Nutritional aspects and possible pathological mechanisms of hyperhomocysteinaemia: an independent risk factor for vascular disease

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Numerous case–control and prospective studies have identified elevated plasma homocysteine as a strong independent risk factor for cerebovascular, cardiovascular and peripheral vascular disease. Homocysteine is formed as a result of the breakdown of the dietary amino acid methionine. Once formed, homocysteine is either remethylated to methionine, or undergoes a trans-sulfuration reaction to form cysteine. The re-methylation of homocysteine to methionine is dependent on three B-vitamins, i.e. riboflavin, vitamin B_{12} and folate. The second pathway of homocysteine metabolism is the trans-sulfuration pathway which requires both vitamin B_6 and riboflavin for its activity. Thus, up to four B-vitamins are required for intracellular homocysteine metabolism. Many studies have noted strong inverse relationships between homocysteine levels and the status of both vitamin B_{12} and folate. However, the relationship between vitamin B_6 status and homocysteine is still uncertain. Similarly, numerous intervention studies have demonstrated effective lowering of homocysteine levels as a result of folate and vitamin B_{12} supplementation, while the homocysteine-lowering ability of vitamin B_6 is unclear. Even though riboflavin plays a crucial role in both the trans-sulfuration and remethylation pathways of homocysteine metabolism, the relationship between riboflavin status and homocysteine levels has not been investigated. The exact mechanism that explains the vascular toxicity of elevated homocysteine levels is unknown at present, studies indicate that it is both atherogenic and thrombogenic. To date, no randomized clinical trial has demonstrated that lowering of homocysteine levels is beneficial in terms of reducing the prevalence of vascular disease. It is probable, however, that optimal B-vitamin status is important in the prevention of vascular disease.

Homocysteine: Vascular disease: Vitamin B

Historical perspective and metabolism

Homocysteine was first discovered in 1932 as a breakdown product of methionine (Du Vigneaud, 1952). Another important milestone in homocysteine's history occurred in Northern Ireland when Carson & Neill (1962) first described the inborn error of methionine metabolism called homocystinuria. Almost simultaneously Gerritsen & Waisman (1964), in the USA, identified homocysteine in the urine of an infant who failed to thrive. After these initial reports, numerous studies began to investigate the biochemical basis and clinical features associated with homocystinuria. Mudd *et al*. (1964) reported that homocystinuria was caused by a lack of the trans-sulfuration enzyme cystathionine β-synthase in the liver and brain. Since this discovery, other rare enzyme defects that also cause homocystinuria have been identified (Fenton & Rosenberg, 1995; Rosenblatt, 1995), and these defects are discussed later in the present review (p. 225).

Once formed, homocysteine is either remethylated to methionine or undergoes a trans-sulfuration reaction to form cysteine. The remethylation pathway involves two critical enzymes. The first enzyme is methionine synthase, which catalyses the remethylation of homocysteine to methionine, and requires vitamin B_{12} in the form of methylcobalamin as cofactor and folate in the form of 5-methyltetrahydrofolate as co-substrate (Finkelstein, 1990). The second critical enzyme in the remethylation pathway is methylenetetrahydrofolate reductase (MTHFR). This enzyme is responsible for the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which then acts as co-substrate for the vitamin B_{12}-dependent remethylation of homocysteine to methionine. Riboflavin in the form of FAD is required as a prosthetic group for MTHFR (Bates &

Abbreviation: MTHFR, methylenetetrahydrofolate reductase.

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Fuller, 1986). During trans-sulfuration homocysteine is irreversibly condensed with serine to form cystathionine. This reaction is catalysed by cystathionine β-synthase, which is pyridoxal 5′-phosphate (vitamin B_6)-dependent (Mudd *et al*. 1995). Pyridoxal 5′-phosphate, the active coenzyme form of vitamin B_6, is formed through pyridoxine oxidation by the FMN-dependent enzyme, pyridoxine phosphate oxidase (McCormick, 1989). Thus, riboflavin is actually required for the activity of both pathways of homocysteine metabolism. S-adenosyl methionine, the breakdown product of methionine, controls the flux of homocysteine through the trans-sulfuration and remethylation pathways (Finkelstein, 1990). High levels of S-adenosyl methionine inhibit the remethylation enzyme MTHFR and activate the trans-sulfuration pathway; alternatively, low levels of S-adenosyl methionine activate the remethylation pathway and inhibit activity of the trans-sulfuration enzyme, cystathionine β-synthase (Selhub & Miller, 1992).

Epidemiological evidence for elevated homocysteine as a risk factor for vascular disease

Retrospective case–control and cross-sectional studies

Kilmer McCully (1969) proposed that the high prevalence of premature vascular disease and thromboembolic events observed among patients with homocystinuria occurred as a direct result of the high homocysteine levels associated with the condition. Since this initial hypothesis, numerous case–control and cross-sectional studies have investigated the relationship between elevated homocysteine levels and vascular disease. There are now approximately seventy published case–control studies. Boushey *et al*. (1995) carried out a meta-analysis of available case–control, cross-sectional and prospective studies. Five cross-sectional and nineteen case–control studies were included in this meta-analysis, and the main conclusion was that elevations in homocysteine levels were considered an independent risk factor for arteriosclerotic vascular disease; the odds ratio for a 5 μmol/l increase in homocysteine was calculated to be 1·6 for men and 1·8 for women. Case–control studies have found elevated homocysteine levels in patients with CHD (Kang *et al*. 1986*a*; Dalery *et al*. 1995; Hopkins *et al*. 1995; Robinson *et al*. 1995; Aronow & Ahn, 1997; Yoo *et al*. 1999), stroke (Brattstrom *et al*. 1984, 1992*b*; Lindgren *et al*. 1995; Markus *et al*. 1997), peripheral vascular disease (Malinow *et al*. 1989; Aronow & Ahn, 1998), myocardial infarction (Malinow *et al*. 1996) and venous thromboembolism (den Heijer *et al*. 1996; Simioni *et al*. 1996; Eichinger *et al*. 1998) compared with matched controls. For example, a study by Clarke *et al*. (1991) found hyperhomocysteinaemia in 42 % of patients with cerebrovascular disease, in 28 % of patients with peripheral vascular disease and in 30 % of patients with coronary artery disease, but no hyperhomocysteinaemia in the controls. Results from the European Concerted Action Project (Graham *et al*. 1997), a large multi-centred case–control study involving 750 cases of atherosclerotic vascular disease and 800 controls, demonstrated that an elevated plasma homocysteine level was an independent risk factor for vascular disease, and that the effect was graded. The relative risk for vascular disease for the top 20 % compared with the bottom 80 % of the control homocysteine distribution was 2·2. Cross-sectional and case–control studies have also demonstrated a significant association between homocysteine levels and the extent of vascular disease in the coronary (Montalescot *et al*. 1997; Verhoef *et al*. 1997*b*), carotid (Malinow *et al*. 1993; Selhub *et al*. 1995; Aronow & Ahn, 1997; Bots *et al*. 1997), aortic (Konecky *et al*. 1997) and peripheral (van den Berg *et al*. 1996) arteries. Taylor *et al*. (1991) studied a cohort of 214 patients with symptomatic lower-extremity arterial occlusive disease and/or symptomatic cerebral vascular disease. They found that clinical progression of lower extremity and coronary disease, but not cerebrovascular disease, was more likely in patients with elevated plasma homocysteine levels than in patients with normal levels. Also, the rate of disease progression was more rapid among individuals with elevated homocysteine levels. Recently, Voutilainen *et al*. (1998) studied 513 healthy men and women aged 45–69 years with no clinical cardiovascular disease, and found elevated plasma homocysteine concentrations in men were associated with early atherosclerosis, as demonstrated by increased wall thickness of the common carotid artery, but found no such association in women.

Prospective studies

Although the number of case–control studies that show a relationship between hyperhomocysteinaemia and vascular disease is quite overwhelming at this stage, this type of study does not demonstrate a causal relationship. Prospective studies, on the other hand, provide stronger support for a causal relationship. There are currently twenty-four prospective studies investigating the relationship between homocysteine levels and vascular disease. These studies can be divided into two types, prospective nested case–control studies and prospective cohort studies, summarized in Tables 1 and 2 respectively. To date, prospective studies have demonstrated that homocysteine is an independent risk factor for myocardial infarction (Stampfer *et al*. 1992; Stehouwer *et al*. 1998; Bots *et al*. 1999; Ridker *et al*. 1999), CHD (Arnesen *et al*. 1994; Moustapha *et al*. 1998; Taylor *et al*. 1999), stroke (Perry *et al*. 1995; Petri *et al*. 1996; Bostom *et al*. 1997*b*, 1999; Stehouwer *et al*. 1998; Bots *et al*. 1999; Ridker *et al*. 1999), venous thromboembolism (Ridker *et al*. 1997; Shemin *et al*. 1999), IHD (Wald *et al*. 1998) and all-cause mortality (Nygard *et al*. 1997*a*; Kark *et al*. 1999), even after adjustment for confounders such as other vascular risk factors.

The relationship appears to be graded, i.e. it is seen across the range of homocysteine value, with no obvious threshold associated with increased risk (Arnesen *et al*. 1994; Perry *et al*. 1995; Nygard *et al*. 1997*a*). Results from the prospective studies however are inconsistent. Among the nested case–control studies, six studies have been negative and seven studies have been positive. Five of the thirteen nested case–control studies were carried out in the US Physician cohort, and only two studies found a positive association between elevated homocysteine levels and vascular disease. However, the US Physician cohort

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Table 1. Results from prospective nested case–control studies of homocysteine (Hcy) and vascular disease of individuals without disease at study entry
(Values for odds ratio (OR) and relative risk (RR) are given with 95 % CI in parentheses)

Study, country, follow-up	Percentage of males	Cases	Controls	Age (years), type of group	End point	Hcy (μmol/l) Cases	Hcy (μmol/l) Control	OR or RR†	Hcy identified as a risk factor?
Stampfer *et al.* (1992) USA, 5 years	100	271	271	40–84 US physicians	MI or CHD death	11·1	10·5*	RR of MI 3·4 (1·3, 8·8)‡	Yes
Alfthan *et al.* (1994) Finland, 9 years	51	265	269	40–64 general pop[n]	Stroke or MI	9·99 9·58	9·82 (males) 9·28 (females)	OR males 1·0 (0·95, 1·06)§ OR females 1·0 (0·95, 1·10)	No
Verhoef *et al.* (1994) USA, 5 years	100	109	427	40–84 US physicians	Ischaemic stroke	11·1	10·6 NS	OR 1·2 (0·7, 2·0)ll	No
Arnesen *et al.* (1995) Norway, 3·5 years	50	123	492	12–61 general pop[n]	CHD event or CHD death	12·7	11·3**	RR for CHD 1·32 (1·05, 1·65)¶	Yes
Perry *et al.* (1995) UK, 12·8 years	100	107	118	40–59 general pop[n]	Fatal and non-fatal stroke	13·7	11·9**	RR of stroke 4·7 (1·1, 20·0)††	Yes
Chasan-Taber *et al.* (1996) USA, 7·5 years	100	333	333	40–84 US physicians	Acute MI or CHD death	NG	NG	RR of MI 1·7 (0·9, 3·3)‡	No
Evans *et al.* (1997) USA, < 7 (MI) or 11–17 years (death)	100 MI Death	240 93 147	472 186 286	35–57 general pop[n]	Non-fatal MI CHD death	12·6 12·8	13·1 NS (MI) 12·7 NS (Death)	OR for CHD death and MI 0·82 (0·55, 1·54)††	No
Ridker *et al.* (1997) USA, 10 years	100	145	646	40–84 US physicians	Incidence VTE	11·5	10·9 NS	RR idiopathic VTE 2·48‡ (0·97, 6·39)	Yes, idiopathic VTE
Verhoef *et al.* (1997*a*) USA, 9 years	100	149	149	40–84 US physicians	Angina pectoris with surgery	10·9	10·4 NS	OR for angina pectoris 1·0‡‡ (0·8, 1·4)	No
Folsom *et al.* (1998) USA, 3·3 years	NG	232	537	45–64 general pop[n]	CHD event or CHD death	8·86	8·53 NS	RR of CHD 1·28 (0·5, 3·2)ll	No
Wald *et al.* (1998) UK, 8·7 years	100	229	1126	35–61 general pop[n]	Death from IHD	13·1	11·8***	OR of death from IHD 2·9†† (2·04, 4·12)	Yes
Bots *et al.* (1999) The Netherlands, 2·7 years	49	224	533	> 55 general pop[n]	MI and stroke	17·3 18·4	15·2 (MI)** 15·2 (stroke)**	OR for MI 2·10 (0·88, 5·03)ll OR for stroke 1·90 (0·80, 4·48)	Yes
Ridker *et al.* (1999) USA, 3 years	0	122	244	59·3 (mean) general pop[n]	MI, stroke, or death due to CV disease	14·1	12·4**	RR of MI or stroke 4·6‡ (1·7, 12·3)	

CV, cardiovascular disease; MI, myocardial infarction; NG, not given; Pop[n], population; VTE, venous thrombotic disease or thromboembolism.
* $P < 0{\cdot}05$, ** $P < 0{\cdot}01$, *** $P < 0{\cdot}001$.
† Multivariate adjusted.
‡ > 95th Percentile *v.* 90th percentile.
§ Unit change in hcy.
ll Quintile 5 *v.* quintile 1.
¶ Increase in hcy of 4 μmol/l.
†† Quartile 4 *v.* quartile 1.
‡‡ Increase in hcy of 5 μmol/l.

represents a relatively-well-nourished sample, and consequently their homocysteine levels may have been lower than those of the US population in general, which possibly reduced the likelihood of finding an association. Of the eleven cohort studies only two failed to find an increased risk of vascular disease associated with elevated homocysteine levels. One of these studies, by Sirrs *et al*. (1999) in haemodialysis patients, actually observed an inverse relationship between homocysteine levels and mortality, suggesting that lower homocysteine levels were actually associated with an increased risk of death. The follow-up period in this study, however, was very short. Bostom *et al*. (1997*a*) and Moustapha *et al*. (1998) both carried out similar studies in patients with end-stage renal disease, and both found elevated homocysteine levels to be an independent risk factor for vascular disease. The follow-up period of these studies, however, was twice as long as that employed by Sirrs *et al*. (1999). Sirrs *et al*. (1999) hypothesized that their findings could have reflected a transient depression in homocysteine levels before death, which was not observed in the studies that used a longer follow-up.

The fact that authors use different statistical methods for calculating odds ratios and relative risks makes interpretation and comparison of prospective studies difficult. In the Boushey *et al*. (1995) meta-analysis referred to earlier only three prospective studies were included, and a

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Table 2. Results from prospective cohort studies of homocysteine (Hcy) and vascular disease (Values for odds ratio (OR) and relative risk (RR) are given with 95 % CI in parentheses)

Study, country, follow-up	Percentage of males	Sample size	No. of events	Age (years), type of patient	End point	Hcy (μmol/l) Event	Hcy (μmol/l) Non-event	OR or RR	Hcy identified as a risk factor?
Petri *et al.* (1996) USA, 4·8 years	7	337	60	23–47 SLE	Stroke, ATE or VTE	10·3	7·41**	OR stroke 2·44 (1·04, 5·75) OR ATE 3·49 (0·97, 12·54)	Yes
Bostom *et al.* (1997*b*) USA, 1·4 years	49	73	16	44–68 ESRD	CV event and CV death	NG	NG	RR 3·9 (1·4, 10·5)[b] age and sex adjusted	Yes
Nygard *et al.* (1997*a*) Norway, 4·6 years	81	587	64	32–80 CAD	All cause mortality	NG	NG	All cause mortality ratio 4·51§ (1·2, 16·6)	Yes
Massy *et al.* (1998) France, 6·75 years	63	79	9	43–59 RTR	CHD events	15·2	13·3 NS	NG	No
Moustapha *et al.* (1998) USA, 1·45 years	56	167	86	41–71 ESRD	CHD events and mortality	43·0	26·9*	RR CHD events and death 1·01 (1·00, 1·01)‡	Yes
Stehouwer *et al.* (1998) The Netherlands, 10 years	100	878	162	64–84 elderly	MI, stroke	NG	NG	RR for CHD death 1·58 (0·93, 2·69)ll	Yes
Sirrs *et al.* (1999) Canada, 0·75 years	63	88	35	55–79 HD	VAF and death	28·5	31·0*	RR of death 0·74 (0·01, 0·61)ll	No
Taylor *et al.* (1999) USA, 2·1 years	63	351	47	40–80 LED or CVD	Progression CHD and death	NG	NG	RR of death 2·02 (1·23, 3·51)§	Yes
Bostom *et al.* (1999) USA, 9·9 years	41	1947	165	59–91 general pop[n]	Stroke	NG	NG	RR 1·82 (1·14, 2·91)§	Yes
Kark *et al.* (1999) Israel, 9–11 years	45	1788	405	>50 general pop[n]	All cause and cause-specific mortality	13·7 11·6	12·1 (men)*** 10·8 (women)** 22·9‡	Hazard ratio for all cause mortality 1·97 (1·31, 2·98)¶	Yes
Shemin *et al.* (1999) USA, 1·5 years	48	84	47	>50 HD	Vascular access thrombosis	26·1		RR 1·04 (1·01, 1·06)†	Yes

ATE, arterial thrombotic disease; CAD, coronary artery disease; CV, cardiovascular disease; CVD, cerebrovascular disease; ESRD, end-stage renal disease; HD, haemodialysis patients; LED, lower-extremity occlusive disease; MI, myocardial infarction; NG, not given; RTR, renal transplant recipient; SLE, systemic lupus erythematosus; VAF, vascular access failure; VTE, venous thrombotic disease or thromboembolism.

* $P<0·05$, ** $P<0·01$, *** $P<0·001$.

† Multivariate adjusted.

‡ Unit change in Hcy.

§ Quartile 4 *v.* quartile 1.

ll Tertile 3 *v.* tertile 1.

¶ Quintile 5 *v.* quintile 1; for details of calculation of hazard ratio, see Kark *et al.* (1999).

substantial number have been published since. Another meta-analysis at this time would make interpretation of prospective studies much easier.

Reference range for normal homocysteine

Adults

The normal range of fasting homocysteine varies widely (Ueland *et al*. 1993), depending on the choice of sample population and statistical cut-off employed. Current reference ranges are defined by measuring homocysteine levels in control populations of individuals who are 'presumed' to be free of vascular disease. A statistical cut-off is then used to define the normal range. This cut-off, however, varies substantially; for example, the 80th, 90th and 95th percentiles, mean plus 2 SD deviations and mean plus 3 SD have all been used to define the normal homocysteine range. The reference range of Kang *et al*. (1992), who defined normal homocysteine level as 5–15 μmol/l, moderate hyperhomocysteinaemia as 15–30 μmol/l, intermediate hyperhomocysteinaemia as 30–100 μmol/l and severe hyperhomocysteinaemia as > 100 μmol/l, is widely accepted. Ubbink *et al*. (1995) suggested that the reference range should be defined according to the homocysteine levels found in a population with an optimum supply of B-vitamin cofactors, because they play such an important role in determining homocysteine levels. Under these conditions, these authors proposed a reference range for normal homocysteine of 4·7–11·7μmol/l, which was based on the concentrations obtained after various B-vitamin supplementation trials. Clearly, more work is needed to establish an internationally-applicable reference range for fasting plasma homocysteine concentrations.

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Children and adolescents

Until recently very little data existed on the homocysteine levels of children and adolescents. However, three studies (De Laet *et al*. 1999; Greenlund *et al*. 1999; Osganian *et al*. 1999), which included between them data on the homocysteine levels of more than 5000 children, have recently been published. Studies on children and adolescents generally agree that between the ages of 2 months and 19 years the homocysteine levels are approximately half those of adult values, and range from 4·9 to 7·4μmol/l (Tonstad *et al*. 1996*b*; Reddy, 1997; Vilaseca *et al*. 1997; De Laet *et al*. 1999; Greenlund *et al*. 1999; Osganian *et al*. 1999). In adulthood, males tend to have higher homocysteine levels than females (Brattstrom *et al*. 1994; Nygard *et al*. 1995; Shimakawa *et al*. 1997). Studies in children and adolescents have found no difference between homocysteine levels of boys and girls (Tonstad *et al*. 1996*a*, 1997; Reddy, 1997; De Laet *et al*. 1999; Greenlund *et al*. 1999) until after puberty (Tonstad *et al*. 1997; Vilaseca *et al*. 1997; De Laet *et al*. 1999), when homocysteine levels tend to increase markedly and a male–female difference becomes apparent, probably as a result of increased muscle mass and sex hormone concentration at this stage of development. In terms of ethnic differences, Greenlund *et al*. (1999) found no differences between black and white children, but Ubbink *et al*. (1996) and Osganian *et al*. (1999) found higher homocysteine levels in blacks *v*. whites. It would be useful to have some longitudinal studies which would assess the potential impact of homocysteine levels in childhood on coronary artery disease risk in later life.

Main determinants of homocysteine levels

Inherited genetic defects

Genetic defects in the key enzymes required for homocysteine metabolism can cause either a severe elevation in homocysteine levels, known as homocystinuria, or a more moderate accumulation of homocysteine, known as hyperhomocysteinaemia.

Homocystinuria. Deficiency of the trans-sulfuration enzyme cystathionine β-synthase is the most common cause of homocystinuria worldwide (Mudd *et al*. 1995). It is inherited as an autosomal recessive trait, the prevalence of which varies from 1:65 000 in Ireland to 1:900 000 in Japan, with an overall prevalence of 1:335 000 (Naughton *et al*. 1998). The most common clinical features include dislocation of the optic lens, osteoporosis, thinning and lengthening of long bones, mental retardation, thromboembolism and premature vascular disease. The extent of these clinical symptoms varies from patient-to-patient owing to considerable genetic heterogeneity. Approximately 50 % of affected patients respond to vitamin B_6 supplementation, which decreases plasma methionine levels to normal and results in virtual elimination of homocysteine from the urine (Mudd *et al*. 1995). The number of possible mutations in the cystathionine β-synthase gene is currently estimated to be sixty-four (Kraus, 1998). The two most common mutations are G307S, which confers pyridoxine non-responsiveness and seems to be Celtic in origin, and 1278T which confers pyridoxine responsiveness. The overall frequency of the G307S mutation amongst patients with homocystinuria is 31 % (although it is found in 71 % of Irish homocystinuric alleles) and the overall frequency of the 1278T mutation is 24 %. Even rarer causes of homocystinuria result from deficiencies of MTHFR (Rosenblatt, 1995) and methionine synthase enzymes (Cb1E or G; Fenton & Rosenberg, 1995), from defective synthesis of methylcobalamin and adenosylcobalamin (Cb1C and D; Fenton & Rosenberg, 1995), and from defective release of hydroxycobalamin from lysosomes (Cb1F; Fenton & Rosenberg, 1995). These severe genetic defects are associated with homocysteine levels as high as 200–400 μmol/l (Goodman *et al*. 1970; Levy *et al*. 1970).

Hyperhomocysteinaemia. All the previously mentioned mutations are very rare, and are associated with severely elevated homocysteine levels presenting as homocystinuria. Several years ago a common enzymic defect in the enzyme MTHFR, which is associated with mild elevations in plasma homocysteine levels (hyperhomocysteinaemia), was identified (Frosst *et al*. 1995). This mutation in MTHFR is autosomal recessive, and is characterized by a C→T substitution at base pair 677 resulting in an alanine to valine substitution. This variant is associated with lower enzyme activity *in vivo* (approximately 30 % wild type), reduced activity after *in vitro* heating (hence, is often referred to as thermolabile MTHFR; Frosst *et al*. 1995) and a propensity for the enzyme to dissociate from its prosthetic group (FAD; Guenther *et al*. 1999) in individuals possessing the TT (homozygous) genotype. The frequency of homozygosity for the C677T polymorphism is generally between 5 and 18 % (Motulsky, 1996; Heijmans *et al*. 1999); thus, in absolute terms this mutation affects a large number of individuals worldwide. Homozygous individuals (TT) seem to be more prone to elevated homocysteine levels than individuals without the thermolabile variant (CC; Harmon *et al*. 1996; Jacques *et al*. 1996; Ma *et al*. 1996; Brattstrom *et al*. 1998; Gudnason *et al*. 1998). This elevation, however, appears to be mediated by folate status (Harmon *et al*. 1996; Jacques *et al*. 1996; Ma *et al*. 1996). Studies have shown that when folate status is good no detectable difference in homocysteine is found between different genotype groups. When folate status is low, however, homocysteine levels tend to be significantly higher in TT individuals compared with CT or CC individuals (Harmon *et al*. 1996; Jacques *et al*. 1996). This finding suggests that TT individuals may have a greater requirement for folate, since a greater intake of folate is needed to normalize homocysteine metabolism and represents a gene–nutrient interaction between folate and thermolabile MTHFR (Molloy *et al*. 1997; Rosenberg & Rosenberg, 1998). It is still unclear if the moderate increase in homocysteine levels associated with the TT genotype is associated with an increased risk for vascular disease. For a comprehensive review of enzymic defects resulting in hyperhomocysteinaemia, see Brattstrom *et al*. (1998) and Bailey & Gregory (1999).

Disease states

There are numerous disease states known to affect homocysteine levels, but it is beyond the scope of the present review to cover them all (for more details, see

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Ueland & Refsum, 1989). Important among such conditions are renal disease, hyperproliferative disorders, organ transplantation and Alzheimer's disease. Hyperhomocysteinaemia is the most common cardiovascular risk factor in end-stage renal disease (Bostom & Culleton, 1999). It is probable that the high prevalence of elevated homocysteine levels in renal disease occurs as a result of impaired renal homocysteine metabolism, since the kidney is estimated to be responsible for approximately 70 % of daily homocysteine metabolism (Refsum *et al*. 1998*a*). A recent stable-isotope study by van Guldener *et al*. (1999) suggests that decreased activity of the remethylation pathway may be responsible for the hyperhomocysteinaemia encountered in renal disease. There is evidence of altered methionine metabolism in hyperproliferative disorders, such as cancer and psoriasis, resulting in elevated homocysteine levels, possibly as a result of a large burden of rapidly-dividing cells, or owing to occasional folate deficiency as a result of drug therapies such as methotrexate (Hoffman, 1985; Refsum *et al*. 1989, 1991; Ueland & Refsum, 1989). Elevated homocysteine levels have also been consistently found in patients following renal or cardiac transplants (Ambrosi *et al*. 1994; Massy *et al*. 1994, 1998; Berger *et al*. 1995; Arnadottir *et al*. 1996; Gupta *et al*. 1998). It is possible that this elevation occurs as a result of impaired B-vitamin status or impaired renal function owing to immunosuppressants, or a combination of both (Jacobsen, 1998). Recently, serum homocysteine has been found to be significantly higher ($P < 0{\cdot}001$) in patients with Alzheimer's disease than in control populations of elderly people with no evidence of cognitive impairment (Clarke *et al*. 1998). This finding has led to the proposal that hyperhomocysteinaemia may play a role in the pathogenesis of Alzheimer's disease (Clarke *et al*. 1998; Diaz-Arrastia, 1998; Fekkes *et al*. 1998; McCaddon *et al*. 1998; Miller, 1999).

Drug therapy

There are many drugs that affect homocysteine metabolism, either directly or indirectly, by altering the metabolism of its vitamin cofactors. It is beyond the scope of the present review to give details of all the relevant drugs (for more details, see Ueland & Refsum, 1989; Refsum *et al*. 1998*b*), and only a few key drugs which affect homocysteine levels are given here. Drugs associated with an increase in homocysteine levels include: methotrexate, a folate antagonist, which inhibits the enzyme dihydrofolate reductase (Refsum *et al*. 1986, Refsum & Ueland, 1990; Quinn *et al*. 1998; Morgan *et al*. 1998); N_2O, which inactivates vitamin B_{12} (Ermens *et al*. 1991; Christensen *et al*. 1993; Badner *et al*. 1998); 6-azauridine triacetate, which is a vitamin B_6 antagonist (Slavik *et al*. 1969, 1982); and anticonvulsant therapy which alters folate metabolism by mechanisms which are not clearly understood (Ueland & Refsum, 1989; Schwaninger *et al*. 1999). Other drugs associated with a reduction in homocysteine levels include aminothiols such as penicillamine and acetylcysteine (Kang *et al*. 1982, 1986*b*; Wiklund *et al*. 1996), and oral contraceptives and hormone therapy (Brattstrom *et al*. 1992*a*; van der Mooren *et al*. 1994; Anker *et al*. 1995).

Age and sex

Premenopausal women tend to have lower homocysteine levels compared with young men. After the menopause female homocysteine levels become more comparable with those of the male (Boers *et al*. 1983; Brattstrom *et al*. 1985, 1990; Kang *et al*. 1986*a*; Wouters *et al*. 1995; Verhoef *et al*. 1999). It is well established that homocysteine levels increase with age (Kang *et al*. 1986*a*; Andersson *et al*. 1992; Brattstrom *et al*. 1992*a*, 1994; Selhub *et al*. 1993; Robinson *et al*. 1995; Nygard *et al*. 1995, 1998; Vilaseca *et al*. 1997); this increase may be due to a decline in renal function (Brattstrom *et al*. 1994; Wu *et al*. 1994) or intake and status of the B-vitamin cofactors (Selhub *et al*. 1993; Brattstrom *et al*. 1994; Koehler *et al*. 1996) with increasing age.

Lifestyle

Recently, data from the Hordaland Homocysteine Study (Nygard *et al*. 1995, 1997*b*, 1998) has shown that smoking, high coffee consumption and lack of exercise are associated with an elevated plasma homocysteine level. Smoking and coffee consumption were found to influence plasma homocysteine concentration even in subjects with high folate status (Nygard *et al*. 1998), and the association between high coffee consumption and elevated plasma homocysteine did not disappear after adjustment for confounding factors such as smoking, lower multivitamin use and lower intake of fruit and vegetables (Nygard *et al*. 1997*b*; Stolzenberg-Solomon *et al*. 1999).

Homocysteine levels are influenced by the status of the B-vitamin cofactors involved in homocysteine metabolism, and such influences will now be discussed in detail.

Treatment and prevention of hyperhomocysteinaemia

Folate

A strong inverse relationship exists between folate status and homocysteine levels (Kang *et al*. 1987; Israelsson *et al*. 1988; Andersson *et al*. 1992; Selhub *et al*. 1993; Ueland *et al*. 1993; Brattstrom *et al*. 1994); this finding is not surprising since folate, in the form of 5-methyltetrahydrofolate, acts as a co-substrate for the remethylation of homocysteine. Hyperhomocysteinaemia has been found in subjects who have a folate status which is normal or low within the normal range, as well as in subjects with a subnormal folate status (Kang *et al*. 1987; Selhub *et al*. 1993; Ueland *et al*. 1993). Folic acid is also a very effective homocysteine-lowering agent. Clarke & Homocysteine Lowering Trialists' Collaboration (1998) recently carried out a meta-analysis of randomized controlled trials that assessed the effects of folic acid-based therapies on homocysteine levels. Results of this meta-analysis clearly showed that of the three B-vitamins most commonly used in these trials (vitamin B_6, vitamin B_{12} and folic acid), folic acid had the most profound homocysteine-lowering effect, resulting in a reduction of 25 % on average. This meta-analysis also showed that the homocysteine-lowering effect was similar when doses ranged from 0·5 to 5 mg/d. Doses of folic acid as low as 200 µg/d appear to effectively lower homocysteine levels in both hyperhomocysteinaemic

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(Guttormsen *et al*. 1996) and normohomocysteinaemic subjects (Ward *et al*. 1997) where this dose was shown to be optimal, although 100 μg/d produced significant ($P < 0{\cdot}001$) lowering of homocysteine levels. However, higher doses of folic acid may be required to lower homocysteine levels in certain disease states such as renal failure (Bostom & Culleton, 1999). It should be noted, however, that subjects will not respond maximally to folic acid supplementation unless vitamin B_{12} status is adequate (Allen *et al*. 1990; Landgren *et al*. 1995); this finding reflects the inter-dependence of folate and vitamin B_{12} as co-substrate and cofactor respectively in homocysteine remethylation. If homocysteine is viewed as a functional indicator of folate status, then it seems probable that current definitions of 'normal' folate status may be inadequate, since folic acid-responsive homocysteine levels are found in individuals who have what is currently defined as 'normal' folate status (Ward *et al*. 1997). Whilst 'normal' folate status, as currently defined, may be adequate to ensure the absence of clinical signs of folate deficiency, it appears to be insufficient with respect to plasma homocysteine levels, and therefore is also not optimal in terms of potential risk of vascular disease. Evidence that an erythrocyte folate level of > 400 μg/l is required to give maximal protection from neural-tube defects (Daly *et al*. 1995) also supports the view that the current definition of 'normal' erythrocyte folate status may be too low. Given this new evidence regarding the relationships between folate status and plasma homocysteine levels and the risk of neural-tube defects, current dietary reference values should be revised.

Vitamin B_{12}

Many studies have been able to demonstrate an inverse relationship between vitamin B_{12} status and plasma homocysteine levels (Israelsson *et al*. 1988; Andersson *et al*. 1992; Selhub *et al*. 1993; Ueland *et al*. 1993; Brattstrom *et al*. 1994), although the strength of the correlation is not as strong as that observed between folate status and homocysteine levels. Vitamin B_{12} also effectively lowers plasma homocysteine levels at doses ranging from 6 μg to 2 mg (Rasmussen *et al*. 1996; Bronstrup *et al*. 1998; Clarke & Homocysteine Lowering Trialists' Collaboration, 1998). An early study by Ubbink *et al*. (1994) found that the reduction in homocysteine levels achieved using a combination of folic acid (0·65 mg), vitamin B_6 (10 mg) and vitamin B_{12} (0·4 mg) supplementation was not significantly different from that achieved using folic acid alone. This finding suggested that there was no additional benefit to be gained from vitamin B_{12} supplementation in terms of lowering homocysteine levels. However, since this study, a meta-analysis by Clarke & Homocysteine Lowering Trialists' Collaboration (1998) has found that vitamin B_{12} supplementation produces an average 7 % reduction in homocysteine levels. Also, Bronstrup *et al*. (1998) recently found that a combination of folic acid (400 μg) and vitamin B_{12} (400 μg) supplementation for 4 weeks resulted in a lowering of homocysteine levels by 18 % *v*. 11 % produced by folic acid alone, again indicating that the combination of vitamin B_{12} and folic acid is more effective than folic acid alone.

Vitamin B_6

Vitamin B_6 has been used for many years to successfully treat pyridoxine-responsive homocystinuria caused by cystathionine β-synthase deficiency (Mudd *et al*. 1985), but its role in the prevention or treatment of hyperhomocysteinaemia is still uncertain. Intervention studies indicate that vitamin B_6 is effective in reducing the abnormally high homocysteine concentrations observed after a methionine load in subjects with hyperhomocysteinaemia (Dudman *et al*. 1993*b*; Franken *et al*. 1994; Bostom *et al*. 1997*a*). However, results from nine studies investigating the effect of vitamin B_6 alone on fasting homocysteine levels are inconclusive (summarized in Table 3). To date, only two studies (Lakshmi & Ramalakshmi, 1998; Mansoor *et al*. 1999) have found a significant lowering of fasting homocysteine levels following vitamin B_6 supplementation. The study of Lakshmi & Ramalakshmi (1998) was carried out in young women who had clinical and biochemical vitamin B_6 deficiency. Ten volunteers received 20 mg pyridoxine hydrochloride daily for 15 d, which resulted in a reduction in fasting homocysteine levels of 19·7 % (from 14·7 to 11·8 μmol/l, $P < 0{\cdot}05$). However, the trial was not conducted 'blind' nor was it placebo-controlled, and there was no washout period. The study of Mansoor *et al*. (1999) was carried out in a group of nine healthy people who took 120 mg vitamin B_6 daily for 5 weeks. This regimen resulted in a significant (17 %; $P = 0{\cdot}011$) lowering of plasma homocysteine levels. All the intervention studies discussed here were carried out using volunteers aged between 18 and 60 years. It is surprising that no vitamin B_6 intervention has been carried out in elderly subjects, as vitamin B_6 status is known to decline with age (Hamfelt & Soderhjelm, 1988; Reynolds *et al*. 1988), and plasma homocysteine levels are known to increase with age (Kang *et al*. 1986*a*; Andersson *et al*. 1992; Brattstrom *et al*. 1992*a*, 1994; Selhub *et al*. 1993; Robinson *et al*. 1995; Nygard *et al*. 1995, 1998; Vilaseca *et al*. 1997). It is possible that the general failure of studies to date to demonstrate a lowering in fasting homocysteine levels in response to vitamin B_6 may have been as a result of suboptimal folate or vitamin B_{12} status, which resulted in low S-adenosyl methionine levels, favouring homocysteine remethylation while inhibiting trans-sulfuration (Selhub & Miller, 1992).

A relationship between suboptimal vitamin B_6 status and vascular disease is well-supported in the literature (Rinehart & Greenberg, 1949; Schroeder, 1955; Robinson *et al*. 1995, 1998; Chasan-Taber *et al*. 1996; Verhoef *et al*. 1996; Rimm *et al*. 1998). It is still unknown if vitamin B_6 exerts its effect on vascular disease directly through its various effects on platelets (Subbarao *et al*. 1979; Konecki & Feinberg 1980; Lam *et al*. 1980), connective tissue (Murray *et al*. 1978; Myers *et al*. 1985) and thromobogenesis (Editorial, 1981; Hladovec, 1979), or indirectly by causing the accumulation of homocysteine (Mudd *et al*. 1995). Many studies have found a significant ($P < 0{\cdot}05$) inverse relationship between homocysteine levels and vitamin B_6 status (Brattstrom *et al*. 1992*b*, Stampfer *et al*. 1992; Selhub *et al*. 1993; Robinson *et al*. 1995, 1998; Verhoef *et al*. 1996, 1997*a*; Graham *et al*. 1997; Osganian *et al*. 1999) and an accumulation of homocysteine in vitamin B_6 depletion–repletion studies

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Table 3. Effect of vitamin B_6 supplementation on fasting homocysteine levels

Study, type of group	*n*	Mean age (years)	Vitamin B_6 (mg/d)	Length of treatment (weeks)	Total homocysteine (μmol/l)		Percentage change in homocysteine	Statistical significance of changes: *P*
					Pretreatment	Post-treatment		
Wilcken *et al.* (1981) renal transplant recipients	11	41·2	100	2	7·3*	6·8*	–6·8	NS
Brattstrom *et al.* (1988) healthy subjects	15	51·0	40	2	12·0	11·4	–5·0	NS
Brattstrom *et al.* (1990) occlusive arterial disease	20	< 55	240	2	23·1	25·6	+ 10·8	NS
Arnadottir *et al.* (1993) dialysis patients	18	51·0	300	16	26·7	32·9	+ 23·2	0·05
Ubbink *et al.* (1994) hyperhomocysteinaemic men	18	34·6	10	6	29·1	27·8	–4·5	NS
Bostom *et al.* (1997*a*) renal transplant recipients	7	41·0	50	6	NG	NG	–12·2	NS
Dierkes *et al.* (1998) healthy young women	34	25·1	2	4	8·2	8·6	+ 4·9	NS
Lakshmi & Ramalakshmi (1998) clinical and biochemical vitamin B_6 deficiency	10	20–45 (mean NG)	20	2	14·7	11·8	–19·7	0·05
Mansoor *et al.* (1999) healthy subjects	9	39·0	120	5	NG	NG	–17·0	0·011

* Measurement of cysteine–homocysteine mixed disulfide not total homocysteine. NG, not given.

(Smolin *et al*. 1983; Shultz & Hansen, 1998), which would support an indirect relationship between vitamin B_6 and vascular disease mediated via homocysteine. However, some studies have failed to find any relationship between vitamin B_6 and homocysteine (Brattstrom *et al*. 1990; Dalery *et al*. 1995; Riggs *et al*. 1996; Ubbink *et al*. 1996; Dierkes *et al*. 1998; Healy *et al*. 1998; Bates *et al*. 1999), and the findings of vitamin B_6 depletion–repletion studies are contradictory (Smolin *et al*. 1983; Miller *et al*. 1992; Stabler *et al*. 1997; Shultz & Hansen, 1998). Thus, currently the relationship between vitamin B_6 and plasma homocysteine remains unclear and requires further study, in particular in elderly populations.

Riboflavin

Riboflavin plays an essential role in both the remethylation and trans-sulfuration pathways of homocysteine metabolism. However, little information is available on the effect of riboflavin status on homocysteine levels. Riboflavin supplementation has only been included in two intervention studies (Olszewski *et al*. 1989; Lakshmi & Ramalakshmi, 1998) where homocysteine was the primary end point. The first study, by Olszewski *et al*. (1989), involved treatment of twenty-one myocardial infarction patients with a combination of choline, troxerutin, vitamin B_6, vitamin B_{12}, folate and riboflavin for 21 d. This study made it impossible to discover what effect, if any, riboflavin alone had on fasting homocysteine levels. The second study (Lakshmi & Ramalakshmi, 1998) was carried out in women with clinical and biochemical riboflavin deficiency (mean erythrocyte glutathione reductase activation coefficient 1·80) who took a pharmacological dose of riboflavin (10 mg) for 15 d. This treatment resulted in an improvement in riboflavin status, but there was no significant change in plasma homocysteine levels. However, this study was poorly designed, was not conducted 'blind', had no placebo group or washout period, and the sample size was very small.

The importance of riboflavin for homocysteine metabolism has recently been highlighted by the characterization of the flavin-dependent enzyme MTHFR from *Escherihia coli* (Guenther *et al*. 1999). The wild-type enzyme and a mutant form of MTHFR, which is commonly known as thermolabile MTHFR, have both been characterized. Guenther *et al*. (1999) demonstrated that the thermolabile enzyme was approximately ten times more likely than the wild-type enzyme to dissociate from its FAD cofactor. They also found that the addition of folates to MTHFR *in vitro* stabilized the binding FAD in both the wild-type and mutant E. *coli* enzymes. Given the intimate involvement of riboflavin in both pathways of homocysteine metabolism and, in particular, the recent characterization of the thermolabile variant of MTHFR, which highlighted the importance of riboflavin, it is timely to investigate the effect of riboflavin supplementation on plasma homocysteine and to examine the interaction between riboflavin status, folate status and homocysteine levels in individuals with the TT genotype.

Possible mechanisms for the vascular toxicity of hyperhomocysteinaemia

The exact mechanism for the vascular toxicity of hyperhomocysteinaemia is still unknown; however, studies indicate it is both atherogenic and thrombogenic.

Atherosclerotic mechanisms

Effects on endothelial and smooth-muscle cells. Studies have shown that homocysteine is directly toxic to endothelial cells in a dose-dependent manner (Wall *et al*.

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1980; De Groot *et al*. 1983; Blann, 1992; Dudman 1999) and that this damage can be prevented by catalase, which indicates that the generation of H_2O_2 possibly from homocysteine auto-oxidation, is important in the pathological process (Starkebaum & Harlan, 1986; Dudman *et al*. 1991; Emsley *et al*. 1999). Studies also indicate that elevated homocysteine is associated with a reduced ability to produce endothelium-derived relaxing factor (NO; Stamler *et al*. 1993; Upchurch *et al*. 1995; Loscalzo, 1996; Keaney & Loscalzo, 1997; Welch *et al*. 1997). Studies using cultured endothelial cells from animals and human subjects have all found impaired endothelium-dependent vasodilatation in association with mild-to-moderate hyperhomocysteinaemia (Celermajer *et al*. 1993; van den Berg *et al*. 1995, Lentz *et al*. 1996; Upchurch *et al*. 1996; Tawakol *et al*. 1997), indicating that homocysteine interferes with the relaxing action of NO on the blood vessel wall. Elevated homocysteine levels have also been shown to stimulate vascular smooth muscle cells to proliferate and synthesize collagen, while at the same time impeding the regeneration of endothelial cells, hallmarks of atherosclerosis (Harker *et al*. 1983; Tsai *et al*. 1994, 1996). Welch *et al*. (1997) proposed that smooth-muscle cell proliferation was induced as a direct result of the inactivation of NO by lipid peroxides, which have themselves been formed as a result of the auto-oxidation of homocysteine (Garg & Hassid, 1989; Marks *et al*. 1995).

Oxidative effects. Several *in vitro* studies indicate increased oxidant stress in response to hyperhomocysteinaemia (Stamler *et al*. 1993; Loscalzo, 1996; Outinen *et al*. 1998; Voutilainen *et al*. 1998). However, *in vivo* studies are contradictory (Dudman *et al*. 1993*a*; Mansoor *et al*. 1995; Young *et al*. 1997). Homocysteine readily undergoes auto-oxidation in plasma (Velury & Howell, 1988; Stamler *et al*. 1993; Andersson *et al*. 1995) forming reactive oxygen species such as H_2O_2 and superoxide, which may themselves cause oxidation of LDL (Heinecke *et al*. 1987), and other products such as cysteine–homocysteine mixed disulfides, and homocysteine thiolactone. It has been proposed that homocysteine thiolactone reacts with LDL to form LDL–homocysteine thiolactone aggregates, which may then be taken up by macrophages and subsequently incorporated into foam cells in early atherosclerotic plaques (Naruszewicz *et al*. 1994; Jakubowski, 1997; Ferguson *et al*. 1999). It has also shown that homocysteine can inhibit the synthesis of glutathione peroxidase (Upchurch *et al*. 1995, 1997) which detoxifies H_2O_2 and lipid peroxides, thereby leaving the cell vulnerable to oxidative damage. Also, work by several authors suggests that lipid peroxidation, as a result of elevated homocysteine levels, may then cause decreased expression of the enzyme nitric oxide synthase and directly degrade NO (Heinecke *et al*. 1987; Chin *et al*. 1992; Liao *et al*. 1995; Blom *et al*. 1995; Domagala *et al*. 1997)

Thrombotic mechanisms

Coagulant pathway. Elevated homocysteine levels *in vitro* have been shown to upset the balance of certain factors within the coagulation pathway, including activation of factors V and X11 (Ratnoff, 1968; Rodgers & Kane, 1986). Elevated homocysteine *in vitro* also increased platelet adhesion and thromboxane production, inhibited prostacyclin synthesis (Harker *et al*. 1974, 1976; Graeber *et al*. 1982; Wang *et al*. 1993), and stimulated tissue factor synthesis (Fryer *et al*. 1993; Rodgers *et al*. 1993).

Anticoagulant pathway. Elevated homocysteine levels *in vitro* are associated with the following effects on the anticoagulant pathway: suppression of protein C activation (Rodgers & Conn, 1990; Lentz & Sadler, 1991; Hayashi *et al*. 1992); down regulation of thrombomodulin expression (Lentz & Sadler, 1991; Hayashi *et al*. 1992; Lentz *et al*. 1996); suppression of heparan sulfate expression (Nishinaga *et al*. 1993); inhibition of von Willebrand factor synthesis (Lentz & Sadler, 1993; Lubec *et al*. 1996; Freyburger *et al*. 1997).

Fibrinolytic pathway. In the fibrinolytic pathway, elevated homocysteine levels block the binding of tissue plasminogen activator to its endothelial cell receptor, annexin II (Hajjar, 1993; Hajjar *et al*. 1998), and enhances the binding between atherogenic lipoprotein lipoprotein (a) and fibrin even at low concentrations (Harpel *et al*. 1992).

The ultimate consequence of all these effects on the coagulant, anticoagulant and fibrinolytic pathways is a shift in the normal balance between coagulation and fibrinolysis, creating a pro-thrombotic environment which facilitates the formation of a thrombus.

There are, however, many problems associated with studies investigating the vascular toxicity of elevated homocysteine. Most studies use very high concentrations of homocysteine, sometimes even higher than those seen in severe homocystinuria, and their relevance to hyperhomocysteinaemia is uncertain. Some studies also use different forms of homocysteine, such as homocysteine thiolactone, which may be more toxic than homocysteine itself. Also, the effects observed in some studies are sometimes not shown to be specific to homocysteine, and may be observed with other S-containing amino acids, such as cysteine (Fryer *et al*. 1993; Nishinaga *et al*. 1993; Stamler *et al*. 1993; Tsai *et al*. 1994; Kokame *et al*. 1996). A further problem is that most of these studies have been carried out *in vitro* or in animals and their applicability in human subjects is unknown. Atherosclerosis is a very slow process which is difficult to mimic using short-term *in vitro* experiments. Finally, there is very little information available for human subjects on the effect on haemostatic factors, and the vasculature in general, of lowering homocysteine levels. Van den Berg *et al*. (1995) reported that lowering homocysteine levels in patients with peripheral arterial occlusive disease following 1 year of folic acid and vitamin B_6 therapy resulted in a lowering of thrombomodulin, von Willebrand factor and endothelin, which were all elevated at baseline, but there was no change in the status of tissue-type plasminogen activator or selectin, which were both normal at baseline. Also, Bellamy *et al.* (1999) in a double-blind placebo-controlled crossover study involving eighteen subjects with homocysteine levels > 13 μmol/l, found that folate supplementation (5 mg/d for 6 weeks) reduced homocysteine levels and enhanced endothelium-dependent responses. In contrast to this finding, van Guldener *et al*. (1998) reported no improvement in thrombomodulin, E-selectin, plasmin activator inhibitor-1 or tissue-type

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plasmin activator endothelin after 1 year of folic acid therapy in a group of thirty peritoneal dialysis patients.

Concluding statement

The present review has highlighted many areas that require further research. Definitions of normal reference ranges for both adults and children require further clarification. More research, especially *in vivo*, is essential to explore the mechanism by which homocysteine damages the vasculature, as well as to examine if lowering homocysteine levels *in vivo* is beneficial in terms of improving haemostatic variables. Further work is also necessary to find the combination of B-vitamins that most effectively lowers homocysteine levels, and to investigate the homocysteine-lowering capacity of riboflavin, which is intimately involved in homocysteine metabolism but has so far received little attention.

The effect of homocysteine on vascular disease is a graded effect (Arnesen *et al*. 1995; Perry *et al*. 1995; Nygard *et al*. 1997*a*); therefore, decreasing homocysteine levels is likely to be beneficial, even if the levels are currently defined as 'normal'. If B-vitamin supplementation is to form part of a public health strategy aimed at vascular disease prevention in the general population, it is vital that the lowest effective dose (and combination) of B-vitamins that will result in the greatest lowering of homocysteine levels is found so that the risk of overexposure is limited. Thus, further intervention trials that investigate the effect of very low doses of B-vitamins on fasting homocysteine levels, are vital. The United States Department of Health and Human Services (Food and Drug Administration, 1996) recently implemented a policy to fortify all grain products with folic acid at a level of 1·4μg/g product. Although the primary aim of this strategy was to prevent neural-tube defects, it is hoped that the fortification policy will also be beneficial in terms of vascular disease prevention. Jacques *et al*. (1999) have already demonstrated a significant improvement in folate status, and a corresponding lowering of plasma homocysteine levels, as a direct result of this fortification policy. The topic of fortification in the UK is still controversial, mainly because of the fear that folic acid supplementation would correct the pernicious anaemia associated with vitamin B_{12} deficiency but allow the neurological damage to progress, thus putting certain subgroups of the population at risk (Savage & Lindenbaum, 1995).

Ultimately what is needed is a large-scale randomized placebo-controlled primary prevention trial which will establish whether lowering homocysteine levels has a favourable effect on the incidence of vascular disease. No such trial is planned currently because of the huge expense involved, but several secondary prevention trials are already underway (Eikelboom *et al*. 1999), and the first results are expected in a few years.

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