B-vitamins, homocysteine metabolism and CVD

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The present review focuses on the B-vitamins, i.e. folate, vitamin B_{12}, vitamin B_6 and riboflavin, that are involved in homocysteine metabolism. Homocysteine is a S-containing amino acid and its plasma concentrations can be raised by various constitutive, genetic and lifestyle factors, by inadequate nutrient status and as a result of systemic disease and various drugs. Hyperhomocysteinaemia is a modest independent predictor of CVD and stroke, but causality and the precise pathophysiological mechanism(s) of homocysteine action remain unproven. The predominant nutritional cause of raised plasma homocysteine in most healthy populations is folate insufficiency. Vitamin B_{12} and, to a lesser extent, vitamin B_6 are also effective at lowering plasma homocysteine, especially after homocysteine lowering by folic acid in those individuals presenting with raised plasma homocysteine. However, riboflavin supplementation appears to be effective at lowering plasma homocysteine only in those individuals homozygous for the T allele of the C677T polymorphism of the methylenetetra-hydrofolate reductase (MTHFR) gene. This gene codes for the MTHFR enzyme that produces methyltetrahydrofolate, which, in turn, is a substrate for the remethylation of homocysteine by the vitamin B_{12}-dependent enzyme methionine synthase. Individuals with the MTHFR 677TT genotype are genetically predisposed to elevated plasma homocysteine, and in most populations have a markedly higher risk of CVD.

Homocysteine: Folate: Vitamin B_{12}: Vitamin B_6: Riboflavin

Epidemiological and experimental evidence is accumulating to indicate a probable role for certain B-vitamins in the prevention of CVD. Active forms of four B-vitamins, i.e. folate, vitamin B_{12}, vitamin B_6 and riboflavin, are involved in the metabolism of a S-containing amino acid, homocysteine. Homocysteine metabolism links the methionine cycle with the folate cycle (Fig. 1). In most tissues and cells the major, or only, pathway for the conversion of homocysteine to methionine is the transfer of a methyl group from 5-methyltetrahydrofolate to homocysteine, catalysed by the vitamin B_{12}-dependent enzyme methionine synthase. One product of this reaction, methionine, can donate the methyl group via the activated form of methionine *S*-adenosylmethionine to a range of substrates, including proteins, phospholipids and DNA. The other product of the methionine synthase reaction, tetrahydrofolate, is reconverted to 5-methyltetrahydrofolate via the folate cycle. The reduction of 5,10-methylenetetra-hydrofolate to 5-methyltetrahydrofolate in the folate cycle is catalysed by the methylenetetrahydrofolate reductase (MTHFR) enzyme, which has a FAD (derived from riboflavin) prosthetic group. The metabolically-active form of vitamin B_6, pyridoxal phosphate, is an enzyme cofactor for cystathionine β-synthase, which is involved in the catabolism of homocysteine to sulfate in the trans-sulfuration pathway (Fig. 1).

Homocysteine and CVD

The current interest in homocysteine stems from a clinical syndrome, homocystinuria, identified >40 years ago (Carson & Neill, 1962; Gerritsen *et al.* 1962). Homocystinuria is an autosomal recessive disorder characterised by abnormalities of the long bones, ocular lens dislocation, mental retardation and aggressive vascular disease, in particular venous thromoembolism (Robinson, 2000). Classical homocystinuria is caused by a deficiency of cystathionine β-synthase. Two other rare genetic disorders involving deficiencies of MTHFR or methionine synthase can also cause plasma homocysteine concentrations to rise 20-fold and homocysteine to be excreted in the urine. Each

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of these enzyme deficiencies results in similar clinical symptoms, including aggressive vascular disease, suggesting that homocysteine might be the pathogenic factor in the vascular complications. It has been hypothesised, therefore, that even mild elevations in plasma total homocysteine (tHcy; hyperhomocysteinaemia) could be causally related to CVD (McCully, 1969). Although the inborn errors of homocysteine metabolism are very rare, hyperhomocysteinaemia is common in many populations. It is more common in males, rises with age and the menopause in women, is influenced by lifestyle factors including smoking and alcohol and can be secondary to systemic disease (e.g. kidney dysfunction) or drug treatment (de Bree *et al*. 2002).

Two recent meta-analyses of observational studies have indicated that tHcy is an independent predictor of CVD and stroke (Homocysteine Studies Collaboration, 2002; Wald *et al*. 2002). The Homocysteine Studies Collaboration (2002) has found stronger associations between tHcy (measured in blood after the onset of disease) and CVD and stroke in retrospective studies than in prospective studies when tHcy is measured initially in blood from individuals who at that time had no history of CVD or stroke. A total of 5073 heart disease events and 1113 stroke events were included in the meta-analysis. After adjustment for known cardiovascular risk factors and regression dilution bias in the prospective studies, it was found that a 25% lower than usual tHcy concentration (approximately 3 µmol/l lower) is associated with an 11% (odds ratio 0·89 (95% CI 0·83, 0·96)) lower heart disease risk and 19% (odds ratio 0·81 (95% CI 0·69, 0·95)) lower stroke risk. Wald *et al*. (2002), in a meta-analysis of prospective studies and genetic studies that yielded similar highly-significant results, have estimated that a 3 µmol/l decrease in tHcy is associated with a 16 (95% CI 11, 20) % decreased risk of heart disease and a 24 (95% CI 15, 33) % decreased risk of stroke. In the genetic studies the prevalence of a mutation in the MTHFR gene in disease cases was determined and it was found that individuals who have a cytosine to thymine substitution (corresponding to an amino acid change of alanine to valine) at base 677 of the gene have decreased MTHFR enzyme activity and higher tHcy (Frosst *et al*. 1995) than those with the wild-type genotype. This mutation is a common polymorphism, with an average prevalence of the TT genotype of 12% (Brattstrom *et al*. 1998). The genetic studies of Wald *et al*. (2002) are supported by those of Klerk *et al*. (2002), who in a further meta-analysis of case–control observational studies (11 162 cases and 12 758 controls) have found that individuals with the MTHFR 677TT genotype have a 16% (odds ratio 1·16 (95% CI 1·05, 1·28)) higher odds of CVD compared with individuals with the CC genotype. The genetic studies of disease risk are susceptible to different sources of error from those associated with the prospective studies of tHcy and disease risk, and give evidence of associations that are neither confounded by the usual lifestyle and socio-economic factors nor diluted by measurement error (Wald *et al*. 2002). Davey-Smith & Ebrahim (2003) have concluded that such triangulation of the associations between genotype, tHcy and CVD risk provides robust evidence of a general casual effect of tHcy on disease risk that would be experienced by the whole population, independent of genotype.

Causality of homocysteine in CVD is further supported by studies that show that B-vitamin lowering of tHcy decreases the rate of restenosis (Schnyder *et al*. 2001) and adverse outcomes among CVD patients with successful angioplasty (Schnyder *et al*. 2002), and improves vascular endothelial function in such patients (Chambers *et al*. 2000).

Various pathophysiological mechanisms have been proposed to explain the effect of tHcy on cardiovascular risk, but there is as yet no consensus. Proposed mechanisms include possible effects of tHcy on endothelium dysfunction, increased arterial intimal–medial thickness, increased arterial stiffness, increased procoagulant activity and age-related immune dysfunction (Mangoni & Jackson, 2002; Dawson *et al*. 2004). An alternative explanation is that elevated tHcy is a reliable marker of low B-vitamin status or decreased methylation capacity of cells; either of these factors might be directly related to disease. Castro *et al*. (2003), for example, have found that there are increased tHcy and *S*-adenosylhomocysteine concentrations in plasma and a lower global DNA methylation status in leucocytes from patients with CVD compared with matched controls. Disturbances in DNA methylation will have effects on the control of gene expression (Frisco *et al*. 2002). The addition of a methyl group to cytosine in the sequence CG is the major modification made to DNA after new strands have been synthesised during cell division (about 80% of the CG is methylated) and methylation-free regions (CpG islands) tend to occur in the promoter (control) region of genes. Methylation of the promoter region of genes or the protein core (chromatin) of DNA determines whether genes are active (unmethylated) or inactive (methylated). Paradoxically, even in the face of global DNA hypomethylation, certain regions of the genome can be hypermethylated, and such perturbations in DNA methylation and gene expression could have profound effects on CVD risk (Dong *et al*. 2002).

The first report of a randomised controlled trial of tHcy lowering in patients to prevent recurrent stroke (Toole *et al*. 2004) does not, however, provide any evidence of causality; albeit the authors found the expected strong prospective association between tHcy at baseline and vascular risk. In this trial a decrease of about 2 µmol tHcy/l achieved by a (once daily) high-dose B-vitamin formulation (*n* 1827) containing 25 mg vitamin B_6, 0·4 mg vitamin B_{12} and 2·5 mg folic acid *v*. a low-dose formulation (*n* 1853) containing 200 µg vitamin B_6, 6 µg vitamin B_{12} and 200 µg folic acid after non-disabling cerebral infarction was found to have no effect on vascular outcomes during the 2 years of follow-up. The authors put forward several reasons for these somewhat disappointing outcomes, which include: coinciding with folic acid fortification of the US grain supply, which profoundly decreased the percentage of the population with folate deficiency and thus high tHcy (from 22 before fortification to 1·7 after fortification; Jacques *et al*. 1999); correction of low serum vitamin B_{12} concentrations in the low-dose
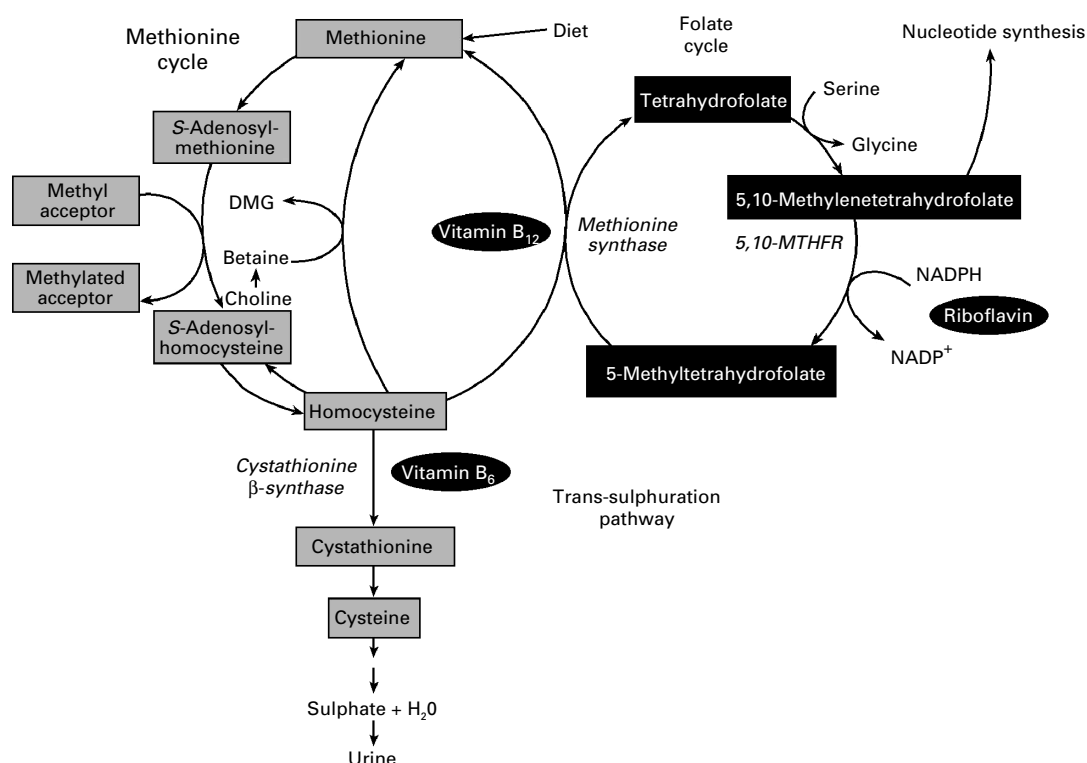
Fig. 1. Pathways for the metabolism of homocysteine. 5,10-MTHFR, 5,10-methylenetetrahydrofolate reductase; DMG, dimethylglycine. (Adapted from Robinson, 2000.)

group, which may have blunted the vitamin effect; a truncated treatment and follow-up period.

Lowering homocysteine with B-vitamins

Folate and folic acid

The folate metabolite 5-methyltetrahydrofolate is a substrate for the enzyme methionine synthase, which remethylates homocysteine to form methionine (Fig. 1). Cross-sectional studies have indicated a strong inverse association between dietary and/or blood folate concentrations and tHcy concentrations (Nygard *et al*. 1998). The Homocysteine Lowering Trialists Collaboration (1998) has shown in a meta-analysis (1114 subjects in twelve randomised trials) that supplements ($0·5–5·0$ mg/d) of folic acid, the synthetic form of the B-vitamin, decrease tHcy by about 25 (95% CI 23, 28) % ($P<0·001$). In addition, there is no difference in tHcy lowering within the range $0·5–5·0$ mg folic acid/d, and folic acid is much more effective than other B-vitamins in lowering tHcy. A more recent study of 316 healthy older (50–75 years) Dutch adults in a randomised double-blind placebo-controlled trial (van Oort *et al*. 2003) has shown that daily supplementation of about $400\,\mu g$ folic acid/d can give 90% of the maximal decrease in tHcy. A similar adequate dose for near-optimal lowering of tHcy by folic acid has been reported previously by Ward *et al*. (1997), who found no further decrease in tHcy with folic acid at $400\,\mu g/d$ compared with $200\,\mu g/d$ in thirty healthy adults aged 34–65 years using a sequential design.

In contrast to these studies in healthy subjects, which have indicated that doses of folic acid of $<0·5$ mg/d (and largely achievable by dietary means) are adequate to effect a maximal decrease in tHcy, Wald *et al*. (2001) have found that in patients with CVD a dosage of $0·8$ mg/d appears to be necessary for maximal lowering of tHcy. However, very recent evidence from patients with heart disease indicates that optimal tHcy lowering may be achievable at much lower folic acid doses (Tighe *et al*. 2004), similar to those shown to be effective in healthy subjects.

Vitamin B_{12}

In addition to 5-methyltetrahydrofolate, the remethylation of homocysteine to methionine requires vitamin B_{12} as a cofactor of the enzyme methionine synthase (Fig. 1). In the meta-analysis of the Homocysteine Lowering Trialists' Collaboration (1998) vitamin B_{12} has been found to be a less-effective agent for lowering tHcy than folic acid, with $0·5$ mg vitamin B_{12}/d producing an additional 7 (95% CI 3, 10) % decrease in tHcy above the 25% lowering achieved by folic acid supplements. The effect of vitamin B_{12} is, in general, diminished by the larger role of folate status in determining tHcy. Quinlivan *et al*. (2002) have recently described the association between tHcy and both folate and vitamin B_{12} status in two groups of healthy volunteers pre- and post-supplementation with folic acid. They have shown that once folate status has been optimised a clear dependence of tHcy on vitamin B_{12} status emerges that is not observed in the presupplementation period and

coincides with the loss of a correlation between tHcy and folate status. These findings are consistent with recent evidence from the USA that shows that in the era of folic-acid fortification of grain foods it is vitamin B_{12} status (and not folate status) that is now the major nutritional determinant of tHcy in that population (Liaugaudas *et al.* 2001).

Although folate status is the major determinant of tHcy in non-folic acid-supplemented populations, subgroups within such populations may exhibit dependence of tHcy on vitamin B_{12} status rather than folate. Mann *et al.* (1999) have found that free-living Australian habitual meat-eaters have lower tHcy and higher serum vitamin B_{12} concentrations than both ovo-lacto vegetarians and vegans. Interestingly, ovo-lacto vegetarians have much lower methionine intakes than the habitual meat-eaters. Similarly, Hung *et al.* (2002) have reported higher tHcy and serum folate levels in Taiwanese vegetarians compared with omnivores, whilst Herrmann *et al.* (2003) have shown that German vegans have metabolic features that indicate vitamin B_{12} deficiency, which lead to a substantial increase in tHcy.

Although several studies have provided dose–response data relating to tHcy lowering by physiological doses of folic acid, intervention studies with vitamin B_{12} are largely restricted to studies undertaken >30 years ago that do not include a measurement of tHcy (Scott, 1997), or more recent vitamin B_{12} intervention studies that do include a tHcy response but mostly focus on high (non-physiological) doses of vitamin B_{12} (Ubbink, 1994; den Heijer *et al.* 1998).

Vitamin B_6

Pyridoxal phosphate, the active form of vitamin B_6, is a cofactor for enzymes involved in amino acid metabolism. These enzymes include cystathionine β-synthase, the first enzyme in the trans-sulfuration pathway that breaks down homocysteine to sulfate (Fig. 1). The meta-analysis published by the Homocysteine Lowering Trialists' Collaboration (1998) has indicated that vitamin B_6 (mean 16·5 mg daily) does not have any marked tHcy-lowering effect that is additional to that of folic acid and vitamin B_{12}. It is possible that an effect of vitamin B_6 on tHcy lowering has been missed in previous trials because of the much greater effects of folic acid and/or vitamin B_{12}. More recently, McKinley *et al.* (2001) have shown in a randomised placebo-controlled trial that a low dose of vitamin B_6 (1·6 mg/d) for 12 weeks results in a further lowering of tHcy in healthy elderly subjects aged 63–80 years after previous repletion with folic acid (and riboflavin). Folic acid supplementation lowers fasting tHcy by 19·6% ($P < 0·001$), and subsequent supplementation with vitamin B_6 (additional to folic acid) in these elderly subjects (n 11) lowers tHcy by a further 7·5% ($P = 0·008$), when compared with the group (n 10) supplemented with folic acid alone.

In the era of widespread folic acid supplementation in the USA low vitamin B_6 status, but not tHcy, has been shown to be strongly associated with CVD in a case–control study (Kelly *et al.* 2003). This finding supports those from studies of vitamin B_6-deficient monkeys and epidemiological studies (Rimm *et al.* 1998) in which an association between low vitamin B_6 status and vascular disease has been reported, which might be explained by a relationship between inflammation and low vitamin B_6 status that has been reported by Kelly *et al.* (2004). Whatever the mechanism, such associations between B-vitamin status and vascular disease suggest that tHcy might be an indicator of low B-vitamin status rather than being directly related to disease.

Riboflavin

The cosubstrate for methionine synthase, 5-methyltetra-hydrofolate, is regenerated via the folate cycle and the MTHFR enzyme, which requires FAD as a prosthetic group (see Fig. 1). In addition, riboflavin in another active form (FMN) is required for the generation of pyridoxal phosphate (the active coenzyme form of vitamin B_6), which serves as a cofactor of cystathionine β-synthase and other enzymes of the trans-sulfuration pathway.

Notwithstanding the close metabolic relationships between riboflavin and the homocysteine and folate cycles, a double-blind randomised placebo-controlled riboflavin-supplementation trial in healthy elderly subjects who had a suboptimal riboflavin status has found no effect of low-dose riboflavin supplementation (1·6 mg/d) for 12 weeks in lowering tHcy (McKinley *et al.* 2002). In this trial the placebo group (n 22) was followed from the initial riboflavin intervention, after which they received folic acid (400 µg/d) for 6 weeks followed by folic acid and riboflavin (1·6 mg/d) for a further 12 weeks and then by a 16-week wash-out period post supplementation. The purpose of the follow-up with supplementation of the original placebo group was to determine whether there is a dependence of tHcy on riboflavin once folate status is optimised and to determine whether these subjects have tHcy that can be lowered by other B-vitamin (folic acid) intervention. It was observed that folic acid supplementation lowers tHcy and that riboflavin supplementation improves riboflavin status. However, it would seem that in these volunteers, who were pre-selected for suboptimal riboflavin status, riboflavin supplementation is not an effective tHcy-lowering agent either before or after folate status optimisation.

Riboflavin supplementation does not appear to lower tHcy in the general population. However, observational studies have shown a dependence of tHcy on riboflavin status in those subjects homozygous (TT) for the MTHFR C677T polymorphism (Jacques *et al.* 2002; McNulty *et al.* 2002) or for the T allele of this polymorphism (Hustad *et al.* 2000). These observations are concordant with the molecular data from *in vitro* studies, which indicate that when compared with the wild-type enzyme the thermolabile MTHFR enzyme has an enhanced propensity to dissociate into monomers and to lose the FAD prosthetic group on dilution (Yamada *et al.* 2001).

The observational epidemiological studies, together with data from *in vitro* studies, have prompted a comparison of the effect of low-dose (1·6 mg/d) riboflavin supplementation across the three different genotypes (CC, CT and TT)

for MTHFR. Subjects with a suboptimal riboflavin status were randomly stratified, by screening the tHcy value within each of the genotype groups, to receive either treatment or a placebo for 12 weeks. Increases in riboflavin status (approximately 10% decrease from baseline in the erythrocyte glutathione reductase activation coefficient) are observed for all three genotypes with riboflavin supplementation (McNulty *et al*. 2003). Only in the TT group (*n* 27), however, is a significant (*P*=0·007) decrease ($-20·8$%) in tHcy observed. The percentage change in tHcy from baseline among the CC (*n* 22) and CT (*n* 23) groups is not significant (*P*=0·650 and *P*=0·327 respectively).

These data for human subjects are, therefore, consistent with the observational data and the current understanding of the molecular aspects relating to the MTHFR mutant. To this end, Stern *et al*. (2003) have provided data on homocysteine methylation in cultured immortalised lymphocytes from individuals homozygous for the MTHFR C677T mutation. Under adequate nutrient (i.e. folate and riboflavin replete) conditions, the activity of the MTHFR enzyme and the proportion of methylated folates in the TT cells is about 50% that of CC cells, yet there is no difference in homocysteine accumulation in the culture medium or methionine synthetic capacity between TT and CC cells. Only folate and riboflavin depletion together result in marked differences between genotypes, with decreased methionine production in TT cells compared with CC cells. The findings, however, involve only one immortalised cell type over a short period and the authors acknowledge that other tissues may respond differently to these and other nutritional perturbations.

One such nutritional perturbation may involve choline, which through its oxidised product, betaine, can remethylate homocysteine via an alternative enzyme to methionine synthase, i.e. betaine–homocysteine *S*-methyltransferase, in a restricted number of tissues (Fig. 1). For example, Schwahn *et al*. (2003) have found a negative correlation between plasma betaine and tHcy in subjects with CVD. They have also reported that hyperhomocysteinaemic MTHFR-compromised mice appear to be much more sensitive to changes in choline and/or betaine intake than wild-type animals. High doses (>6 g/d) of betaine have long been used to lower tHcy in hyperhomocystinuria, and Olthof *et al*. (2003) have recently shown that much lower doses of betaine ($\geq$1 g/d) lead to immediate and long-term lowering of tHcy in healthy men and women.

Conclusions

Elevated tHcy is strongly implicated in CVD, but a cause-and-effect relationship remains to be proven. Although hyperhomocysteinaemia is very responsive to lowering with folic acid in most populations, other metabolically-related B-vitamins, particularly vitamin B_{12} but also vitamin B_6, have a role in preventing the elevation of tHcy. A fourth B-vitamin, riboflavin, appears to be important in genetic predisposition (C677T MTHFR polymorphism) to low folate status and/or high tHcy.

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