels (3.2 μ M) in the combination vitamin group, there was no significant reduction in the risk of the primary end point (a composite of death from cardiovascular causes, MI and stroke), although there was a marginally significant 25% (95% CI: 3–41%, $P = 0.03$) reduction in stroke in patients receiving vitamins compared with those on placebo (75). A small increase in unstable angina admissions was noted with vitamin therapy. A Bayesian analysis of vitamin therapy using data from the NORVIT and HOPE 2

studies suggested that there is little effect of supplements on the rates of cardiovascular events, mortality or MI, although there may be a beneficial effect on rate of stroke (76). In a smaller study ($n = 205$), treatment with the combination of folic acid, vitamin B₆ and vitamin B₁₂ for six months was shown to reduce significantly the rate of re-stenosis (19.6% vs. 37.6% on placebo, $P = 0.01$) and the need for revascularisation of the target lesion (10.8% vs. 22.3% on placebo, $P < 0.05$) after coronary angioplasty (77). In an extension of this study, that included 553 subjects who had undergone successful angioplasty of at least one significant stenosis, vitamin treatment was associated with a significant decrease in the incidence of the composite end point of major adverse events (i.e. death, non-fatal MI and need for repeat vascularisation) after a mean follow-up of 11 months (relative risk 0.68, 95% CI: 0.48–0.96, $P = 0.03$) (78). However, another study showed that vitamin treatment may increase the rate of stenosis after coronary stenting (79). Most recently, a meta-analysis of 12 randomised controlled studies of folic acid supplementation, including data from 16,958 subjects with pre-existing vascular disease, showed that folic acid supplementation did not significantly reduce cardiovascular risk or all-cause mortality (80). The overall relative risks for subjects treated with folic acid supplementation compared with controls were 0.95 (95% CI: 0.88–1.03) for cardiovascular disease, 1.04 (95% CI: 0.92–1.17) for CHD, 0.86 (95% CI: 0.71–1.04) for stroke and 0.96 (95% CI: 0.88–1.04) for all-cause mortality (80). A wide range of conditions are known to increase plasma Hcy levels (► Table 6) (81); in addition, other cardiovascular risk factors such as smoking and elevated blood pressure are also associated with increased Hcy levels (82). Furthermore, individuals with pre-existing atherosclerosis have higher Hcy levels than those without (83). Thus, it has been suggested by the HOPE 2 Investigators that Hcy is a marker, rather than a cause, of vascular disease (75), and therefore epidemiological data could be the result of residual confounders.

A randomised controlled trial devoted to the effect of homocysteine lowering on mortality and vascular disease in advanced chronic kidney disease (estimated creatinine clearance ≤ 30 ml/min) ($n = 1,305$) or end-stage renal disease ($n = 751$) has been published (84). In this double-blind randomised controlled trial, carried out in 36 US Department of Veterans Affairs medical centers (median follow-up was 3.2 years), the primary outcome was all-cause mortality. Secondary outcomes included MI, stroke, amputation of all or part of a lower extremity, a composite of these three plus all-cause mortality, time to initiation of dialysis, and time to thrombosis of arteriovenous access in haemodialysis patients. The 2,056 participants aged 21 years or older with high homocysteine levels ($\geq 15 \mu\text{M}$) received a daily capsule containing 40 mg of folic acid, 100 mg of pyridoxine hydrochloride (vitamin B₆), and 2 mg of cyanocobalamin (vitamin B₁₂) or a placebo. Mean homocysteine levels were lowered by 25.8% ($P < 0.001$) in the vitamin group and by 1.7% ($P = 0.14$) in the placebo group at three months. However, high doses of folic acid and B vitamins did not improve survival or reduce the incidence of vascular disease. There was no significant effect on mortality (hazard ratio [HR], 1.04; 95% CI: 0.91–1.18); nor significant effects were demonstrated for MI (HR,

Table 6: Determinants of homocysteine plasma levels.

GENETIC	
Transsulphuration defects	
	Cystathionine β -synthase defect (chromosome 21): homozygote: 1/340,000 born heterozygote: 0.5% whole population
	heterozygote mutation 844ins68: 10–15% whole population in association with other risk factors
Remethylation defects	
	5,10-methylenetetrahydrofolate reductase (MTHFR) defect: Homozygote: 1/3,350,000 born Heterozygote: 0.5% whole population
	Thermolabile variant of MTHFR C677T (50% activity): Homozygote: 5–20% whole population
	Methionine synthase defect A2756G
	Cobalamin/methylcobalamin conversion defect (cbl C,D,E,F,G)
AGE/SEX	
	Increasing age
	Male sex
	Menopause
NUTRITIONAL	
	Folate+vit. B ₁₂ deficiency (elderly, pregnancy, malignancy)
	Vit. B ₆ deficiency
	Lifestyle: Abnormal coffee and alcohol intake
DISEASES	
	Bowel: malabsorption of Vit. B ₁₂
	Liver failure
	Renal failure; renal transplantation
	Psoriasis: folate reduction
	Lymphoblastic leukaemia, malignancy
	Hypothyroidism
	Diabetes mellitus
	Arterial hypertension
PHARMACOLOGICAL	
	Methotrexate: 5-methyl-tetrahydrofolate reduction
	Estrogens: vit. B ₆ deficit
	Diuretics: interference with folate
	Anticonvulsants (carbamazepine, isoniazide, fentoin): interference with folate
	Folate antagonists
	Vitamin B ₁₂ antagonists (e.g. nitrate)
	Metformin
	Glitazones (some)
	Lipid-lowering drugs (colestipol, nicotinate, fibrates)

0.86; 95% CI: 0.67–1.08), stroke (HR, 0.90; 95% CI: 0.58–1.40), and amputations (HR, 1.14; 95% CI: 0.79–1.64). In addition, the composite of MI, stroke, and amputations plus mortality ($P = 0.85$), time to dialysis ($P = 0.38$), and time to thrombosis in haemodialysis patients ($P = 0.97$) did not differ between the two groups.

The effect of folic acid and B vitamins on a composite endpoint of MI, stroke, coronary revascularisation, or mortality among women at high risk for cardiovascular disease has been evaluated in 5,442 US health professionals, 42 years or older, with either a history of cardiovascular disease or 3 or more coronary risk factors (85). These women were randomised, in a double-blind placebo-controlled trial, to receive a daily intake of a combination pill of 2.5 mg of folic acid, 50 mg of vitamin B₆, and 1 mg of vitamin B₁₂ or a matching placebo. After 7.3 years of treatment, mean plasma homocysteine level was decreased by 18.5% (95% CI: 12.5%–24.1%; $P < 0.001$) in the active group over that observed in the placebo group (95% CI: 1.54–2.96 μM). In spite of this, women receiving active vitamin treatment had similar risk than the placebo group for the composite primary end point (relative risk [RR], 1.03; 95% CI: 0.90–1.19; $P = 0.65$), as well as for the secondary outcomes including MI (RR, 0.87; 95% CI: 0.63–1.22; $P = 0.42$), stroke (RR, 1.14; 95% CI: 0.82–1.57; $P = 0.44$), and CVD mortality (RR, 1.01; 95% CI: 0.76–1.35; $P = 0.93$).

Mortality and cardiovascular events have also been evaluated in a randomised controlled trial (86) in patients treated with homocysteine-lowering B vitamins after coronary angiography. In this trial, the effect of treatment with folic acid and vitamin B₁₂ and the effect of treatment with vitamin B₆ as secondary prevention in patients with coronary artery disease or aortic valve stenosis was evaluated. The primary end point was a composite of all-cause death, non-fatal acute MI, acute hospitalisation for unstable angina pectoris, and non-fatal thromboembolic stroke. A total of 3,096 adult participants undergoing coronary angiography (20.5% female; mean age: 61.7 years) were randomised. Using a 2 x 2 factorial design, participants were randomly assigned to 1 of 4 groups receiving daily oral treatment with folic acid, 0.8 mg, plus vitamin B₁₂, 0.4 mg, plus vitamin B₆, 40 mg; folic acid plus vitamin B₁₂; vitamin B₆ alone; or placebo. After one year of treatment, mean plasma total homocysteine concentration was reduced by 30% in the groups receiving folic acid and vitamin B₁₂. During a median 38 months of follow-up, the primary end point was experienced by 14.2% participants receiving folic acid/vitamin B₁₂ vs. 13.1% not receiving such treatment (HR, 1.09; 95% CI: 0.90–1.32; $P = 0.36$) and by 13.0% participants receiving vitamin B₆ vs. 14.3% not receiving vitamin B₆ (HR, 0.90; 95% CI: 0.74–1.09; $P = 0.28$).

During the revision process of the present report, a systematic review by Martí-Carvajal et al. (87) appeared concerning the clinical effectiveness of homocysteine-lowering interventions in people with or without pre-existing cardiovascular. Eight randomised clinical trials (CHAOS 2002; FOLARDA 2004; GOES 2003; HOPE-2 2006; NORVIT 2006; WAFACS 2008; WENBIT 2008), involving 24,210 participants with a low risk of bias in general terms were included in the evaluation. In participants at risk or with established cardiovascular disease, when compared with placebo or standard care, the homocysteine-lowering interventions evalu-

ated, in the form of supplements of vitamins B₆, folate or B₁₂, given alone or in combination, at any dosage, did not reduce the risk of non-fatal or fatal MI, stroke, or death by any cause (pooled RR 1.03, 95% CI: 0.94–1.13, I₂: 0%; pooled RR 0.89, 95% CI: 0.73–1.08, I₂: 15%); and pooled RR 1.00 (95% CI: 0.92–1.09, I₂: 0%), respectively. Thus, the authors concluded that the results from available published trials suggest that there is no evidence to support the use of homocysteine-lowering interventions to prevent cardiovascular events.

5. Homocysteine and cardiovascular disease: Intervention studies with lipid-lowering agents (Table 7)

Recent results indicate that some lipid-modifying agents, including nicotinic acid, colestipol and fibrates, may cause elevated plasma tHcy levels (88–90). The most likely mechanism for this increase is an alteration of creatine–creatinine metabolism and changes in methyl transfer (88). In contrast, statins have no effect on plasma Hcy concentrations (88). Other agents commonly prescribed in patients with cardiovascular disease affect Hcy levels (Table 7). In keeping with rises in creatinine, thiazide diuretics are associated with a 16% increase in plasma Hcy (91). An Hcy-raising effect of metformin has been known since 1971, associated with a deficiency in vitamin B₁₂ due to reduced uptake (92). As a matter of fact, metformin reduces vitamin B₁₂ levels by 10–12% and folate by 8% and raises Hcy by 13% (93). The effects of metformin on Hcy levels can be ameliorated through the use of calcium supplements (94). More recently, significant 20% increases in Hcy have been described with rosiglitazone (94) whereas sulphonylureas have been shown to decrease Hcy (89). Combinations of metformin and glitazones are associated with varying effects with reduction in Hcy seen with pioglitazone compared with rosiglitazone (95, 96). Antacids are also associated with reductions in acid-induced cobalamin release from food and hence secondary decreases in absorption. Both fenofibrate and bezafibrate have been shown to induce elevation in plasma levels of Hcy (88). In a direct comparative study, in which patients were randomised to treatment with fenofibrate or atorvastatin for six months (after an initial six-week placebo run-in period), fenofibrate induced a significant 35% increase in Hcy levels (from 12.3 [3.9] μM to 16.4 [4.6] μM ; $P < 0.0001$), whereas there was no significant change in the group receiving atorvastatin (97). More recently, elevated plasma total Hcy levels associated with treatment with fenofibrate were noted in both the Diabetes Atherosclerosis Intervention Study (DAIS) (98, 99) and the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) trial (100). In DAIS, in 418 patients with type 2 diabetes, treatment with fenofibrate 200 mg/day was associated with a 55% increase in plasma tHcy levels (from 11.5 [5.6] to 16.5 [10.7] μM , $P < 0.001$). This increase was not related to changes in factors known to modulate plasma Hcy levels, including serum levels of vitamin B₁₂ and folate, or renal dysfunction. Subsequent analysis showed that baseline,

Table 7: Homocysteine and vascular disease: Major epidemiological evidence – studies with lipid-lowering drugs.

Author (ref)	Study description/Results/Conclusion/Comments
Giral P et al. (97)	<p>Study description: Direct comparative study, in which patients were randomised to receive fenofibrate or atorvastatin for six months (after an initial six-week placebo run-in period).</p> <p>Results: Fenofibrate induced a significant 35% increase in Hcy levels (from 12.3 μM to 16.4 μM; $P < 0.0001$). There was no significant change in the group receiving atorvastatin.</p> <p>Conclusion: Fenofibrate administration induces a significant rise in Hcy levels.</p> <p>Comments: Limitations: short-term study.</p>
DAIS study (98)	<p>Study description: DAIS, Diabetes Atherosclerosis Intervention Study in 418 patients with type 2 diabetes mellitus treated with 200 mg/day fenofibrate.</p> <p>Results: Treatment with fenofibrate was associated with a 55% increase in plasma total Hcy levels (from 11.5 to 16.5 μM, $P < 0.001$). This increase was not related to changes in factors known to modulate plasma Hcy levels, including serum levels of vitamin B₁₂ and folate, or renal dysfunction. Subsequent analysis showed that baseline, but not end-of-study, elevated plasma Hcy levels decreased the beneficial effect of fenofibrate on angiographic determinants of focal CHD.</p> <p>Conclusion: The fenofibrate-mediated increase in plasma total Hcy levels did not attenuate the beneficial effects of fenofibrate on coronary artery disease or clinical events (99).</p> <p>Comment: In the fenofibrate group, there was no significant correlation between plasma total Hcy levels and minimal lumen diameter, % stenosis or adverse clinical events.</p>
FIELD study (100)	<p>Study description: FIELD Fenofibrate Intervention and Event Lowering in Diabetes trial included 9,795 patients with type 2 diabetes (78% without prior cardiovascular disease) who were randomised to treatment with fenofibrate 200 mg/day or placebo following a 16-week run-in period, comprising four weeks of dietary modification, six weeks of single-blind placebo and six weeks of single-blind fenofibrate therapy.</p> <p>Results: At the completion of the therapy (mean duration of follow-up, five years) plasma Hcy levels were on average 35% higher in the fenofibrate group than in the placebo group (median concentrations 15.1 μM vs. 11.2 μM, $P < 0.001$).</p> <p>Conclusion: Although fenofibrate does increase plasma total Hcy levels, this effect is reversible following withdrawal of therapy and does not compromise the beneficial effects of treatment.</p> <p>Comment: In a subset of fenofibrate-treated patients who were re-studied after study completion, plasma Hcy levels fell from a median of 15.0 μM to 9.5 μM, indicating that the effect of treatment was reversible.</p>

but not end-of-study, elevated plasma Hcy levels decreased the beneficial effect of fenofibrate on angiographic determinants of focal coronary artery disease. Furthermore, Hcy levels at the end of the study correlated negatively with coronary artery disease progression when data from all study patients were included in the analysis. In the fenofibrate group, there was no significant correlation between plasma total Hcy levels and minimal lumen diameter, percent stenosis or adverse clinical events. Thus, the DAIS Investigators concluded that the observed fenofibrate-mediated increase in plasma total Hcy levels did not attenuate the beneficial effects of fenofibrate on coronary artery disease or clinical events. FIELD included 9,795 patients with type 2 diabetes (78% without prior cardiovascular disease) who were randomised to treatment with fenofibrate 200 mg/day or placebo following a 16-week run-in period, comprising four weeks of dietary modification, six weeks of single-blind placebo and six weeks of single-blind fenofibrate therapy. The mean duration of follow-up in the study was five years. At the end of the study, plasma Hcy levels were on average 35% higher in the fenofibrate group than the placebo group (median concentrations 15.1 μM vs. 11.2 μM , $P < 0.001$). However, in a subset of fenofibrate-treated patients who were restudied after study completion, plasma Hcy levels fell from a median of 15.0 μM to 9.5 μM , indicating that the effect of treatment was reversible (100). This effect remains the subject of ongoing subgroup analyses by the FIELD Management Committee.

Conclusions

As a number of confounders may have affected the results of epidemiological studies, the association between elevated Hcy levels and cardiovascular risk does not prove the existence of a causal relation. Individuals with pre-existing atherosclerosis have higher Hcy levels than those without; some drugs may increase Hcy levels, suggesting the potential for attenuation of their clinical benefit; the effect of some drugs on Hcy is reversible following discontinuation of treatment.

On the other hand, in spite of its biological plausibility, large, randomised clinical trials have shown that, even though vitamin supplementation reduced Hcy levels, there was no significant effect on cardiovascular risk. This is consistent with the possibility that tHcy lowering is ineffective as a (secondary) prevention strategy for cardiovascular disease. The discrepancy between evidence from epidemiological and retrospective and prospective case-control studies and the results of these more recent clinical trials may well be due to inherent limitations in the observational studies.

While data from ongoing studies are still awaited to establish whether Hcy is a marker rather than a cause of cardiovascular disease, the data presently available do not provide support for routine screening for and treatment of elevated Hcy to prevent CVD.

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