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Homocysteine and vitamins in cardiovascular disease

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On the basis of recent retrospective and prospective studies, it is now widely accepted that increased total plasma homocysteine is a risk factor for cardiovascular disease. Impaired enzyme function as a result of genetic mutation or deficiency of the essential B vitamins folic acid, B_{12}, and B_6 can lead to hyperhomocysteinemia. Oxidized forms of homocysteine account for 98–99% of total plasma homocysteine. Although there is uncertainty as to whether increased homocysteine is causal or merely a proxy for cardiovascular disease, several lines of evidence suggest that it may play a role in atherothrombotic disease. Homocysteine appears to alter the anticoagulant properties of endothelial cells to a procoagulant phenotype. Mildly increased homocysteine causes dysfunction of the vascular endothelium. Folic acid effectively lowers homocysteine concentration in the plasma. Intervention studies are urgently needed to determine if lowering homocysteine is effective in decreasing the morbidity and mortality of cardiovascular disease.

Cardiovascular disease (CVD)[1] is the leading cause of mortality in the United States and in most Western countries. The emergence of new risk factors for heart disease and other major human illnesses are often greeted with skepticism in the scientific community. McCully and Wilson *(1)* proposed the "homocysteine theory of arteriosclerosis" in 1975 on the basis of pathological examinations of autopsy material from children with homocystinuria *(2)*. However, only within the past 5 years has homocysteine taken its place among other major risk factors such as cholesterol, smoking, and obesity. The fact that homocysteine is now widely accepted as a major independent risk factor for cardiovascular, cerebrovascular, and peripheral vascular disease is because, at least in part, of methodological advances in the determination of total plasma homocysteine that have occurred in the past decade and to a greater appreciation of the role of micronutrients in health and well-being.

Nearly 80 retrospective and prospective clinical studies have been published on homocysteine and vascular disease in the past 10 years. The vast majority of these studies support the observation that increased total plasma homocysteine is associated with an increased risk of CVD *(3–10)*. However, whether homocysteine is causal in atherogenesis and thrombogenesis, or merely a proxy for the disease itself, remains to be determined. Secondary intervention trials that will effectively lower total plasma homocysteine in patients with CVD followed by the assessment of mortality and morbidity outcomes are only now beginning. It may be several years before the answer is known. In the meantime, it may be prudent to understand the relationship between homocysteine and folic acid, vitamin B_{12}, and vitamin B_6 because, in most individuals, optimal nutriture with respect to these vitamins is an effective means of maintaining lower concentrations of total plasma homocysteine.

The determinants of total plasma homocysteine are complex and involve demographic, genetic, and acquired factors. Acquired factors include both state-of-health and life-style considerations. Although the roles that homocysteine plays in atherogenesis, atherosclerosis, and thrombosis are unknown, several recent studies suggest that hyperhomocysteinemia can disrupt functions of the vascular endothelium that may lead to conversion of its usually anticoagulant surface to one that is procoagulant. Treatment of hyperhomocysteinemia and the design of clinical trials is considered with respect to the current policy to fortify the food supply with folic acid. Is homocysteine the "new cholesterol" of risk factors? Time will tell.

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[1] Nonstandard abbreviations: CVD, cardiovascular disease; CBS, cystathionine β-synthase; MTHFR, methylenetetrahydrofolate reductase; MS, methionine synthase; and Δ-MTHFR, thermolabile methylenetetrahydrofolate reductase.

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Total Plasma Homocysteine and Its Determination

In this review, it is important that the term "total plasma homocysteine" be understood in a biochemical sense, because this will have relevance in the discussions that follow on methodology and on pathophysiological mechanisms. Human plasma contains both reduced and oxidized species of homocysteine (Fig. 1). Chemical definitions were established by Butz and du Vigneaud over 65 years ago *(11)*. The sulfhydryl or reduced form is called homocysteine and the disulfide or oxidized form is called homocystine. Disulfide forms also exist with cysteine and with proteins containing reactive cysteine residues (protein-bound homocysteine). The latter oxidized forms are referred to as mixed disulfides. The oxidized forms of homocysteine usually comprise 98–99% of total plasma homocysteine in human plasma, 80–90% of which is protein-bound (Fig. 1). There is no consensus as yet to designate multiple forms of homocysteine in plasma *(12)*. Some investigators write "homocyst(e)ine and hyperhomocyst(e)inemia" to designate multiple plasma forms. This somewhat awkward and archaic usage should be abandoned. First, it is impossible to pronounce the parentheses in speaking, and we certainly don't use "cholester(o)l" when we refer to its multiple forms in plasma. No, we say and write "total cholesterol", or identify a specific chemical form by use of appropriate prefixes. Thus, "total homocysteine", "reduced homocysteine", "protein-bound homocysteine", "homocystine", and "homocysteine-cysteine mixed disulfide" are easily understood. Homocysteine, like cholesterol, has ever increasing generic meaning, and this should not be discouraged. Total homocysteine, therefore, is the sum total of all forms of homocysteine that exist in plasma or serum.

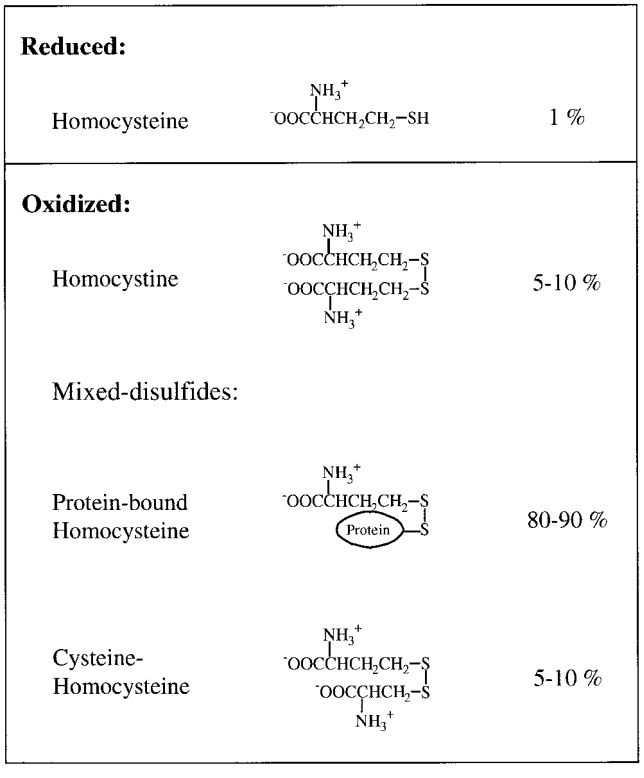

Fig. 1. Constituents of total plasma homocysteine and percentage of composition.

On the basis of work carried out by many investigators, the range of total homocysteine concentration in plasma from "healthy adults" is 5–15 μmol/L (Table 1) *(13)*. However, one must be cautious in the use of so-called "normal ranges" for homocysteine. We have shown that risk for coronary artery disease is represented by a continuum of total homocysteine concentration, with substantial risk occurring between 10 and 15 μmol/L *(14)*. Perhaps the upper limit of normal will be 10 μmol/L or even lower, because it may be possible to achieve this "desirable level" by optimal nutriture with respect to folic acid, B_{12}, and B_6 (*vide infra*). Individuals with homocystinuria because of rare inborn errors of homocysteine metabolism have severe hyperhomocysteinemia, with total homocysteine concentrations approaching 500 μmol/L. It has been reported that up to 20% (~100 μmol/L) of the total homocysteine in these individuals is reduced homocysteine *(15)*. Subjects with coronary artery, cerebrovascular, and peripheral vascular disease usually present with mild hyperhomocysteinemia (15–25 μmol/L) *(3, 6, 8)*. However, if renal function is impaired, or if the subject has end-stage renal disease, total homocysteine can reach intermediate concentrations (25–50 μmol/L) *(16, 17)*.

Methodologies to determine total plasma (or serum) homocysteine were first developed in the mid to late 1980s *(18–23)*. Free homocysteine must first be generated in the plasma sample by chemical reduction of disulfide bonds. Commonly used reductants include 2-mercaptoethanol *(19)*, dithiothreitol *(24)*, sodium borohydride *(25)*, *n*-tributylphosphine *(21)*, and more recently, the water-soluble phosphine tris(2-carboxyethyl)phosphine *(26)*. Homocysteine is then resolved from other low molecular weight thiols (cysteine, cysteinylglycine, and glutathione) by reversed-phase HPLC and determined directly by electrochemical detection *(20)*, or derivatized with a fluorochromophore, resolved by HPLC, and detected fluorometrically *(27–29)*. Alternatively, after reductive generation, homocysteine can be derivatized for capillary gas chromatography and detected by mass spectrometry *(19)*. Although there is relatively good agreement between different laboratories *(13)*, standardized reference ranges do not exist, and there is no consensus on the use of a standardized calibrator. The differences seen between

Table 1. Total plasma homocysteine: working ranges.

Normal range	5–15 μmol/L
Desirable (?)	<10 μmol/L
Hyperhomocysteinemia	
Mild	15–25 μmol/L
Intermediate	25–50 μmol/L
Severe	50–500 μmol/L

laboratories may be because of efficiency of disulfide bond reduction and use of different internal standards and calibrators. Immunoassays for total homocysteine were recently reported by Shipchandler and Moore *(30)* and by Frantzen et al. *(31)*. Both of these assays use a mouse monoclonal antibody directed against *S*-adenosylhomocysteine, which is formed when homocysteine, again generated by reductive cleavage of plasma disulfides, is allowed to react with adenosine in the presence of *S*-adenosylhomocysteine hydrolase. Advances in the development of high-throughput assays for total homocysteine are likely to continue over the next several years.

Hyperhomocysteinemia and Links to CVD

Classical homocystinuria, a monogenic defect of homocysteine metabolism, was discovered in 1962 by Carson and Neill *(32)* and Gerritsen et al. *(33)*. This rare disease, occurring in ~1 in every 100 000 to 200 000 births, is caused by deficiencies of cystathionine β-synthase (CBS), methylenetetrahydrofolate reductase (MTHFR), and methionine synthase (MS) and is inherited through an autosomal recessive mechanism. Individuals who are homozygous for CBS deficiency are unable to catabolize homocysteine through the transsulfuration pathway (Fig. 2). Homozygous deficiency of either MTHFR or MS prevents conversion of homocysteine back to methionine through the methionine cycle (Fig. 2). The clinical hallmarks of homocystinuria include mental retardation, skeletal abnormalities, ectopic lenses, and premature atherothrombotic disease *(34)*. These individuals excrete large amounts of homocystine in the urine and have total homocysteine concentrations in the plasma from 50 to 500 μmol/L. The extremely rapid onset of arteriosclerosis and atherosclerosis seen in these subjects suggests that there may be a dose-time relationship between total plasma homocysteine and the onset and progression of CVD.

The association between mild hyperhomocysteinemia and CVD has been documented in numerous retrospective case-control studies. In 1976, before development of assays for total plasma homocysteine, Wilcken and Wilcken *(35)* reported higher concentrations of homocysteine-cysteine mixed disulfide in plasma from subjects with coronary artery disease 4 h after methionine loading compared with controls. Kang et al. *(36)* found that protein-bound homocysteine was higher in subjects with documented coronary artery disease. In an early study

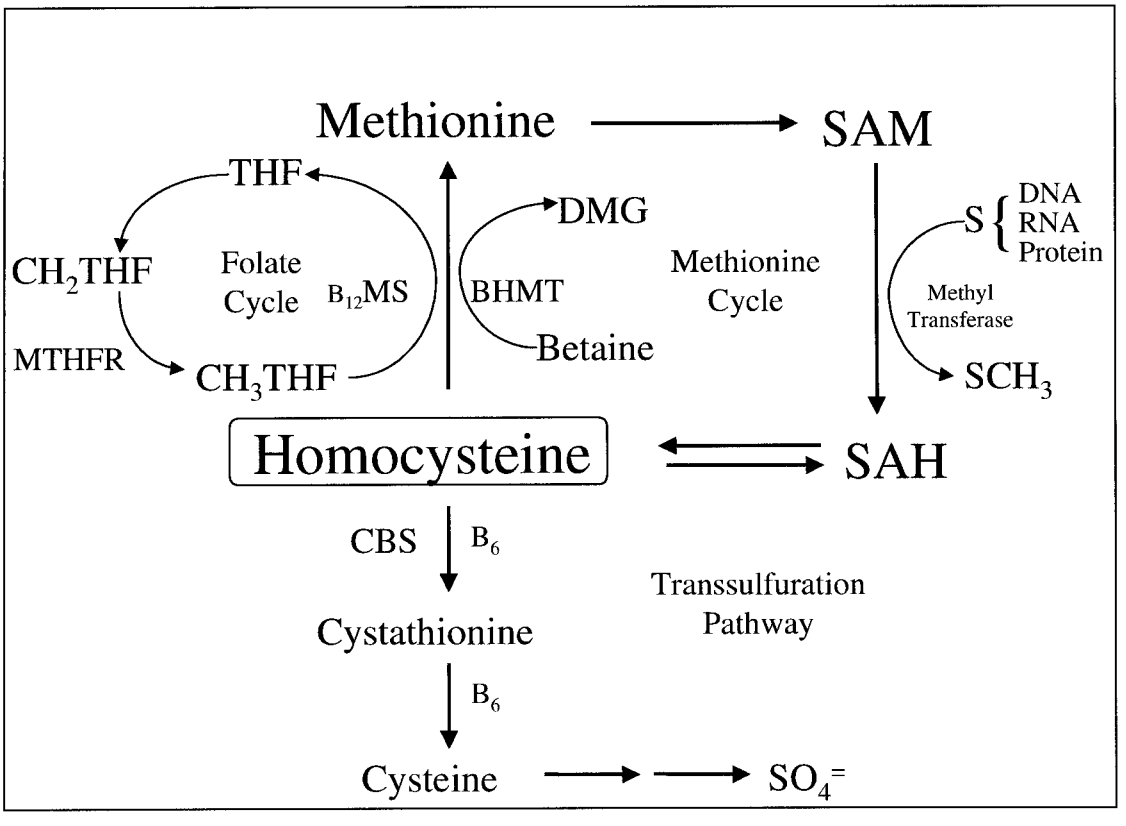

Fig. 2. Metabolism of homocysteine.

Remethylation: homocysteine is remethylated to methionine by B_{12}-dependent MS (*$B_{12}MS$*) in the presence of 5-methyltetrahydrofolate (*CH_3THF*). The latter is the product of 5,10-methylenetetrahydrofolate (*CH_2THF*) reduction by MTHFR. Homocysteine can also be remethylated by betaine:homocysteine methyltransferase (*BHMT*) in the liver and kidney. In the methionine cycle, dietary methionine is converted to *S*-adenosylmethionine (*SAM*), which serves as a methyl group donor substrate for methyltransferases. The other product of this reaction is *S*-adenosylhomocysteine (*SAH*), which is hydrolyzed by SAH hydrolase to homocysteine and adenosine. The methionine and folate cycle enzymes are widely distributed. Homocysteine also enters the catabolic transsulfuration pathway. The first enzyme in this pathway is B_6-dependent CBS. Cystathionine is converted to cysteine by B_6-dependent cystathionase. Cysteine is further catabolized to inorganic sulfate, which is excreted in the urine. The transsulfuration pathway has somewhat limited tissue distribution (liver, kidney, pancreas, and brain).

using basal total plasma homocysteine, Israelsson et al. *(37)* found an incidence of 24% of hyperhomocysteinemia in men who had suffered a myocardial infarction before the age of 55. Boushey et al. *(38)* did a metaanalysis on 27 studies (up to early 1995) relating homocysteine to CVD and 11 studies on folate as a determinant of plasma homocysteine. Increased total homocysteine was an independent graded risk factor for CVD, with odds ratios of 1.6 and 1.8 for a 5 μmol/L increment in men and women, respectively. A strong inverse correlation was found between total homocysteine and serum folate. The authors speculated that increasing folate intake from foods, or folic acid by supplementation would substantially reduce annual mortality from coronary artery disease. More recently, Graham et al. *(39)* reported that increased total homocysteine was as strong as smoking and hyperlipidemia as an independent risk factor for CVD on the basis of a large multicenter European study involving 750 cases and 800 controls.

Results of prospective studies attempting to link hyperhomocysteinemia to CVD have been mixed. In the US Physicians' Health Study, Stampfer et al. *(40)* found higher total plasma homocysteine in cases compared with controls (11.1 ± 4.0 vs 10.5 ± 2.8 μmol/L, $P = 0.03$); however, the relative risk for myocardial infarction was 3.1 for men in the ≥95th percentile, suggesting a threshold effect. Arnesen et al. *(41)*, on the other hand, found that total plasma homocysteine was an independent risk factor for coronary artery disease, with no threshold concentration. Recently, total plasma homocysteine was found to be a strong predictor of mortality in Norwegian patients with coronary artery disease *(42)*. However, prospective studies conducted by Alfthan et al. *(43)* and by Evans et al. *(44)* were negative.

Secondary intervention studies are only just beginning in the United States and Europe. These studies, designed to lower total plasma homocysteine in patients with CVD by supplementation with combinations of folic acid, B_{12}, and B_6, will assess long-term mortality and morbidity outcomes in treatment and placebo groups. It may be several years before the results of these clinical trials are known. A complicating factor for trials in this country is the addition of folic acid, a major nutritional determinant of total homocysteine, to the nation's food supply, as discussed below.

Determinants of Hyperhomocysteinemia

The determinants of total plasma homocysteine are complex and involve demographic, genetic, and acquired factors (Table 2). It is likely that gene-nutrient interactions will be important determinants in subjects who carry one or more mutations in genes that regulate homocysteine metabolism. Thus, genetic background, nutrition, state-of-health, life-style, gender, and age influence the homeostasis of homocysteine.

Table 2. Determinants of hyperhomocysteinemia.

Demographic
Age
Sex
Ethnic origin
Genetic
MTHFR
MS
CBS
Acquired
B-vitamin deficiency (folate, B_{12}, and B_6)
State of health
Impaired renal function
End-stage renal disease
Heart and other organ transplants
Hypothyroidism
Lifestyle
Smoking
Alcohol (excessive)
Lack of exercise
Coffee (excessive)

DEMOGRAPHIC FACTORS

Total plasma homocysteine appears to increase throughout life. In Norwegian children, ages 8–12 years, total homocysteine concentrations were 5–6 μmol/L, with no reported differences for gender *(45)*. Nygård et al. *(46)* found that in Norwegian men and women 40–42 years old, geometric mean values for total homocysteine were 10.8 and 9.1 μmol/L, respectively. In Norwegians 65–67 years old, the values increased to 12.3 and 11.0 μmol/L. In a predominantly female group of French centenarians, mean total plasma homocysteine was 26.7 ± 9.6 μmol/L *(47)*. It is now clear that men have higher total plasma homocysteine concentrations than women of the same age *(46)*; however, the reasons for this difference and when the divergence begins (presumably after puberty) are unknown. South African black children (7–15 years old) had significantly higher homocysteine concentrations than South African white children (5.8 ± 1.8 vs 5.1 ± 0.8 μmol/L, respectively); however, Ubbink et al. *(48)* found that black men had significantly lower concentrations than white men (9.7 ± 3.4 vs 12.0 ± 6.7 μmol/L, respectively) and that young black men were able to metabolize homocysteine more efficiently than young white men, based on methionine-loading studies *(49)*. Comparable studies have not been reported in this country.

GENETIC FACTORS

Studies in the early 1990s established that genetic factors were important contributors to the hyperhomocysteinemia seen in patients with coronary artery disease *(50–53)*. Recent evidence suggests that genetic factors, in combination with acquired or environmental factors, lead to increased total plasma homocysteine *(46, 54)*.

MTHFR converts 5,10-methylenetetrahydrofolate to

5-methyltetrahydrofolate in the presence of NADPH (Fig. 2). 5-Methyltetrahydrofolate in turn serves as a substrate for B_{12}-dependent methionine synthase. MTHFR is a flavin-dependent enzyme consisting of two identical 77-kDa subunits with N-terminal catalytic domains and C-terminal regulatory domains *(55)*. Approximately 50 cases of severe MTHFR deficiency have been reported *(56)*, with neurological or vascular complications appearing in the first or second decade of life. The gene for the enzyme has been cloned *(57)*, and 14 mutations causing severe deficiency have been described *(58*, *59)*. A thermolabile variant of the enzyme, designated Δ-MTHFR in this review, was first reported by Kang et al. *(60)*. Subsequent studies by the same group found a high incidence of Δ-MTHFR in patients with coronary artery disease *(61*, *62)*. In a more recent study by Engbersen et al. *(63)*, 28% of hyperhomocysteinemic patients with premature vascular disease had the thermolabile enzyme. A C677T gene mutation, which produces an alanine-to-valine substitution, is the cause of Δ-MTHFR. The allele frequency of this mutation is widespread: 38% in the French Canadian population *(64)* and 25–39% in other populations *(65–72)*, but only 10% in African Americans *(73)*. Many reports support the hypothesis that individuals who are homozygous for Δ-MTHFR may be at greater risk for CVD *(65–68*, *71)*, but others do not *(69*, *72)*, irrespective of folate status *(70*, *74)*. Although Ma et al. *(75)* found no increased risk for myocardial infarction in men because of Δ-MTHFR, they did find higher total homocysteine concentrations in homozygous men with low folate status. A similar gene-nutrient interaction was found by Jacques et al. *(76)* who studied 365 individuals from the NHLBI Family Heart Study. In patients with end-stage renal disease, Bostom et al. *(77)* found 33% higher total homocysteine in pooled homozygote and heterozygote patients who had plasma folate values below the median when compared with patients with wild-type MTHFR genotype.

Vitamin B_{12}-dependent MS is found in most cells and tissues *(78)*. Inborn errors that affect B_{12} absorption, systemic transport, intracellular transport, and conversion to the coenzyme form (methyl-B_{12}) will produce loss of MS activity and hyperhomocysteinemia *(56*, *79)*. Mutations in the structural gene for MS itself are only now being described *(80–82)*. Human MS cDNA contains an open reading frame of 3798 nucleotides encoding a polypeptide of 1265 amino acids (predicted molecular mass, 140 kDa). On the basis of complementation studies using patient fibroblast cell lines, two types of MS-associated genetic diseases, *cblE* and *cblG*, have been described *(83)*. The *cblE* class may involve a reducing component of the MS system *(84)*, whereas *cblG* is thought to be caused by defective MS apoenzyme *(85*, *86)*. Several MS mutations have now been identified in *cblG* patients *(81*, *87)*; however, the prevalence of these mutations in the general population and their contribution to hyperhomocysteinemia in heterozygous individuals remain to be determined.

Entry of homocysteine into the transsulfuration pathway is catalyzed by B_6-dependent CBS, which has limited tissue distribution *(78)*. Several hundred cases of CBS deficiency, the most common form of homocystinuria, have been described *(34)*. The human cDNA for CBS, cloned by Kraus et al. *(88)* in 1993, contains 2554 nucleotides encoding a subunit of 551 amino acids with a predicted molecular mass of 63 kDa. When bacterial *(89*, *90)* and yeast *(91*, *92)* expression systems have been used, close to 40 mutations have been identified in the CBS gene. Obligate heterozygotes for CBS deficiency might be at greater risk for CVD because of possible increases in basal and postmethionine load total homocysteine *(35*, *51*, *93)*. However, the reliability of assessing heterozygosity based on CBS enzyme activity measurements and postmethionine-loading studies has been questioned *(94)*. A number of genotyping studies have been done; however, they have failed to identify CBS polymorphisms that correlate with CVD *(68*, *95*, *96)*. Clearly, additional studies are needed to assess the role of CBS polymorphism in atherosclerotic disease.

ACQUIRED AND LIFE-STYLE FACTORS

The source of homocysteine in the diet is L-methionine. Fruits and vegetables generally contain 0.9–1.2 g of methionine per 100-g serving. Peaches and grapes (3.6 g/100 g) are exceptions. Nuts and cereal grains contain 1.4–1.8 g/100 g, except Brazil nuts, which contain 5.8 g/100 g. Sources of animal protein have a higher methionine content: meat and fish, 2.7 g/100 g, eggs, 3.2 g/100 g, and cow's milk, 2.9 g/100 g. By comparison, human milk has only 1.4 g/100 g.

Dietary insufficiency or malabsorption of folate, vitamin B_{12}, or vitamin B_6 will lead to hyperhomocysteinemia and an increased risk of CVD *(97–103)*. Even in well-nourished generally healthy populations, serum folate and B_{12} inversely correlate with total homocysteine *(29*, *104)*. The B vitamins drive homocysteine metabolism, with 5-methyltetrahydrofolate serving as substrate for B_{12}-dependent MS in the remethylation of homocysteine back to methionine and vitamin B_6 (as pyridoxal-5-phosphate) as a cofactor for CBS in the transsulfuration pathway (Fig. 2). The large Hordaland study in Norway found that increased total plasma homocysteine was associated with smoking, high blood pressure, increased cholesterol, and sedentary life-style *(46)*. Alcoholics have higher total homocysteine concentrations, perhaps because of malnourishment and malabsorption *(105*, *106)*. Heavy coffee consumption is associated with higher homocysteine concentrations as well *(54)*.

Certain disease states produce higher total homocysteine concentrations. Patients with end-stage renal disease have intermediate hyperhomocysteinemia and an increased risk for vascular disease *(16*, *17)*. Heart transplant recipients have mild to intermediate hyperhomocysteine-

mia *(107, 108)*, which may in part be related to renal insufficiency *(109)*. Hypothyroidism produces increased total plasma homocysteine *(110)*, but treatment with L-thyroxine will normalize homocysteine concentrations *(111)*.

Mechanisms of Blood Vessel Injury

It is usually assumed that reduced homocysteine is the atherogenic form of homocysteine in circulation. But recall that it contributes only 1% of the basal total plasma homocysteine in normocysteinemic and mildly hyperhomocysteinemic individuals (Fig. 1). However, in addition to basal concentrations, we should also consider the transient hyperhomocysteinemia that occurs after eating. Within 2 h after methionine loading, reduced homocysteine reaches a maximum and then declines as oxidized forms (homocystine and mixed disulfides) become increased and reach peak values in 6 h *(112)*. The transient hyperhomocysteinemia seen after methionine loading also occurs after meals, and its magnitude is proportional to protein consumption *(113)*. Abnormal methionine loading in which peak total plasma homocysteine concentrations exceed an overall reference range have been seen in up to 40% of subjects, predominantly female *(37, 39, 114)*. Silberberg et al. *(115)*, however, recommend that age- and gender-specific reference ranges be used to prevent overdiagnosis.

It can be hypothesized that reduced homocysteine directly alters vascular cell function. Because reduced homocysteine undergoes oxidation in vivo, one could argue that the homocysteine oxidation products such as hydrogen peroxide, superoxide anion radical, and other reactive oxygen species are the injurious agents. Thus, a second hypothesis is that homocysteine is acting indirectly through its oxidation and formation of reactive oxygen species.

Many of the in vitro studies using cultured endothelial and smooth muscle cells from human and animal vessels during the past 10 years used exceedingly high concentrations of reduced homocysteine (5–10 mmol/L), far exceeding physiological and pathophysiological concentrations of reduced homocysteine (and for that matter total plasma homocysteine). Another problem is that many of these studies failed to demonstrate specificity for homocysteine and in many cases found a "general thiol effect". Keep in mind that total plasma cysteine is 20- to 30-fold higher than total plasma homocysteine and that the concentration of reduced cysteine is ~70-fold higher than reduced homocysteine (5.0 ± 3.6 vs 0.07 ± 0.02 μmol/L) *(112)*. There is little or no evidence in the literature that cysteine is atherogenic. Nevertheless, it is widely believed that homocysteine can alter the surface properties of endothelial cells by changing their phenotype from anticoagulant to procoagulant *(116)*.

Wang et al. *(117)* recently reported that 10–50 μmol/L D,L-homocysteine (but not L-cysteine or L-cystine) in the presence of 50 μmol/L adenosine inhibited the growth of vascular endothelial cells by a mechanism involving decreased carboxymethylation of $p21^{ras}$. This is one of the few reports in which physiologically relevant concentrations of homocysteine have been used and in which thiol specificity has been demonstrated. Upchurch and coworkers reported that homocysteine modulated the expression of glutathione peroxidase *(118)* and nitric oxide synthase *(119)* in bovine aortic endothelial cells; however, relatively high concentrations of homocysteine were used. Tsai and co-workers *(120, 121)* reported that homocysteine was mitogenic to smooth muscle cells by a mechanism involving synergistic induction of cyclin A mRNA expression with serum. However, other thiols, such as cysteine and glutathione, could replace homocysteine, suggesting a general thiol effect on the stimulation of smooth muscle cell proliferation. Matrix proteins accumulate in atherosclerotic plaques, and recently Majors et al. *(122)* reported that relatively low concentrations of homocysteine (50–300 μmol/L) stimulate collagen production in cultured rabbit aortic smooth muscle cells.

There have been some interesting in vivo studies in both humans and animal models that support the hypothesis of impaired endothelial cell function in the presence of hyperhomocysteinemia. Van den Berg et al. *(123)* assessed endothelial dysfunction in young patients with peripheral arterial occlusive disease and mild hyperhomocysteinemia by measuring plasma von Willebrand factor, thrombomodulin, and tissue plasminogen activator. Plasma concentrations of the first two were above reference values at baseline but decreased after treatment of the hyperhomocysteinemia with pyridoxine and folic acid. Tawakol et al. *(124)* observed impaired endothelial-dependent vasodilation in subjects with mild hyperhomocysteinemia. Linear regression analysis revealed that total plasma homocysteine was the only meaningful predictor of flow-mediated vasodilation. No attempt to correct impaired vasodilation with cofactor therapy was reported. Similar results were obtained by Woo et al. *(125)* in subjects with intermediate hyperhomocysteinemia.

Using an animal model, Lentz et al. *(126)* recently tested the hypothesis that diet-induced mild hyperhomocysteinemia would lead to vascular dysfunction. In cynomolgus monkeys on a diet with high methionine and low folate for 4 weeks, total plasma homocysteine was 10.6 ± 2.6 μmol/L. Monkeys on a regular diet had total plasma homocysteine concentrations of 4.0 ± 0.2 μmol/L. Impaired endothelial-mediated vasodilation was observed in the hyperhomocysteinemic animals. However, in atherosclerotic animals maintained on an atherosclerotic diet for 17 months, normalization of their moderate hyperhomocysteinemia by administration of folic acid, B_{12}, and B_6 was insufficient to restore normal vascular function *(127)*.

Work in our laboratory has focused on the metabolism of homocysteine in vascular cells and tissues. Using direct enzyme assays and Western and Northern blotting, we found that cultured human aortic endothelial cells do not express an active form of CBS *(128)* and thus apparently

cannot initiate homocysteine catabolism through the transsulfuration pathway (Fig. 2). Their inability to express a major pathway for homocysteine could render them highly susceptible to mild increases of total homocysteine seen in the hyperhomocysteinemia of CVD. Our laboratory has also investigated the role of homocysteine in possible early events in atherogenesis. We found that extremely low concentrations of reduced homocysteine (10–50 μmol/L) up-regulated the expression of the chemokine monocyte chemoattractant protein 1 in cultured human aortic endothelial cells and that the up-regulation was specific for homocysteine *(129)*. At maximal dose–response for homocysteine (50 μmol/L), cysteine, cystine, methionine, and homocystine had no effect.

Treatment of Hyperhomocysteinemia

The severe hyperhomocysteinemia seen in subjects with classical homocystinuria often responds to treatment. Approximately 50% of homocystinurics with CBS deficiency respond to pyridoxine (vitamin B_6) *(34)*. In patients with congenital deficiency of transcobalamin, the serum protein that delivers B_{12} to cells throughout the body, weekly injections of vitamin B_{12} will normalize their hyperhomocysteinemia and allow them to lead relatively normal lives. Deficiencies of MTHFR and MS are usually more refractory to treatment *(56, 130)*. Nevertheless, many of these homozygous monogenic diseases respond quite well to treatment. Can the mild hyperhomocysteinemia associated with vascular disease be treated? The answer is emphatically yes.

The treatment of mild hyperhomocysteinemia is based on the hypothesis that the nutritional status of the micronutrients that drive homocysteine metabolism, folate, B_{12}, and B_6, is suboptimal and that by dietary supplementation with folic acid, cyanocobalamin (vitamin B_{12}), and pyridoxine hydrochloride (vitamin B_6), either singly or in combination, one can optimize homocysteine metabolism and lower not only basal total plasma homocysteine, but abnormal methionine-loading hyperhomocysteinemia as well. This hypothesis has been confirmed by many investigators. In 1988, Brattström et al. *(131)* showed that 5 mg of folic acid per day for 14 days was very effective in lowering total homocysteine in healthy subjects, whereas 40 mg of pyridoxine HCl or 1 mg of cyanocobalamin had little or no effect when overall effects of the three treatment groups were compared. Brattström et al. *(132)* later reported that administration of 10 mg of folic acid plus 240 mg of pyridoxine HCl per day for 4 weeks reduced mean total plasma homocysteine by 53% and abnormal methionine loading by 39% in 20 patients with premature cerebral and peripheral occlusive disease. The same group showed that patients who had suffered a myocardial infarction were able to lower their total homocysteine concentrations with 2.5 mg of folic acid daily for 6 weeks *(133)*.

Folic acid is clearly the most effective form of supplementation to reduce total homocysteine concentrations in subjects with mild hyperhomocysteinemia. But is it safe for all individuals and are lower doses effective as well? Before 1986, the recommended daily allowance for folic acid was 400 μg per day. The recommended daily allowance was then changed to 180 μg per day for women and 200 μg per day for men. Women who have folate deficiency at the time of conception are at much greater risk for giving birth to children with neural tube defects. For this reason, the Food and Drug Administration in this country has approved the addition of folic acid to cereals and flour products *(134)*. Individuals will now be consuming on average an additional 100 μg of folic acid a day. Will this have an impact on total plasma homocysteine? Recent studies from John Scott's laboratory in Dublin suggest that it will. Ward et al. *(135)* showed that even 100 μg of folic acid daily for 6 weeks can lower total homocysteine concentrations in healthy individuals, but that 200 μg per day was near optimal. These doses are clearly safe for most individuals. However, the elderly with undiagnosed B_{12} deficiency could experience adverse effects by folic acid supplementation. Stabler et al. *(136)* have recently reviewed the problem that could affect millions of individuals in this country. To protect against unrecognized B_{12} deficiency, cyanocobalamin could be combined with folic acid supplementation. Approximately 1–2% of an oral dose of cyanocobalamin is absorbed by passive diffusion, even in subjects with pernicious anemia. Bostom et al. *(137)* found that patients with renal failure require much more aggressive B-vitamin therapy to achieve total plasma homocysteine lowering.

Studies on classical homocystinuria provide strong evidence that homocysteine is causal; however, the role that homocysteine plays in the usually mild hyperhomocysteinemia associated with coronary artery disease, cerebrovascular disease, and peripheral vascular disease is still unclear. Clinical trials are urgently needed to determine if lowering total plasma homocysteine will reduce the mortality and morbidity of CVD.

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