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 B12 and folate. The second pathway of homocysteine metabolism is the trans-sulfuration pathway which requires both vitamin B6 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 B12 and folate. However, the relationship between vitamin B6 status and homocysteine is still uncertain. Similarly, numerous intervention studies have demonstrated effective lowering of homocysteine levels as a result of folate and vitamin B12 supplementation, while the homocysteine-lowering ability of vitamin B6 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 B12 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 B12-dependent remethylation of homocysteine to methionine. Riboflavin in the form of FAD is required as a prosthetic group for MTHFR (Bates &
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 B₆)-dependent (Mudd et al. 1995). Pyridoxal 5'-phosphate, the active coenzyme form of vitamin B₆, 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 remethyl-ation 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 (Sellhub & 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 also demonstrated a significant association between homocysteine levels and the extent of vascular disease in the coronary (Montalescot et al. 1997; Verhof et al. 1997), carotid (Malinow et al. 1993; Sellhub 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. 1997b, 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. 1997a; 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. 1997a). 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...
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)

<table>
<thead>
<tr>
<th>Study, country, follow-up</th>
<th>Percent-age of males</th>
<th>Cases</th>
<th>Controls</th>
<th>Age (years), type of group</th>
<th>Hcy (µmol/l)</th>
<th>Cases</th>
<th>Control</th>
<th>OR or RR†</th>
<th>Hcy identified as a risk factor?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stampler et al. (1992) USA, 5 years</td>
<td>100 271</td>
<td>271</td>
<td>40–84 US physicians</td>
<td>MI or CHD death</td>
<td>11·1</td>
<td>10·5*</td>
<td>RR of MI 3·4 (0·9, 3·3)</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Alfthan et al. (1994) Finland, 9 years</td>
<td>51 265</td>
<td>269</td>
<td>40–64 general popn</td>
<td>Stroke or MI</td>
<td>9·99</td>
<td>9·82 (males)</td>
<td>OR males 1·0 (0·95, 1·06)</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Verhoef et al. (1994) USA, 5 years</td>
<td>100 109</td>
<td>427</td>
<td>40–84 US physicians</td>
<td>Ischaemic stroke</td>
<td>11·1</td>
<td>10·6 NS</td>
<td>OR 1·2 (0·7, 2·0)II</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Arnesen et al. (1995) Norway, 3-5 years</td>
<td>50 123</td>
<td>492</td>
<td>12–61 general popn</td>
<td>CHD event or CHD death</td>
<td>12·7</td>
<td>11·3**</td>
<td>RR for CHD 1·32 (1·05, 1·65)</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Perry et al. (1995) UK, 12-8 years</td>
<td>100 107</td>
<td>118</td>
<td>40–59 general popn</td>
<td>Fatal and non-fatal stroke</td>
<td>13·7</td>
<td>11·9**</td>
<td>RR of stroke 4·7 (1·1, 1·8)</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Chasan-Taber et al. (1996) USA, 7-5 years</td>
<td>100 333</td>
<td>333</td>
<td>40–84 US physicians</td>
<td>Acute MI or CHD death</td>
<td>NG</td>
<td>NG</td>
<td>RR of MI 1·7 (0·9, 3·3)</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Evans et al. (1997) USA, &lt;7 (MI) or 11–17 years (death)</td>
<td>100 240</td>
<td>472</td>
<td>35–57 general popn</td>
<td>Non-fatal MI</td>
<td>12·6</td>
<td>13·1 NS</td>
<td>OR for CHD death and MI 0·82 (0·55, 1·54)</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>American Physicians’ Health Study, USA, 10 years</td>
<td>100 145</td>
<td>646</td>
<td>40–84 US physicians</td>
<td>Incidence VTE</td>
<td>11·5</td>
<td>10·9 NS</td>
<td>RR idiopathic VTE 2·48† (0·97, 6·39)</td>
<td>Yes, idiopathic VTE</td>
<td></td>
</tr>
<tr>
<td>Verhoef et al. (1997a) USA, 9 years</td>
<td>100 149</td>
<td>149</td>
<td>40–84 US physicians</td>
<td>Angina pectoris with surgery</td>
<td>10·9</td>
<td>10·4 NS</td>
<td>OR for angina pectoris 1·0± (0·8, 1·4)</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Folsom et al. (1998) USA, 3-3 years</td>
<td>NG 232</td>
<td>537</td>
<td>45–64 general popn</td>
<td>CHD event or CHD death</td>
<td>8·86</td>
<td>8·53 NS</td>
<td>RR of CHD 1·28 (0·5, 3·2)II</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>McDowell et al. (1998) UK, 8-7 years</td>
<td>100 229</td>
<td>1126</td>
<td>35–61 general popn</td>
<td>Death from IHD</td>
<td>13·1</td>
<td>11·8***</td>
<td>OR of death from IHD 2·9† (2·04, 4·12)</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Bots et al. (1999) The Netherlands, 2-7 years</td>
<td>49 224</td>
<td>533</td>
<td>&gt;55 general popn</td>
<td>MI and stroke</td>
<td>17·3</td>
<td>15·2 (MI)**</td>
<td>OR for MI 2·10 (0·88, 5·03)II</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Ridker et al. (1999) USA, 3 years</td>
<td>0 122</td>
<td>244</td>
<td>59·3 (mean) general popn</td>
<td>MI, stroke, or death due to CV disease</td>
<td>14·1</td>
<td>12·4**</td>
<td>RR of MI or stroke 4·6† (1·7, 12·3)</td>
<td>Yes</td>
<td></td>
</tr>
</tbody>
</table>

CV, cardiovascular disease; MI, myocardial infarction; NG, not given; Popn, population; VTE, venous thrombotic disease or thromboembolism.

* P < 0·05, ** P < 0·01, *** P < 0·001.
† Multivariate adjusted.
‡‡ >95th Percentile v. 90th percentile.
§ Unit change in hcy.
¶ Quintile 5 v. quintile 1.
‖ Increase in hcy of 4 µmol/l.
¶¶ 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. (1997a) 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.
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 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 \( \mu \text{mol/l} \), moderate hyperhomocysteinaemia as 15–30 \( \mu \text{mol/l} \), intermediate hyperhomocysteinaemia as 30–100 \( \mu \text{mol/l} \) and severe hyperhomocysteinaemia as > 100 \( \mu \text{mol/l} \), 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 4·7–11·7 \( \mu \text{mol/l} \), 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.
**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 7.4 µmol/l (Tonstad et al. 1996b; 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. 1996a, 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 B6 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 vitro (approximately 30% wild type), reduced activity after in vivo 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 homozgyosity 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...
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., 1998a). 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 < 0.001) 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. 1998b), 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); N2O, which inactivates vitamin B12 (Ermens et al. 1991; Christensen et al. 1993; Badner et al. 1998); 6-azauridine triacetate, which is a vitamin B6 antagonist (Slavik et al. 1969, 1982); and anticonvulsant therapy which alters folate metabolism by mechanisms which are not clearly understood (Ueland & Refsum, 1989; Schwangerer et al. 1999). Other drugs associated with a reduction in homocysteine levels include aminothiols such as penicillamine and acetylcysteine (Kang et al. 1982, 1986b; Wiklund et al. 1996), and oral contraceptives and hormone therapy (Brattstrom et al. 1992a; 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 tend to become more comparable with those of the male (Boers et al. 1983; Brattstrom et al. 1985, 1990; Kang et al. 1986a; Wouters et al. 1995; Verhoeef et al. 1999). It is well established that homocysteine levels increase with age (Kang et al. 1986a; Andersson et al. 1992; Brattstrom et al. 1992a, 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, 1997b, 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. 1997b; 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 B6, vitamin B12 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
(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 < 0·001) 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 B12 status is adequate (Allen et al. 1990; Landgren et al. 1995); this finding reflects the interdependence of folate and vitamin B12 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 B12

Many studies have been able to demonstrate an inverse relationship between vitamin B12 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 B12 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 B6 (10 mg) and vitamin B12 (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 B12 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 B12 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 B12 (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 B12 and folic acid is more effective than folic acid alone.

Vitamin B6

Vitamin B6 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 B6 is effective in reducing the abnormally high homocysteine concentrations observed after a methionine load in subjects with hyperhomocysteinaemia (Dudman et al. 1993b; Franken et al. 1994; Bostom et al. 1997a). However, results from nine studies investigating the effect of vitamin B6 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 B6 supplementation. The study of Lakshmi & Ramalakshmi (1998) was carried out in young women who had clinical and biochemical vitamin B6 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 < 0·05). 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 B6 daily for 5 weeks. This regimen resulted in a significant (17%; P = 0·011) 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 B6 intervention has been carried out in elderly subjects, as vitamin B6 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. 1986a; Andersson et al. 1992; Brattstrom et al. 1992a, 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 B6 may have been as a result of suboptimal folate or vitamin B12 status, which resulted in low S-adenosyl methionine levels, favouring homocysteine remethylation while inhibiting transsulfuration (Selhub & Miller, 1992).

A relationship between suboptimal vitamin B6 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; Verhoeef et al. 1996; Rimm et al. 1998). It is still unknown if vitamin B6 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 < 0·05) inverse relationship between homocysteine levels and vitamin B6 status (Brattstrom et al. 1992b, Stampfer et al. 1992; Selhub et al. 1993; Robinson et al. 1995, 1998; Verhoeef et al. 1996, 1997a; Graham et al. 1997; Osganian et al. 1999) and an accumulation of homocysteine in vitamin B6 depletion–repletion studies.
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 B₆, vitamin B₁₂, 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 Escherichia 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, 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. 1989)
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$_2$O$_2$ 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 endothelial-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; Outilainen et al. 1998). However, in vivo studies are contradictory (Dudman et al. 1993a; 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$_2$O$_2$ 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$_2$O$_2$ 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 prostacycllin 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 (Harpe 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
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. 1997a); 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 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 1–4 µg/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 B12 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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