Homocysteine as a risk factor for cardiovascular and related disease: nutritional implications

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Abstract

The objectives were firstly to assess the evidence that homocysteine is a significant and independent risk factor for vascular disease with special reference to cardiovascular disease, and secondly to evaluate the evidence that a food staple fortified with folic acid will reduce this problem on a population basis.

The structure of plasma homocysteine (tHcy) is described. Homocysteine, a highly reactive compound, is synthesized from the amino acid, methionine, and is metabolized by two pathways, the catabolic transsulphuration route *via* cystathionine β-synthase (EC 4.2.1.22) and the remethylation path using 5-methyltetrahydrofolate polyglutamate, the product of 5,10-methylenetetrahydrofolate reductase (MTHFR; EC 1.1.1.171), *via* the cobalamin dependent enzyme, methionine synthase (MS; EC 2.1.1.13).

The mechanisms whereby hyper-tHcy is produced include both increased rates of synthesis and decreased metabolism. The latter may occur owing to nutritional deficiency of the vitamin cofactors which are necessary for the normal function of the metabolic enzymes. In particular, folate is required for methylene reductase, pyridoxal phosphate for cystathionine synthase and cobalamin for methionine synthase. When these vitamins are deficient hyper-tHcy is induced and this occurs especially in the elderly. Alternatively, a variant form of methylene reductase has recently been described which occurs in nearly 10 % of the normal population. This variant is associated with hyper-tHcy, especially in situations associated with a low folate nutritional status.

Meta-analysis of both retrospective case-control studies, nested prospective case-control surveys and a secondary trial of mortality in postmyocardial infarct patients have shown that the association of hyper-tHcy with vascular disease is beyond doubt. This has been further supported by direct assessments of the degree of vascular disease in the carotid brachial and aortic arteries in relation to tHcy levels. Furthermore, treatment with a cocktail of the vitamin cofactors has produced lowering of tHcy levels and regression of the vascular disease in the carotid arteries of affected individuals.

Suggested pathogenic mechanisms in vascular disease induced by hyper-tHcy include vascular endothelial cell dysfunction, smooth muscle proliferation and derangements of normal intravascular regulation mechanisms. A variety of clinical conditions are known to be associated with a high incidence of thromboembolic complications. Some of these are associated with hyper-tHcy.

Low physiological doses of folic acid, as well as pharmocological doses, lower tHcy. However, because of the poor bioavailability of food folate (50 %) and the

considerable chemical instability of the naturally occurring reduced forms of folate, in most people it would require unacceptably high consumption of green vegetables to accomplish the necessary increase in intracellular folate and reduction in tHcy. Accordingly, folic acid, the nonreduced synthetic form of the vitamin, which is 100 % bioavailable and chemically extremely stable, should be added to a food staple such as flour to ensure maximum protection for most of the population.

Introduction

Homocystinuria was first recognized in 1962 (Carson & Neill, 1962; Gerritsen *et al.* 1962). The children they described had multiple congenital abnormalities which included severe thromboembolic disease and mental deficiency. Mudd and colleagues (1964) subsequently identified the cause of the homocystinuria as a lack of cystathionine β-synthase (CβS) (Fig. 2). McCully (1969) then described a child with an inborn error of cobalamin metabolism which resulted in defective methionine synthase (MS) activity, who also had homocystinuria and died of severe vascular disease. As a consequence, McCully & Wilson (1975) proposed that elevated levels of homocysteine might be pathogenically associated with atheromatous disease of the vasculature. Wilcken & Wilcken (1976) observed that homocysteine-cysteine mixed disulphide blood levels after a methionine load were raised in patients who had coronary artery disease. This observation has subsequently been confirmed by innumerable studies from many different countries (Boushey *et al.* 1995).

There are excellent reviews by Green & Jacobsen (1995), Rosenblatt (1995), Mudd *et al.* (1995), D'Angelo & Selhub (1997), Vasikaran & von Bockxmeer (1997) and Refsum *et al.* (1998). This review selects and discusses the principal studies published since 1994 with special reference to the nutritional aspects of these developments and their relation to clinical practice.

Structure and properties of homocysteine

Homocysteine in human plasma is formed by demethylation of the dietary amino acid, methionine, the average dietary intake of which is >1.8 g/d (Mayer *et al.* 1996). Homocysteine is present in only trace amounts in the diet and is complexed to various thiols. Dietary homocysteine does not, under normal circumstances, appear to affect the plasma homocysteine level (Green & Jacobsen, 1995). At the first international meeting on homocysteine it was agreed that total plasma homocysteine concentrations would be denoted by the symbol tHcy. Intracellular homocysteine has a short half-life and is rapidly excreted (Ueland & Refsum, 1989). Whether in the normal range, or considerably elevated (hyperhomocysteinaemia; hyper-tHcy) tHcy is present as a mixture of the dimer homocystine, where two homocysteines are linked *via* their sulphurs, and mixed disulphides of homocysteine and other thiols such as cysteine or glutathionine (Green & Jacobsen, 1995) (Fig. 1). Eighty percent of tHcy is protein bound through disulphide links with plasma proteins, especially albumin; less than 5 % of tHcy is free, reduced and nonprotein bound (Araki & Sako, 1987). Accordingly, chemical reduction is an essential initial step in the measurement of tHcy (Ueland *et al.* 1993).

The normal range of tHcy in our laboratory is 6·0–12·0 μg/l in women and 8·0–14·0 μg/l in men (Wald *et al.* 1998) but there is some variation between laboratories. tHcy is tightly controlled by metabolic pathways described below. Homocysteine is either degraded *via* cystathionine to cysteine and pyruvate, or remethylated to methionine. Remethylation can occur *via*
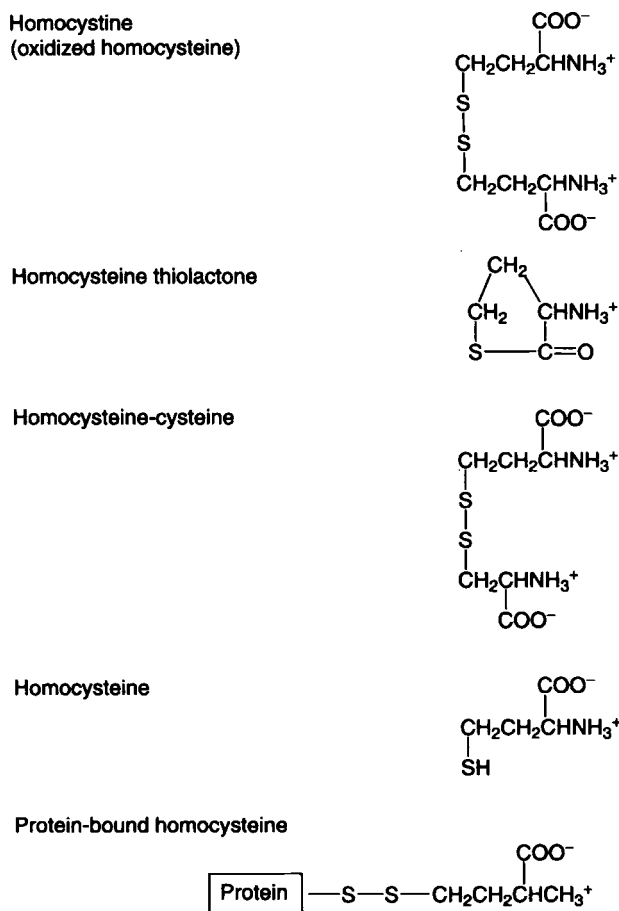
Figure 1 Compounds which comprise total plasma homocysteine (derived from Green & Jacobsen, 1995).

the vitamin B_{12} dependent enzyme methionine synthase or the enzyme betaine homocysteine methyltransferase (EC 2.1.1.5) (Fig. 2). The latter is restricted to liver and kidney in humans (McKeever *et al.* 1991) and depends on provision of the cosubstrate betaine, a breakdown product of choline.

Ingestion of a very high so-called loading dose of methionine in normal subjects leads to a temporary rise in tHcy, which returns rapidly to baseline. This has been utilized as a functional assay of the 'transsulphuration pathway' for homocysteine catabolism (Fig. 2) and is called the oral methionine loading test (Green & Jacobsen, 1995). The basis of this test is that CβS, which controls entry into the transsulphuration pathway, has a high K_m for homocysteine and thus becomes active only at high homocysteine concentrations. By contrast, methionine synthase has a low K_m and is active all the time. The methionine loading test is thus a test of CβS malfunction, such as may occur in heterozygotes of CβS deficiency (Boers *et al.* 1985) or vitamin B_6 deficiency. Keeping subjects being tested on a low protein diet is not necessary (den Heijer *et al.* 1996).
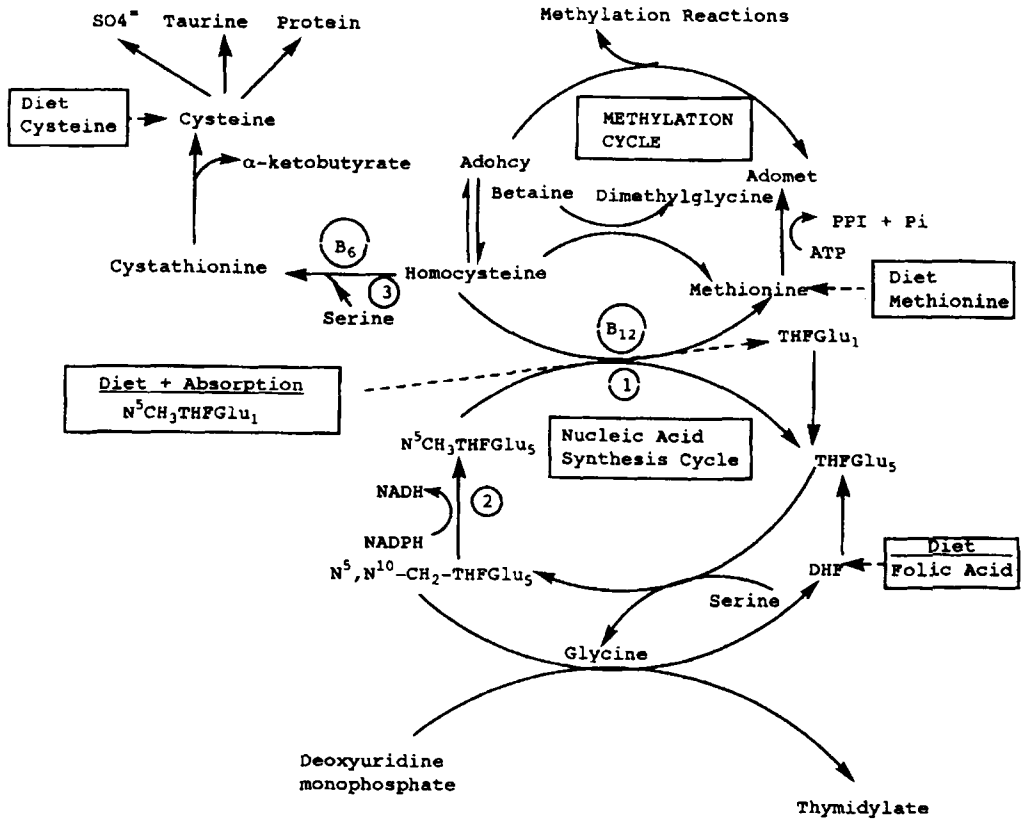
Figure 2 Biochemical pathways associated with homocysteine metabolism.

tHcy levels are higher in men than in women, both fasting and post methionine loading and usually higher in postmenopausal rather than premenopausal women (Boers *et al.* 1983; Brattström *et al.* 1984; Kang *et al.* 1986; Wouters *et al.* 1995). Postmenopausal women may have levels which exceed those of men of the same age, especially in post methionine loading as compared with fasting tHcy (Wouters *et al.* 1995). The higher tHcy level in men than in women may be due to lower concentrations of B vitamins in men than women (Selhub *et al.* 1993), but also to the increased muscle mass in men than in women, as 80 % of S-adenosylmethionine (SAM) is used for creatine–creatinine synthesis which inevitably leads to S-adenosylhomocysteine (SAH) and homocysteine (Andersson *et al.* 1992*a*) (Fig. 2).

tHcy also rises with age (Andersson *et al*. 1992*b*; Ueland *et al*. 1993; Selhub *et al*. 1993; Brattström *et al*. 1994; Rasmussen *et al*. 1996; Verhoef *et al*. 1997), even when allowance is made for age related increase in creatinine and decreasing vitamin levels (Verhoef *et al*. 1997). The rise may be related to age induced reduction in the function of MS (Dudman *et al*. 1993) or CβS (Nordstrom & Kjellstrom, 1992). tHcy is also higher in the erect than in the supine position for unknown reasons (C.C. Cronin *et al*. pers. comm.).

Low concentrations of homocysteine are known to occur in pregnancy (Kang *et al*. 1986; Andersson *et al*. 1992*b*) and in postmenopausal women on hormone replacement drugs (van der Mooren *et al*. 1994). Homocysteine concentrations are also low in Down's syndrome patients, who have trisomy of chromosome 21 and a low incidence of coronary artery disease (CAD) (Brattström *et al*. 1987). Since chromosome 21 carries the allele for CβS (Skovby, 1985), the extra allele increases the level of the enzyme which enhances the degradation of homocysteine (Chadefaux *et al*. 1988; Brattström *et al*. 1989). Low levels have also been recorded in insulin-dependent diabetics who have not progressed to develop renal and other vascular complications (Robillon *et al*. 1994).

Biochemical pathways associated with homocysteine

The concentration of homocysteine in the cell is closely controlled by five enzymes: S-adenosylhomocysteine hydrolase (EC 3.3.1.1), which controls its rate of synthesis, and CβS, MS, methylenetetrahydrofolate reductase (MTHFR) and betaine homocysteine methyl transferase, which control its metabolism.

Synthesis of homocysteine

Homocysteine is synthesized from dietary methionine, which is an essential amino acid, using the methylation cycle. SAM is first synthesized from methionine using ATP and methionine adenosyltransferase (EC 2.5.1.6). This enzyme is present in three forms, two of which are present in the liver: the α form has a low K_m and the β form a high K_m. The α form is present in extrahepatic fetal tissues (Finkelstein, 1990). This enzyme has been cloned (Corrales *et al*. 1991, 1992). It has been demonstrated that when oxidation overload occurs glutathione is lowered and methionine adenosyltransferase, which is sensitive to oxidation, is inhibited.

The product of the SAM mediated methylation reactions is SAH. Just as SAM is the universal methylator (Finkelstein, 1990; Weir & Scott, 1995), so SAH, its product, is its main inhibitor (Weir & Scott, 1995). The ratio of SAM to SAH (methylation ratio) determines whether the methyl transfer will occur, although different methylation reactions are not equally inhibited by a particular ratio and the degree of inhibition is not linear with the fall in the ratio (Weir & Scott, 1995). This is relevant to hyper-tHcy, since all causes of raised tHcy will automatically produce high levels of SAH (de la Haba & Cantoni, 1959). Although SAH is normally converted to adenosine and homocysteine by SAH hydrolase, the equilibrium of the reaction favours SAH formation (de la Haba & Cantoni 1959). Thus, the flow of SAH through the hydrolase to homocysteine requires the continuous removal of either adenosine or homocysteine. Elevation of either will inevitably increase SAH if the other product is also present. Whatever may be the effect of homocysteine on fetal nerve and vascular endothelial cells, prolonged hyper-tHcy could produce toxic effects by elevating intracellular and circulating SAH which would, in turn, switch off one or more of the methyltransferase reactions.

Metabolism of homocysteine

As explained above, homocysteine is either degraded via the transsulphuration pathway using CβS, or is remethylated back to methionine either by MS which uses the product of MTHFR, 5-methyltetrahydrofolate, or by betaine homocysteine methyltransferase (Fig. 2).

Cystathionine β-synthase (CβS)

The biochemistry of this enzyme (Fig. 2, no. 3) is described in detail elsewhere (Finkelstein, 1990; Mudd *et al*. 1995). This is a catabolic reaction, it transsulphurates homocysteine using serine, and in so doing produces cystathionine and subsequently cysteine and α-ketobutyrate, both of which are pyridoxal phosphate (PLP) dependent reactions. Cysteine is then used for protein biosynthesis or it can be used by cystathionine γ-lyase (EC 4.4.1.1) to make glutathione.

The genes encoding CβS have been cloned and are found on chromosome 2 (Kraus *et al*. 1993). The enzyme was isolated from human liver (Kraus, 1994) and was found to be a tetramer with subunits of 63 kDa. Post-translation, this becomes a dimer, which has a sixty-fold lower K_m for homocysteine and is thirty times more active (Skovby *et al*. 1984). Defects are known to occur in the CβS enzyme and the genes coding for it have also been cloned and sequenced (Tsai *et al*. 1996*a*). Cumulatively, the heterozygosity for the different genetic variants for the CβS defect occurs in only 1/200 of the general population.

5,10-Methylenetetrahydrofolate reductase

A detailed discussion of the properties of this enzyme has been given by Matthews (1984). It catalyses the reduction of 5,10-methylenetetrahydrofolate using NADPH in a two step process. The first synthesis is to the quinonocol form of 5-methyldihydrofolate, followed by its reduction by FADH to 5-methyltetrahydrofolate (Matthews & Haywood, 1979). The enzyme is inhibited by SAM (Kutzbach & Stokstad, 1971). This is important because the enzyme really controls the provision of methyl groups into the methylation cycle. It has been sequenced and cloned and several mutations and one variant have been identified at the genomic level (Goyette *et al*. 1994).

Methionine synthase

A detailed discussion of properties of this enzyme has been given by Matthews (1984). The mammalian form requires methyl cobalamin as a cofactor. The enzyme reaction uses the methyl group attached to the cofactor to methylate homocysteine. The cobalamin form that is generated in this reaction is called Cob(I)alamin. It has an unpaired electron and is very unstable. Usually it immediately takes the methyl group from 5-methyltetrahydrofolate to regenerate the original and stable form, methylcobalamin, and this goes on to repeat the cycle. However, about every fifty cycles of the enzyme, Cob(I)alamin is oxidized by molecular oxygen to Cob(II)alamin. The enzyme with this form of the cofactor cannot accept the methyl group from 5-methyltetrahydrofolate, which effectively inactivates the enzyme. However, Cob(II)alamin can be reactivated by accepting a methyl group not from 5-methyltetrahydrofolate but from SAM. This latter process also requires a reduction of the cobalamin cofactor. The anaesthetic gas nitrous oxide (N_2O) inactivates methionine synthase. It seems clear that

this oxidation does not follow the pattern of oxidation with molecular oxygen. Instead a hydroxy radical is produced which attacks the protein structure of the enzyme causing its irreversible denaturation (Drummond & Matthews, 1994).

Mechanisms of hyperhomocysteinaemia

Hyper-tHcy can result either from enhanced production, or from reduced rates of metabolism of tHcy.

Enhanced homocysteine synthesis

This occurs acutely when a subject eats a protein meal which contains a large quantity of methionine (Fig. 2). This forms the basis of the methionine loading test. Normally the elevated tHcy level is present for only a short period. Prolonged elevation suggests that there is a defect in the transsulphuration pathway *via* CβS.

Reduced homocysteine metabolism

A reduced rate of homocysteine metabolism occurs when there is a defect in any of the enzymes or deficiency of their vitamin cofactors. Thus, reduction in activity of CβS, MTHFR or MS, or availability of their cofactors, pyridoxine, folate and cobalamin respectively, can cause hyper-tHcy (Table 1). Enzymes may malfunction due to inherited mutations or to dietary deficiencies, or perhaps secondary to other acquired metabolic diseases. Defects of betaine homocysteine methylene transferase have not been described.

Inherited mutations
The mechanisms of these defects are discussed in detail by Boers *et al.* (1985), Fenton & Rosenberg (1989), Green & Jacobsen (1995), Rosenblatt (1995) and Tsai *et al.* (1996*a,b*). Inherited defects result from less than a dozen mutations of the gene for MTHFR. Such

Table 1b. Causes of Hyperhomocysteinaemia

Enhanced Production	
High dietary methionine	Methionine loading test
Reduced Clearance	
Defective enzyme	Vitamin cofactor deficiency
Transsulphuration–catabolism pathway	
◆ Cystathionine synthase	Pyridoxine (B$_6$)
◇ Mutant (rare)	
Remethylation Pathway	
◆ Methylene reductase	
◇ Mutant (rare)	$N^5N^{10}CH_2THF$ Glu$_5$ (folate)
◇ MTHFR C-T variant	
◆ Methionine synthase	Cobalamin (B$_{12}$)
	Cobalamin E
	Cobalamin G

mutations are extremely rare, patients who are homozygous for such mutations numbering less than one hundred. More recently an unstable thermolabile variant form of MTHFR has been described (Kang *et al.* 1988*a*). Subsequently the relevant abnormality in the MTHFR gene where a cytosine to thymine switch in the 677 base resulted in transcriptions of alanine instead of valine has been recognized (Frosst *et al.* 1995). Nearly half of most populations studied are heterozygous, and 5–20 % homozygous (van der Put *et al.* 1997). The very high prevalence of this genetic abnormality means that it is called a variant rather than the classically recognized mutants of low frequency.

Nutritional deficiencies

Deficiency of one or other of the three vitamins (folate, B_6 and B_{12}; Table 1) is common, especially among the elderly (Joosten *et al.* 1993; Selhub *et al.* 1993; Naurath *et al.* 1995). This is especially true of folate, although cobalamin and to a lesser extent PLP deficiency also occur in the elderly. Selhub and colleagues (1993) studied 1160 survivors of the Framingham Heart Study cohort, 67–96 years old. They showed that tHcy increased with age after controlling for vitamin concentrations. There was a strong inverse relationship between tHcy and plasma folate after controlling for age, sex and the levels of the other vitamins. Weaker inverse associations were also found with plasma cobalamin and PLP. There was an association between tHcy and the dietary intake of folate and PLP, but not of B_{12}. The authors concluded that inadequate plasma concentrations of one or more B vitamins contributed to 67 % of the cases of high tHcy. tHcy levels were elevated in patients with dietary folate intakes up to 280 µg/d, which is in excess of the current recommended dietary allowance or reference nutrient intake of 200 µg/d (Department of Health, 1991). In a carefully controlled study of normal male volunteers in a metabolic ward, Jacob and coworkers (1994) demonstrated that tHcy increases as plasma folate decreases and that restriction of dietary methyl groups (methionine) did not increase dietary folate requirements.

    tHcy and methylmalonic acid concentrations rise when plasma levels of folate and cobalamin are in excess of what are conventionally considered to be the lower limits of normal (Kang *et al.* 1988*b*; Stabler *et al.* 1990; Allen *et al.* 1993; Joosten *et al.* 1993; Selhub *et al.* 1993; Naurath *et al.* 1995). tHcy and methylmalonyl CoA are the substrates for the two mammalian cobalamin-dependent enzymes, MS and methylmalonyl CoA mutase (EC 5.4.99.2). Along with N^5CH_3-THF-Glu$_5$, tHcy is the substrate for MS (Fig. 2). Dysfunction of the mutase enzyme results in elevation of methylmalonyl CoA which degrades to methylmalonic acid and is excreted in the urine. Thus, a rise in these substrates gives a clear indication of one or other vitamin deficiency. Methylmalonic acid and tHcy will be elevated in B_{12} deficiency, while only tHcy is raised in folate deficiency.

    What is an adequate intracellular concentration of a vitamin such as folate has been questioned by Daly and coworkers (1995). They re-analysed data derived from an earlier study of 50 000 women who attended antenatal clinics in the Dublin maternity hospitals in the late 1980s (Kirke *et al.* 1993). Of these women, 81 subsequently delivered a child with neural tube defect (NTD). When the maternal red cell folate (RCF) levels were assessed it was apparent that the risk of delivering an NTD baby was present throughout the range of RCF values, but the prevalence progressively increased the lower the RCF level. The accepted cutoff level of normal RCF at present is 150 µg/ml or above. This level, because of the shape of the curve, results in a prevalence of 5–6 NTD births per thousand live births (Daly *et al.* 1995). The prevalence drops rapidly as the RCF increases with progressively less reduction of risk as the

level exceeds 400 g/l. This level at which the risk of an NTD is reduced tenfold from the levels seen at 150 g/l, has been used as a compromise target for dietary folate therapy, i.e. to reduce NTD as far as possible without exposing the population to the potential risks of too high levels of folic acid (Daly *et al.* 1997; Kelly *et al.* 1997).

The question of how to ensure that all women attain an RCF level in excess of 400 μg/ml is thus very relevant. Recent evidence suggests that for the population to attain such a level and lower tHcy maximally would be difficult by dietary folate alone, because dietary folate is only 50 % bioavailable (Gregory, 1995). The only effective way is to take folic acid which is 100 % bioavailable, either as tablets or as food staples such as flour or breakfast cereals fortified with folic acid (Cuskelly *et al.* 1996). Folic acid is extremely stable chemically, unlike the natural folate where the pteridine ring is reduced to the tetrahydro form, making oxidative cleavage of pteridine to the *p*-aminobenzoic acid–glutamic acid residue very possible (Blakley, 1969). Since the fetal neural tube closes 24–28 d after conception, well before the mother is aware that she is pregnant, it is important to ensure that she is taking the folic acid supplement peri-conceptually (i.e. during any period when she might become pregnant). Numerous studies have shown that young women, even if they are aware of the risk, are poor compliers at taking folic acid tablets daily (Wild *et al.* 1996*a,b*). This is because in most societies over half of all pregnancies are unplanned. The only effective way to ensure that the majority of the population are benefiting maximally is to fortify a food staple such as flour (Oakley, 1997*a*). Following the recommendations of the Food and Drug Administration and the Folic Acid Working Party Group of the Centres for Disease Control and Prevention, it is now a law in the USA that flour will be fortified with 140 μg folic acid/100 g cereal grain product which, it is calculated, will ensure that most Americans will receive at least 100 μg folic acid/d (Tucker *et al.* 1996; Oakley, 1997*a*).

Is 100 μg folic acid/d enough to achieve the necessary red cell folate levels and lower tHcy to the maximum extent? It is well known that pharmacological doses of folic acid will achieve levels of RCF >400 μg/l (Brattström *et al.* 1988, 1990; Malinow *et al.* 1990; Landgren *et al.* 1995) but only at the expense of the unphysiological form of folate, folic acid, appearing in the blood (Kelly *et al.* 1997).

Methyl folate (N^5CH_3THF Glu_1), the normal circulating form of folate in the blood, enters cells by a process controlled by cobalamin dependent MS (Fig. 2). In B_{12} deficient patients methyl folate cannot enter the cell whereas folic acid enters by a noncobalamin dependent process (Fig. 2). Folic acid can thus maintain the supply of intracellular folate required for the synthesis of thymidylate and DNA. Accordingly, folic acid will treat the megaloblastic anaemia that occurs in cobalamin deficiency, but will not affect the neurological complications which require the synthesis of SAM (Scott *et al.* 1981). Thus, folic acid might by treating the anaemia allow the irreversible neurological disease to progress undiagnosed, which would not occur if the anaemia were correctly treated with B_{12} (Scott & Weir, 1981).

Recent evidence suggests that small physiological doses of folic acid, which are converted by the intestine to methyl folate and are therefore not likely to mask the neuropathy (Kelly *et al.* 1997), will also reduce tHcy (Ward *et al.* 1997) and elevate RCF (Daly *et al.* 1997; Malinow *et al.* 1998). Ward and coworkers (1997) gave 30 normal adult volunteers with tHcy levels within the normal range varying levels of folic acid: 100 μg/d for six weeks, followed by 200 μg/d for six weeks, followed by 400 μg/d for six weeks and finally six weeks with no added folic acid. The group was divided into tertiles depending on their initial tHcy. After 100 μg folic acid/d there was a significant fall in the tHcy levels in the upper and middle tHcy tertiles, but no significant effect in the lowest tertile. After 200 μg folic acid/d there was a further significant fall in the upper and middle tertiles, but none in the lowest. After 400 μg/d there was no further

significant fall, and following a further six weeks without folic acid, in the upper and middle tertiles there was a significant rise to former levels once again. These results show that a significant effect was obtained with 100 μg/d and a maximal effect with 200 μg/d over periods of six weeks. The authors argued that 100 μg folic acid/d taken for a lifetime in a food staple such as flour would be likely to have as complete an effect as 200 μg folic acid/d over six weeks did in this study (Ward *et al.* 1997).

In a study conducted on 747 elderly subjects 67 to 96 years old who were members of the Framingham Heart Study group (Tucker *et al.* 1996) it was demonstrated that folic acid fortification of flour significantly increased both the folate content of the diet and the RCF levels and lowered the tHcy values. They argued that at a level of fortification of 140 μg/100 g cereal grain product the ensuing benefits would greatly outweigh the harm induced by the potential masking of the onset of the neurological complications of vitamin B_{12} deficiency. They suggested that much of this problem could be alleviated by adding B_{12} as well as folate to the staple diet. Recently the results of a randomized double-blind controlled trial have been published (Daly *et al.* 1997). Participants were randomly allocated to receive 0, 100 or 400 μg of folic acid daily for a period of six months and compliance was strictly monitored. Whereas 400 μg folic acid caused a doubling of the RCF, a significant increase was also obtained with both 100 and 200 μg doses. This study suggests that the US policy of fortifying flour with sufficient folic acid to ensure an increase of 100 μg folic acid/d is appropriate, since this dose attained the 400 μg/ml RCF level which was suggested by Daly *et al.* (1995) as sufficient to produce maximal practical protection from NTD. This is likely to have a dramatic effect on the incidence of both NTD and vascular related deaths in the USA over the next decade.

PLP deficiency is also associated with raised tHcy (Robinson *et al.* 1995, 1997; Morrison *et al.* 1996) and may be the reason for the high percentage of cardiac patients with abnormal post-methionine load tests found in the European COMAC Group (Robinson *et al.* 1997). Once again, the risk of vascular disease decreased with rising PLP values. Although PLP deficiency did not appear to influence the tHcy level in the same way as folate deficiency, it was nevertheless an independent risk factor for arteriosclerotic vascular disease.

Evidence for a pathogenic association between mild hyperhomocysteinaemia and vascular disease

It is known that in patients with homocystinuria and tHcy levels >100 μg/ml, reduction of the tHcy level will reduce the incidence of vascular events (Mudd *et al.* 1995). However, in order to prove that the reduction of mild hyper-tHcy (i.e. tHcy <100 μg/ml) will prevent the development of atherosclerotic vascular disease would require a primary prevention study showing that when tHcy levels are lowered there is a reduction in the mortality and morbidity from vascular events such as coronary thrombosis or strokes, when compared with suitable controls. Such a study has never been conducted as the numbers of subjects and time required to establish such a cause and effect, combined with ethical considerations, would be prohibitive.

Nevertheless, secondary prevention trials are under way which could prove that lowering tHcy in subjects who have already had a vascular event will prevent a recurrence (Vasikaran & van Bockxmeer, 1997). The biggest trial of all will be the effect in the USA on the prevalence of vascular events following the introduction in 1998 of compulsory fortification of flour with 140 μg folic acid/100 g (Oakley, 1997a). This, it has been estimated, will save in the region of 50 000 lives per annum (Boushey *et al.* 1995). Only time will tell.

Retrospective and prospective case-control studies

In the meantime, evidence has accumulated from both retrospective and prospective (nested case-control) studies that a raised tHcy is associated with atheromatous and thrombotic vascular events. It is known that mild homocysteinaemia is associated with coronary artery disease (Boushey *et al.* 1995) and cerebral (Brattström *et al.* 1992; Perry *et al.* 1995) and peripheral occlusive arterial disease (Brattström *et al.* 1990; Aronson *et al.* 1994) in relatively young patients (under 60 years). Hyper-tHcy is also associated with thromboembolic disease of the venous system. The evidence is fully reviewed by D'Angelo & Selhub (1997). The syndrome is characterized by recurrent episodes of thrombosis at a relatively young age, after minimal provocation and in unusual sites. Ten studies have so far been reported, of which eight have found a significantly positive association (see D'Angelo & Selhub, 1997 for references). Hyper-tHcy is also an independent risk factor for hyperlipaemic patients (Glueck *et al.* 1995). These studies and others (Pancharuniti *et al.* 1994; Morrison *et al.* 1996) show that there is usually a correlation between tHcy and the plasma concentrations of cobalamin, PLP and folate. The level of tHcy always responds to treatment with these vitamins albeit when given in pharmacological doses. The risk of cardiovascular disease mortality was also correlated with the mean tHcy level in randomly chosen men in 11 different countries (Alfthan *et al.* 1997).

Retrospective case-control studies

A quantitative meta-analysis was conducted by Boushey and colleagues (1995) using mostly retrospective case-control studies. They evaluated 27 studies relating tHcy to arterial vascular disease and eleven studies of the effect of folic acid therapy on tHcy.

Effect on coronary artery disease

In 14 of 17 studies into CAD tHcy was a significant risk factor. A 5 µmol/l rise in tHcy was associated with CAD in men (odds ratio (OR) 1·6; 95 % confidence interval (CI) 1·4–1·7) and in women (1·8; 1·3–1·9). They calculated that 10 % of all CAD risk in the population was due to tHcy. The vascular response to tHcy was graded throughout the range of homocysteine values, showing no threshold association. This is similar to the association between serum cholesterol and vascular disease.

Effect on cerebrovascular events

Cerebrovascular disease (CVD) also showed a positive association with tHcy in nine of eleven studies (2·5; 2·0–3·0). A significant fall in the incidence of CVD is likely with a fall of 5 µmol/l in fasting tHcy (2·3; 1·8–2·0). If only the three best studies are analysed, the effect of tHcy is still significant (1·5; 1·3–1·9) (Boushey *et al.* 1995). These effects were apparent even taking into account the temporary lowering of tHcy in the acute phase following a stroke.

Peripheral vascular disease

All nine studies which examined the effect of tHcy on this form of vascular disease gave a positive association (6·8; 2·9–15·8) (Boushey *et al.* 1995).

Other retrospective case-control studies

A major study reported from the European Concerted Action Project by Graham and colleagues (1997) assessed 750 patients with cardiac, cerebral and peripheral vascular disease from 19

separate European centres. These subjects were below the age of 60 years, included both sexes and were matched by 800 appropriate controls. The results showed that there was a significantly higher incidence of vascular disease in the upper 20 % as opposed to the lower 80 % (2·2; 1·6–2·9). The results of the methionine loading test, when added to those of the fasting tHcy, added a further 27 % of cases to those deemed to be at risk. A dose response was noted between the tHcy level and the risk of vascular disease, which was independent of the other risk factors such as smoking and high blood pressure and was of similar magnitude. The relative risk per 5 μmol/l tHcy increment was remarkably similar and as significant as that calculated in the meta-analysis of Boushey *et al.* (1995).

In both sexes there was a multiplicative effect of high tHcy, smoking and high blood pressure. No such effect was found for plasma cholesterol concentration. As in other studies, RCF, plasma cobalamin and PLP concentrations were inversely related to tHcy. People who took vitamin tablets were at a substantially lower risk of vascular disease and this was associated with lower tHcy.

The authors concluded that hyper-tHcy increases the risk of vascular disease to the same extent as smoking and hyperlipidaemia and that both smoking and high blood pressure markedly enhance the risk of hyper-tHcy.

Prospective (or nested case-control) studies in vascular disease
Primary prospective studies in ischaemic heart disease (IHD).

Although, as described above, retrospective case-control studies show a clear relationship with IHD (Boushey *et al.* 1995), primary prospective cohort studies have been less conclusive. Of the four studies conducted to date, two have shown a negative relationship with tHcy (Stampfer *et al.* 1992; Alfthan *et al.* 1994) and two have been positive (Arnesen *et al.* 1995; Wald *et al.* 1998).

The US Physicians' Health Study (Stampfer *et al.* 1992) reported on the development of 271 IHD events, both fatal and nonfatal in their cohort of 14 916 US male physicians. These were matched by 271 controls; average age at the time of the IHD event was 62. Initially there was an association between IHD and tHcy in the upper 5 % of the tHcy distribution, compared with the lower 90 % (3·1; 1·4–6·9). However, after a longer follow-up period, this difference fell to 1·3 which was no longer significant (Verhoef & Stampfer, 1995). A further report (Chasan-Taber *et al.* 1996) showed that both folate and pyridoxine deficiency were commoner, although not significantly so, in those who subsequently went on to develop a myocardial infarct. This was present even when controlled for diabetes, angina, hypertension, body mass index and lipoprotein cholesterol. Adding the effect of tHcy to this model did not add to the significance of the prediction values, except as described above in the early period of the study. The authors still concluded that these data are compatible with the hypothesis that low dietary intakes of folate and/or vitamin B_6 contribute to the risk of MI. One interesting factor in this study was that hypertension was found to correlate positively with tHcy ($P \leq 0.003$) and is similar in this respect to the study by Graham and colleagues (1997).

Alfthan and colleagues (1994) in Finland studied 191 patients with IHD, both fatal and non-fatal and compared them with 269 controls. Males and females were included and their average age was 61 years. The OR was 1·00 (0·77–1·29).

In contrast, a study by Arnesen and colleagues (1995) demonstrated a significantly positive association between cardiovascular disease and tHcy. In this nested case-control study in Tromso, of 21 626 subjects 12–61 years old, 123 developed IHD within a mean follow-up period of four years. Ninety percent of these cases were males and for each case identified, four

controls were selected and matched for age, sex and number of hours since last meal when venesection occurred. The level of tHcy in cases and controls was 12.7 ± 4.7 v. $11.3 \pm 3.7 \mu mol/l$ (mean $\pm$ S.D.; $P < 0.002$). The relative risk of a $4 \mu mol/l$ rise in tHcy was 1.41 ($1.16-1.71$). When adjustment was made for potential factors, the OR was 1.32 ($1.05-1.65$). There was no threshold level of tHcy at which the risk of IHD emerged. Thus, tHcy was an independent risk factor for IHD, the association was not as high as for total and very low density lipoprotein cholesterol and smoking, but was higher than for diabetes mellitus and systolic blood pressure.

A nested case-control study from London and Dublin on blood samples collected from members of the British United Provident Association (BUPA) reported on 21 520 men 35–64 years old who had no evidence of IHD at baseline (Wald *et al.* 1998). Subsequently, 229 men died of IHD during a mean follow-up of 8.7 years. Five age matched controls with no evidence of IHD were selected for each case, giving a total of 1126. tHcy was higher in cases than controls (mean 13.1 v. 11.8 $\mu mol/l$, $P < 0.001$). The risk of IHD in the highest quartile of tHcy was 3.7 times that in the lowest quartile (95 % CI 1.8–4.7), or 2.9 times after adjusting for other risk factors. For each 5 $\mu mol/l$ rise in tHcy there was a continuous dose response relationship which increased risk by 41 % (95 % CI 22–59).

In a meta-analysis of the eight previous restrospective studies of the relationship between tHcy and IHD, carried out by Wald *et al.* (1998), the age adjusted association was stronger. For each 5 $\mu mol/l$ increase in tHcy, the rate of IHD risk increase was 84 % (95 % CI 52–123). The reason why the retrospective, as compared with the prospective, analyses show a stronger association is not immediately apparent, but may relate to the mean age of subjects in the prospective trials being ten years older than in the retrospective studies. The association is strongest when IHD occurs in young subjects (Verhoef & Stampfer, 1995; Wald *et al.* 1998). A similar phenomenon has been reported for other IHD risk factors, such as cholesterol (Law *et al.* 1994), blood pressure (Neaton & Wentworth, 1992) and smoking (Kahn, 1966). Alternatively, there are clear methodological differences between the prospective and retrospective studies. In the former, there is no possibility of the cases being different in any significant way from the controls with respect to collection and analysis. However, in the latter questions arise such as: was the elevation of homocysteine in the cases a result, rather than a cause? Were the controls truly matched to the cases? Were the samples treated identically? (e.g. blood on standing releases significant homocysteine). Were collection methods identical in cases and controls?

Prospective study in cerebrovascular disease.

A study by Perry and colleagues (1995), from the British Regional Heart Study Group, who had obtained serum samples from 5661 men 40–59 years old from 18 towns in the UK, looked prospectively at the relationship of tHcy to the development of a stroke. During a follow up period of a mean 12.8 years, 141 incidences of stroke occurred in men who had no history of stroke at screening. tHcy was obtained in 107 cases and 118 matched controls. tHcy was significantly higher in cases than controls (13.7; 12.7–14.8 v. 11.9; 11.3–12.6 $\mu mol/l$; $P = 0.004$).

Once again the relative risk of stroke was graded throughout the quartiles of tHcy. The relative risk of second, third and fourth quartiles with respect to the first gives an OR respectively of 1.3, 1.9 and 2.8 ($P = 0.005$). No association was found with body mass index, hypertension, smoking, alcohol intake, diabetes, high density lipoprotein cholesterol or creatinine. The authors concluded that tHcy is a strong and independent risk factor for stroke.

A similar study by Verhoef and colleagues (1994) in 109 cases and 427 controls, from an initial total of 14 916 subjects followed for 5 years, showed a mild but insignificant association

with tHcy (cases *v.* controls 11·1 ± 4·0 SD *v.* 10·6 ± 3·4, P = 0·12). However, the duration of the trial was less than that of Perry *et al.* (1995).

Direct association of vascular damage and homocysteine

The relationship of the level of tHcy to the degree of arteriosclerotic thickening of the carotid wall was assessed by an ultrasound method by Malinow and colleagues (1993). Pairs of asymptomatic adults (287) were selected from a probability sample of 15 800 men and women 45–65 years old. Subjects with thickened intimal-medial carotid walls had higher tHcy levels than controls (P < 0·001). The relative risk of having a thickened carotid artery wall for those with tHcy > 10·5 µmol/l as compared with those < 5·88 µmol/l was 3·15 (1·57–6·33).

A similar study was conducted by Selhub and colleagues (1995) on 1041 elderly subjects 67–96 years old who were members of the Framingham Heart Study Cohort. Using ultra-sonography, they found that 43 % of men and 34 % of women had > 25 % carotid artery stenosis. The incidence of > 25 % occlusion of the carotid artery was higher when tHcy was > 14·4 µmol/l, than when < 9·1 µmol/l. The OR was 2·0 (1·4–2·9) after adjusting for sex, age, high density lipoprotein cholesterol, systolic blood pressure and smoking. Plasma folate, pyridoxine and dietary folate intake were inversely associated with the degree of carotid artery stenosis after adjusting for the risk factors.

More recently, Konecky and colleagues (1997) used transoesophageal echocardiography to evaluate 156 patients with atheromatous plaques in their thoracic aorta. tHcy was significantly associated with aortic disease (P < 0·001) and was independently predictive when other risk factors were excluded. Interestingly, only plasma vitamin B_6 correlated with the degree of atheromatous change.

Following an acute rise in tHcy induced by a methionine loading test in normal volunteers, a significant reduction in the mean brachial artery dilatation associated with the rate of blood flow was found, suggesting an acute and direct effect of homocysteine on endothelial function (Chambers *et al.* 1998). Brachial artery flow is endothelium dependent and dilatation is largely mediated by NO. These findings suggest that hyper-tHcy impairs the response to endothelial NO in healthy subjects.

Finally, in 38 patients in whom carotid artery atheromatous plaque size had been demonstrated to be enlarging prospectively and whose tHcy was > 14 µmol/l, it has been shown that treatment with folic acid, pyridoxine and B_{12} over a mean period of 4·4 years produced a significant regression of the plaque size (Petersen & Spena, 1998).

These studies give direct support for a positive relationship between tHcy and vascular disease as measured in the carotid arteries and the aorta in asymptomatic living subjects. In all studies, the pathogenic effect of homocysteine was present throughout the range of tHcy values, including the 'normal range' (5–15 µmol/l).

Secondary prospective study

Recently, 587 Norwegian patients with angiographically confirmed CAD were followed prospectively for a median period of 4·6 years and during this time 64 patients died (10·9 %). They found a strong graded relationship between tHcy levels and overall mortality; levels were only weakly associated with the degree of CAD, but more strongly associated with a previous history of myocardial infarction, left ventricular ejection fraction and serum creatinine. The association of tHcy with mortality remained strong, even when the effects of these and other

potential aetiological factors were accounted for. As with other studies, the effect of the association with tHcy was seen throughout the range of tHcy values (P < 0·02). If only those patients who died of CAD were included (50), the association with tHcy was even stronger (Nygard *et al.* 1997).

Effect of sex and menopause on the relationship of tHcy to vascular disease

Since the majority of the foregoing studies have predominantly looked at the effect of raised tHcy in men, it is important to summarize the comparative evidence between women and men, and the effect of the menopause. Most studies have shown higher tHcy levels in post- than in pre-menopausal women (Boers *et al.* 1983; Brattström *et al.* 1985; Wouters *et al.* 1995) but not all (Andersson *et al.* 1992*a*).

In a further study from the European Concerted Action Project, Verhoef and colleagues (1997) looked at 750 cases with vascular disease before the age of 60 years, compared with 800 controls. tHcy levels rose progressively with age in both sexes; and fasting levels were 15 % (1·1–20) higher in men than in women, and in post methionine loading tests by 8 % (4–13). After the menopause, tHcy levels were higher in women than men in post methionine loading tests, but not in fasting levels. However, the tHcy levels were unchanged after adjusting for vitamin levels and serum creatinine. In men, after adjusting for age and conventional risk factors, the OR for the association between tHcy and vascular disease were 1·5 (1·2–1·8) in fasting samples and 1·3 (1·1–1·6) post methionine loading. The comparative values in women were 1·6 (1·3–2·5) fasting and 1·6 (1·2–2·0) post methionine loading. The association of tHcy levels with vascular disease was similar, whether the female subjects were pre- or post-menopausal. The authors concluded that tHcy is an independent risk factor for vascular disease in men, and both before and after the menopause in women.

Pathogenesis of vascular disease related to hyperhomocysteinaemia

The vascular disease associated with hyper-tHcy is related to endothelial dysfunction, smooth muscle proliferation and abnormalities of coagulation and related factors (reviewed by D'Angelo & Selhub, 1997).

Vascular endothelial dysfunction

Endothelial cells express MTHFR but not betaine homocysteine methyl transferase (Wang *et al.* 1992). There is controversy as to whether they contain CβS (Green & Jacobsen, 1995). This is important because in CβS deficiency endothelial cells certainly get damaged. If they do not have this enzyme, the damage must by done by extracellular rather than intracellular homocysteine in patients with hyper-tHcy. Patients with CβS and MTHFR deficiency not only develop hyper-tHcy but also have endothelial cells that are peculiarly susceptible to damage induced by homocysteine (Wang *et al.* 1992). The reactivity of the sulphydryl group of homocysteine has been implicated. In the presence of NO and endothelium-derived relaxing factor, thiols react to form S-nitrosothiols which are potent vasodilatory and antiplatelet aggregation factors (Stamler *et al.* 1993). Under normal circumstances endothelial cells modulate the potential adverse effects of homocysteine by releasing endothelium-derived relaxing factor and forming S-NO-homocysteine. The adverse vascular effects of homocysteine

may be due to inability to maintain S-NO formation as the homocysteine levels increase (Stamler *et al.* 1993). This in turn leads to the generation of superoxide anion radicals and hydrogen peroxide which inactivate NO and lead to endothelial cell damage (Loscalzo, 1996), causing platelet adhesion, induction of tissue factor and reduced plasminogen activation.

Vascular smooth muscle proliferation

Hyper-tHcy also causes vascular damage by enhancing proliferation and growth of vascular smooth muscle cells (Tsai *et al.* 1994), possibly induced by platelet factors (Harker *et al.* 1976).

Coagulation abnormalities

Homocysteine also enhances intravascular thrombosis. It induces tissue factor procoagulant activity by increasing tissue factor mRNA in endothelial cells, thus causing a promotion of the initiation of vascular coagulant mechanisms, possibly mediated by hydrogen peroxide (Fryer *et al.* (1993). tHcy also activates factors XII and IV and suppresses protein C (Rodgers & Conn, 1990).

   tHcy inhibits the processing and secretion of von Willebrand factor from the endoplasmic reticulum of the endothelial cells, which suggests that intracellular transport of multiple endothelial cell proteins may be altered in patients with hyper-tHcy (Lentz & Sadler, 1993) and could be a mechanism of altered endothelial cell resistance to thrombosis. For example, the modulation by homocysteine of tissue plasminogen activates binding to endothelial cell membrane receptors which impairs the antithrombotic properties of the blood vessel wall (Hajjar, 1993).

Summary of current evidence that elevated tHcy causes cardiovascular disease

Taken as a whole the evidence from both retrospective and prospective case-control trials supports a primary association between tHcy and atherosclerotic vascular disease. The reasons why two of the early prospective trials (Stampfer *et al.* 1992; Alfthan *et al.* 1994) failed to show an association between tHcy and IHD, while two studies did (Arnesen *et al.* 1995; Wald *et al.* 1998), are discussed in detail elsewhere (Wald *et al.* 1998). When taken in conjunction with the studies showing a direct association between the degree of arterial disease and tHcy (Malinow *et al.* 1995; Selhub *et al.* 1995; Konecky *et al* 1997) the evidence is persuasive. The recent publications showing regression of atheromatous plaques when treated with cobalamin, folate and PLP are particularly convincing (Peterson & Spena, 1998).

   It seems that disordered platelet function is primarily responsible for the development of atheromatous disease of the arterial endothelium, while abnormal coagulation and/or fibrinolysis produces thromboembolic disease.

Hyperhomocysteinaemia due to defective enzymes causing impaired metabolism

Defects associated with cystathionine β-synthase

This subject has been reviewed by D'Angelo & Selhub (1997). The evidence suggests that being heterozygous for the CβS gene defect does not enhance the likelihood of developing

heart attacks or strokes (Mudd *et al.* 1995). Doppler studies on arterial disease have given conflicting results. Furthermore the prevalence of CβS mutations is not elevated in CVD patient populations when compared with controls (Kluijtmans *et al.* 1996).

Defects associated with dysfunction of methylene tetrahydrofolate reductase

Severe mutations of MTHFR

Rare, but severe, mutations of the MTHFR which have been cloned and sequenced (Goyette *et al.* 1994, 1995) are associated with neurological abnormalities and severe vascular disease (Rosenblatt, 1995). Even among the heterozygous carriers they lead to premature death. However, these defects are too rare to account for the prevalence of mild hyper-tHcy present in the community.

Thermolabile variant MTHFR C677T

An unstable thermolabile variant form of this enzyme has also been described (Kang *et al.* 1988*a*). This enzyme was originally characterized by partial denaturation at 46°C for five minutes (van der Put *et al.* 1995). Its malfunction leads to moderately raised levels of tHcy (Kang *et al.* 1988*a*, 1991; Jacques *et al.* 1996; Harmon *et al.* 1996). This thermolabile variant was shown to be significantly associated with CHD (Kang *et al.* 1988*a*, 1991). A responsible gene has recently been isolated (Frosst *et al.* 1995) and the defect shown to be a CβT mutation in the 677 nucleotide, which codes for an enzymic substitution of valine for alanine. Several studies have suggested that this polymorphism may be the only mutation that accounts for this thermolabile MTHFR (van der Put *et al.* 1995; Kluijtmans *et al.* 1996; Christensen *et al.* 1997). All studies showed distinct thermolability for the homozygous mutant genotype. The efficiency of the homozygous thermolabile mutant is around fifty percent of the homozygous wild type and the heterozygote was intermediate between the two. This is because the enzyme is deficient owing to its thermolability, rather than to malfunction of what enzyme remains (R.G. Matthews, pers. comm.).

A positive association between the presence of the MTHFR mutant and raised tHcy has been reported (Kang *et al.* 1991, 1993; Ma *et al.* 1996). However, it appears that the tHcy level is elevated only in those subjects with low plasma folates (Harmon *et al.* 1996; Jacques *et al.* 1996; Ma *et al.* 1996).

The Hordaland homocysteine study (Guttormsen *et al.* 1996) screened 18 043 subjects 40–67 years old and found 67 cases with tHcy ⩾ 40 μmol/l. When compared with 329 controls, the cases had lower plasma folate and cobalamin levels and consumed less vitamin supplements. Homozygosity for the MTHFR C677T was present in 73·1 % of cases and in 10·2 % of controls. Two years later, 58 of the 67 cases were reinvestigated and 41 still had tHcy > 20 μmol/l, 92 % of whom were homozygous for the MTHFR C677T mutant. This study suggests that the C677T mutant is strongly associated both with low plasma folate and high tHcy.

A recent meta-analysis has shown that the prevalence of this defect varies in different communities; while being relatively uncommon in Africans (Delport *et al.* 1997), the homozygous form was present in 235 out of 2542 western Caucasian controls (9·2 %) (van der Put *et al.* 1997).

The importance of the C677T variant is that its homozygous form is both relatively common in the normal population and has been reported to be associated with certain diseases. These include NTD and vascular disease, in particular CAD in some studies, but not in others.

Neural tube defects and MTHFR C677T

The association of the variant with NTD is perhaps its most convincing link to a clinical state. It has now been demonstrated by four separate groups (Ou *et al*. 1995; Van der Put *et al*. 1995; Whitehead *et al*. 1995; Papapetrou *et al*. 1996). However, it still accounts for only 13 % of the attributable risk in the population (Whitehead *et al*. 1995). How the variant increases NTD risk is still to be determined; it may be by reducing the folate status in the embryo and perhaps also in the mother. This has recently been shown in normal individuals homozygous for the MTHFR C677T variant (Molloy *et al*. 1997).

The MTHFR C677T variant is associated with an increased tHcy and lower plasma (Harmon *et al*. 1996) and red cell (Molloy *et al*. 1997) folate. However, when mothers who were homozygous for the MTHFR C677T variant were excluded, there was no significant effect on the relationship of the remainder between NTD prevalence and maternal RCF levels (Molloy *et al*. 1998). This suggests that the primary effect of the variant is not so much to elevate tHcy as to lower intracellular folate.

Vascular disease and MTHFR C677T

Vascular disease has also been associated with the homozygous C677T thermolabile enzyme (Kang *et al*. 1992; Frosst *et al*. 1995; Gallagher *et al*. 1996; Kluitjmans *et al*. 1996). This would seem likely, since MTHFR (C677T) produces a moderate rise in tHcy (Engbersen *et al*. 1995; Harmon *et al*. 1996) which is a risk factor for cardiovascular, peripheral and CVD (see above). Gallagher and colleagues (1996) studied 111 patients with proven heart disease and 105 matched controls. The defective allele was higher in cases than controls (OR 1·6; 95 % CI 1·1–2·4; P = 0·02). Being homozygous for the MTHFR variant was 17 % higher in cases compared with 7 % in controls (2·9; 1·2–7·2; P = 0·02) and tHcy levels were significantly matched to disease status.

Other studies have challenged the view that there is any significant association between the MTHFR C677T and CVD, in Australia (Wilcken *et al*. 1996), the Netherlands (van Bockxmeer *et al*. 1997), USA (Ma *et al*. 1996) and Britain (Adams *et al*. 1996), even to the extent of an outright denial of any relationship (Wilcken, 1997). The increased tendency to CAD in subjects with raised tHcy may include *inter alia* an increased tendency to atheroma and an enhanced liability to thrombosis. Thus, the criteria for choosing cases and controls may be of fundamental importance. Kang and coworkers (1991), in choosing their control group, excluded subjects with any history of thrombosis, whereas Adams and coworkers (1996) compared their patients with controls from the general population, regardless of whether or not they had had a previous vascular event.

Furthermore, the MTHFR C677T variant is associated with a raised tHcy only in the presence of low folate levels (Harmon *et al*. 1996; Jacques *et al*. 1996; Ma *et al*. 1996). At folate levels in the middle and upper quartiles, the prevalence of the genotypes did not vary. tHcy was only significantly elevated in homozygous subjects in the low folate group. This was

especially true for men under 60 years old (Ma *et al.* 1996). The MTHFR C677T variant is therefore likely to be associated with vascular disease only in communities with a low dietary folate intake (Scott & Weir, 1996). Ma and colleagues (1996) found a nonsignificant increase in the risk of myocardial infarct among those with the MTHFR C677T variant in those who had a low folate level and were under 60 years old; however, the number with low folate was small and the CI was wide.

The differences between the findings of Kang and co-workers (1988*a*) when assessing the frequency of the thermolabile enzyme in patients with CVD which were very significant, and the findings of the majority of studies of the MTHFR C677T variant which found a minimal association and only in situations of folate deficiency, may relate to the presence of other as yet undiscovered genetic variants of the MTHFR enzyme. It is also important to remember that tHcy is only the marker of an intracellular event. While excess homocysteine may be produced in all cells of the body when severe vitamin B nutritional deficiency or severe mutant defects of MTHFR occur (Mudd *et al.* 1995), it is possible that the raised tHcy associated with the C677T variant reflects disordered metabolism only in certain specific organs such as the liver but not in the endothelial cells lining the blood vessels. It is quite possible that defective enzyme activity might manifest itself only in cells with a high flux of folate through the MTHFR enzyme, i.e. cells with a low turnover and a high requirement for SAM mediated methyl groups (Weir & Scott, 1995).

The MTHFR C677T is associated with low serum folate in most (Harmon *et al.* 1996), though not all, (Kluijtmans *et al.* 1996) studies. Some groups found that the RCF level was high (van der Put *et al.* 1995), while others found that it was low (Molloy *et al.* 1997). There are currently two methods used to measure RCF, the radiometric assay (Levine, 1993) and the biological assay using *Lactobacillus casei* (Molloy & Scott, 1998). The radiometric assay is known to measure RCF at lower levels overall than the microbiological assays (McGown *et al.* 1978; Gunter *et al.* 1996). However, when subjects were divided into those homozygous for the C677T variant (TT), heterozygous (CT) or homozygous wildtype (CC), it was found that the radiometric assay gave results which were consistently and significantly higher than the microbiological assay in the TT group, while the levels in the CT and CC groups were significantly lower as expected (Molloy *et al.* 1998). This strongly suggests that the radiometric assay is measuring a biologically inactive folate compound or degradation product in the TT group, that cannot be measured by the microbiological assay. Whatever its nature, it could be of relevance to the pathogenesis of hyper-tHcy seen in homozygous MTHFR C677T subjects (Molloy *et al.* 1998). Folate is required to stabilize the function of MTHFR (Frosst *et al.* 1995), which is supported by the finding that folic acid therapy normalizes hyper-tHcy induced by the thermolabile MTHFR enzyme (Kang *et al.* 1988*a,b*). It may be that the biologically inactive folate compound in some way destabilizes the function of methylene reduction producing hyper-tHcy. It is also possible that the inactive compound represents a catabolic product of folate and that the TT group is in some way more at risk of intracellular folate catabolism.

Other reasons why the hyper-tHcy found in homozygous carriers of the MTHFR C677T variant was not associated with an increased prevalence of CAD may relate to smoking and the prevalence of high blood pressure. Hypertension, an independent risk factor for myocardial infarction, was found to correlate positively with tHcy levels (Levenson *et al.* 1994; Chasan-Taber *et al.* 1996; Graham *et al.* 1997). Smoking also correlated with tHcy in the European cohort study (Graham *et al.* 1997).

Clinical conditions associated with hyperhomocysteinaemia and coronary artery disease

Chronic renal failure (CRF)

Hyper-tHcy is a common finding in patients with CRF in whom tHcy tends to be in excess of 25 μmol/l (Wilcken *et al.* 1980; Bostom *et al.* 1996*a*; Dennis & Robinson, 1996). Since in CRF cardiovascular disease accounts for more than a third of all deaths (Parfrey, 1993), tHcy has been under close scrutiny as a possible cause (Bachmann *et al.* 1995). The important point is that dialysis, while reducing blood urea and creatinine levels, has little effect on tHcy. The resulting levels of tHcy, hyperfibrinogenaemia and lipoprotein (a) excess may well account for the high incidence of vascular disease in CRF patients (Bostom *et al.* 1996*b*). It is also well known that plasma folate concentrations show a significant negative correlation with tHcy levels, both before and after dialysis (r = −0·53, P < 0·003; and r = −0·58, P < 0·001 respectively). This was also true for RCF pre- and post-dialysis and occurred despite what appeared to be an adequate dietary folate intake (Bostom *et al.* 1996*a*; Tamura *et al.* 1996). Raised tHcy is usually easily reduced by taking vitamin B supplements; however, it appears that CRF patients require higher than normal levels of folate to reduce tHcy levels and prevent the associated vascular complications (Bostom *et al.* 1996*a*; Robinson *et al.* 1997). As reviewed by Perna and colleagues (1996), raised tHcy levels in CRF may be caused by reduced urinary excretion, however, the total amounts of tHcy excreted are trivial when considered against the total quantity produced by the body. The main reason probably relates to decreased renal metabolism, since the tHcy levels are lower in the renal vein than the renal artery (Bostom *et al.* 1995; Arnadottir *et al.* 1996).

Connective tissue disease

In systemic lupus erythematosus it has been shown prospectively that elevated tHcy relates to the risk of stroke and thromboembolic events (Petri *et al.* 1996). In this study 337 systemic lupus erythematosus patients were followed for a mean of 4·8 years. Twenty-nine patients had a stroke and 31 had arterial thrombotic events. tHcy was significantly associated with the thrombotic events (OR 3.74; 95 % CI 1·96–7·32; P = 0·001) and with stroke (2·24; 1·22–4·13; P = 0·001). When established risk factors were adjusted, tHcy remained an independent risk factor for both thrombosis and stroke.

In rheumatoid arthritis (RA) tHcy was higher than control values in both the fasting and post methionine challenge tests (Roubenoff *et al.* (1997). Paradoxically, the patients on treatment with methotrexate had normal tHcy values. Roubenoff and colleagues suggested that the raised tHcy in their RA patients were associated with low plasma concentrations of PLP. It would also explain their high incidence of cardiovascular mortality.

We have found similar results in RA patients treated with a variety of different therapies. In particular, methotrexate was found to lower tHcy, but gold therapy was associated with high values (Mahmud *et al.* 1998*a*).

Diabetes

Paradoxically Type I insulin dependent diabetes mellitus was associated with lower tHcy than in controls. This was true for males and females (Robillon *et al.* 1994).

We have confirmed these findings, although the rate of rise of the tHcy levels with age in both sexes is quicker in patients with insulin dependent diabetes mellitus than in normal controls. This may indicate that the increased rate of renal perfusion in insulin dependent diabetes mellitus may initially lead to a more rapid rate of tHcy metabolism by the kidneys, which may deteriorate with age (C.C. Cronin *et al.* 1996).

It has also been reported that the prevalence of the MTHFR C677T variant is higher in diabetes with than without retinopathy (Neugebauer *et al.* 1997). It was suggested that this might have pathogenic significance.

Chronic inflammatory bowel disease

Thromboembolic disease is common in chronic inflammatory bowel disease (Talbot *et al.* 1986), which may relate to a hypercoagulable state (Edwards *et al.* 1987). A recent study has demonstrated that prothrombotic agents are not involved (Jackson *et al.* 1997); however, higher tHcy is (Andus *et al.* 1996, Mahmud *et al.* 1998). The hyper-tHcy is especially associated with the MTHFR C677T variant (Molloy *et al*, 1997; Mahmud *et al.* 1998*b*).

Conclusions

There is now very strong evidence that tHcy correlates with increased risk of cardiovascular disease, stroke and a wide range of other thromboembolytic conditions. It is clear from some of these studies, particularly the two definitive prospective studies of Arnesen *et al.* (1995) and Wald *et al.* (1998), that this risk associated with homocysteine is continuous right down to the lowest levels encountered in plasma. The absence of a plateau suggests that there is increased benefit from lowering homocysteine, not just in the abnormally high range, but in what would formerly have been considered the normal range. This begs the question of the probable percentage of the population who have tHcy that can be lowered and whether such lowering could be achieved by nutritional means. It seems clear that most people and particularly the elderly have tHcy that can be reduced principally by the addition of folic acid to the diet (Selhub *et al.* 1993). Vitamin B_6 and/or B_{12} could also contribute. This applies to populations with folate intakes not significantly below the existing recommended dietary allowance in the UK and the USA of 200 µg food folate/d. Even in younger apparently well fed adult males the majority have tHcy that could be reduced signifcantly by small increased levels of dietary folic acid (100 to 200 µg/d) (Ward *et al.* 1997). This, together with evidence that tHcy is related to risk of CVD and stroke without an apparent plateau, suggests that it may be desirable to raise the recommended dietary allowance (recommended nutrient intake) for folate. We suggest that because of poor bioavailability and perhaps more importantly the poor stability of the natural forms of folate in food, this objective will not easily be achieved by an increase in food folates. The alternative is to fortify food with a stable and bioavailable synthetic form, namely folic acid. This seems the only realistic way of optimally lowering homocysteine in most individuals and populations.

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