

MINIREVIEW

Impaired Homocysteine Metabolism and Atherothrombotic Disease

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SUMMARY: Based on recent retrospective, prospective, and experimental studies, mild to moderate elevation of fasting or postmethionine-load plasma homocysteine is accepted as an independent risk factor for cardiovascular disease and thrombosis in both men and women. Hyperhomocysteinemia results from an inhibition of the remethylation pathway or from an inhibition or a saturation of the transsulfuration pathway of homocysteine metabolism. The involvement of a high dietary intake of methionine-rich animal proteins has not yet been investigated and cannot be ruled out. However, folate deficiency, either associated or not associated with the thermolabile mutation of the N^{5,10}-methylene tetrahydrofolate reductase, and vitamin B₆ deficiency, perhaps associated with cystathionine β -synthase defects or with methionine excess, are believed to be major determinants of the increased risk of cardiovascular disease related to hyperhomocysteinemia. Recent experimental studies have suggested that moderately elevated homocysteine levels are a causal risk factor for atherothrombotic disease because they affect both the vascular wall structure and the blood coagulation system. The oxidant stress that results from impaired homocysteine metabolism, which modifies the intracellular redox status, might play a central role in the molecular mechanisms underlying moderate hyperhomocysteinemia-mediated vascular disorders. Because folate supplementation can efficiently reduce plasma homocysteine levels, both in the fasting state and after methionine loading, results from further prospective cohort studies and from on-going interventional trials will determine whether homocysteine-lowering therapies can contribute to the prevention and reduction of cardiovascular risk. Additionally, these studies will provide unequivocal arguments for the independent and causal relationship between hyperhomocysteinemia and atherothrombotic disease. (*Lab Invest* 2001, 81:645–672).

Cardiovascular diseases remain the leading cause of mortality in Western populations. Hyperlipoproteinemia, hypertension, diabetes, obesity, and tobacco smoking are the main risk factors for atherosclerosis and its thrombotic complications. However, these factors alone cannot account for all of the deaths caused by vascular pathologies.

As early as 1969, clinical studies conducted in homocystinuric children revealed the importance of severe hyperhomocysteinemia in premature development of atherosclerosis and thromboembolism (McCully, 1983). According to numerous retrospective case-controlled studies, moderate increases in plasma homocysteine, which can be precisely quantitated by high performance liquid chromatography

(HPLC), raise the risk for cardiovascular disease 2-fold, after adjusting for classic risk factors. Moreover, up to 20% to 40% of patients with vascular pathologies present moderate to intermediate hyperhomocysteinemia. However, results from prospective studies are inconsistent. Additionally, molecular mechanisms underlying hyperhomocysteinemia-induced vascular disease are poorly understood.

In the present review, we describe homocysteine metabolism and its regulation by tissue folate and S-adenosylmethionine levels. We also discuss the metabolic abnormalities leading to hyperhomocysteinemia and the importance of moderate hyperhomocysteinemia in the incidence of cardiovascular pathologies. Specific attention is given to understanding the inconsistencies in the prospective studies. Finally, we focus on the potential mechanisms involved in vascular disorders caused by hyperhomocysteinemia and we describe the possible vitamin treatments.

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Homocysteine Metabolism and Its Regulation

Homocysteine: An Intermediate Product of Methionine Metabolism

Homocysteine is formed from methionine, which is the main source of protein sulfur atoms (Fig. 1). The demethylation of S-adenosylmethionine (SAM) to S-adenosylhomocysteine (SAH) (Fig. 1, reaction 2), results from the activation of methionine by ATP (Fig. 1, reaction 1). This activation is accompanied by vital methylation reactions that allow the synthesis of creatine and phosphatidylcholine and the methylation of DNA, RNA, and a large number of neuromediators. The “nonproductive” methylation of glycine to sarcosine, which is physiologically inert, allows an alter-

native demethylation of SAM (Fig. 1, reaction 3). Although the equilibrium constant of the reaction favors the formation of SAH, under normal metabolic conditions, low intracellular concentrations of homocysteine and adenosine, SAH formed by transmethylation reactions is hydrolyzed into homocysteine and adenosine (Fig. 1, reaction 4). This transmethylation is the sole pathway for homocysteine synthesis in the body.

The resulting homocysteine is either catabolized into cystathionine or remethylated into methionine. The homocysteine remethylation is catalyzed by methionine synthase, which uses N⁵-methyltetrahydrofolate (5-methylTHF) as the methyl donor and cobalamin as the cofactor (Fig. 1, reaction 7). The

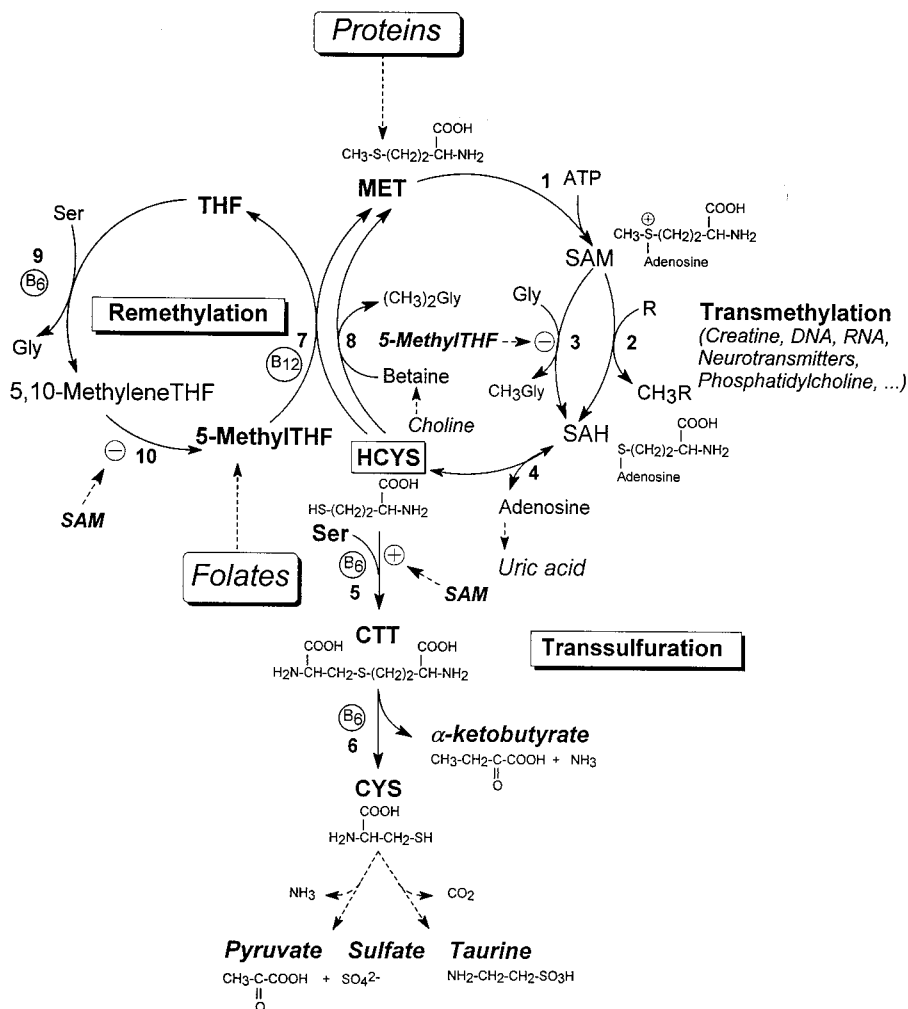


Figure 1.

Homocysteine metabolism. (1) Activation of methionine (MET) by ATP in the presence of methionine S-adenosyl-transferase (EC 2.5.1.6). (2) S-adenosylmethionine (SAM) demethylation into S-adenosylhomocysteine (SAH) coupled with methylation of an acceptor R into RCH₃. (3) Alternative demethylation catalyzed by glycine N-methyltransferase (EC 2.1.1.20), which converts glycine (Gly) into sarcosine (CH₃Gly). (4) Hydrolysis of SAH into homocysteine (HCYS) and adenosine catalyzed by S-adenosylhomocysteine hydrolase (EC 3.3.1.1). (5) Condensation of serine (Ser) with HCYS to form cystathionine (CTT) through the action of vitamin B₆-derived pyridoxal-5'-phosphate-dependent cystathionine β-synthase (CBS) (EC 4.2.1.22). (6) Conversion of CTT into cysteine (CYS) and α-ketobutyrate catalyzed by pyridoxal-5'-phosphate-dependent γ-cystathionase (EC 4.4.1.1). (7) N⁵-methyltetrahydrofolate (5-methylTHF) demethylation into THF and HCYS remethylation into MET by methionine synthase (EC 2.1.1.12) dependent on vitamin B₁₂-derived methylcobalamin. (8) In the liver, remethylation of HCYS by betaine-homocysteine methyltransferase (EC 2.1.1.5) in the presence of choline-derived betaine. (9) Synthesis of N^{5,10}-methylene tetrahydrofolate (5, 10-methyleneTHF) from THF coupled with the conversion of Ser into Gly through the action of pyridoxal-5'-phosphate-dependent serine hydroxymethyltransferase (EC 2.1.2.1). (10) Reduction of 5,10-methyleneTHF into 5-methylTHF catalyzed by N^{5,10}-methylene tetrahydrofolate reductase (MTHFR) (EC 1.5.1.20). EC, enzyme classification number of the International Union of Biochemistry.

formation of 5-methylTHF depends on N^{5,10}-methylene tetrahydrofolate reductase (MTHFR), which catalyzes the reduction of N^{5,10}-methylene tetrahydrofolate (5, 10-methyleneTHF) (Fig. 1, reaction 10) obtained from THF (Fig. 1, reaction 9). A minor remethylation pathway working independently of folates and cobalamin uses the conversion of betaine (derived from choline) to N, N-dimethylglycine (Fig. 1, reaction 8). This pathway, catalyzed by betaine-homocysteine methyltransferase, mainly occurs in the liver. In folate and/or cobalamin deficiencies, this pathway maintains the tissue concentration of methionine necessary for the SAM synthesis. Homocysteine catabolism is conducted by cystathionine β -synthase (CBS), an enzyme dependent on pyridoxal-5'-phosphate (derived from vitamin B₆), which allows the condensation of homocysteine with serine (Fig. 1, reaction 5). The resulting cystathionine is converted into cysteine, a precursor of glutathione, and α -ketobutyrate by γ -cystathionase, which is also dependent on pyridoxal-5'-phosphate (Fig. 1, reaction 6). Cysteine is finally converted into sulfates, which are excreted in the urine. Of note are the preferential distributions of the transsulfuration pathway and the catabolizing enzyme glycine N-methyltransferase in mammalian tissues such as liver and kidney (Finkelstein, 1990). These tissues may be essential to take up (via carriers, receptors, or channels) and metabolize excess transient circulating homocysteine (Blom, 2000). Tissues lacking this pathway require exogenous cysteine to synthesize glutathione.

The Regulation of Homocysteine Metabolism

SAM is an important determinant in the regulation of homocysteine metabolism. Recent data suggest that SAM coordinately regulates homocysteine remethylation and catabolism by inhibiting methionine-conserving enzymes with low K_m values and activating methionine-catabolizing enzymes with higher K_m values (Finkelstein, 1990; Selhub and Miller, 1992). When the tissue SAM level is sufficient to maintain vital transmethylation reactions, SAM reduces the rate of remethylation of homocysteine into methionine by 5-methylTHF, through an inhibition of MTHFR activity (Fig. 1, reaction 10) (Finkelstein, 1990; Fowler, 1997). SAM also regulates homocysteine hepatic remethylation by inhibiting betaine-homocysteine methyltransferase activity (Fig. 1, reaction 8). Moreover, SAM limits its own synthesis by inhibiting low K_m type I and type II extrahepatic methionine S-adenosyltransferase activity (Fig. 1, reaction 1). Conversely, SAM stimulates high K_m hepatic methionine S-adenosyltransferase III and the alternative transmethylation pathway by activating glycine methyltransferase (Fig. 1, reaction 3), whereas SAH inhibits most other SAM-dependent transmethylases (Fig. 1, reaction 2) (Finkelstein, 1990). Additionally, to avoid homocysteine accumulation, which is potentially cytotoxic, SAM stimulates homocysteine catabolism by increasing CBS activity (Fig. 1, reaction 5) and γ -cystathionase activity (Fig. 1, reaction 6) (Finkelstein, 1990; Fowler, 1997). With a high

dietary supply of methionine-rich animal proteins, an increase in tissue concentrations of SAM occurs, and 70% of homocysteine is catabolized by CBS through the transsulfuration pathway (Fig. 1, reaction 5). In contrast, under conditions of restricted protein supply, the remethylation pathway is favored by an acceleration of the MTHFR-dependent folate mobilization (Fig. 1, reaction 10). In this case, only 10% of the homocysteine pool is catabolized through the CBS transsulfuration pathway. Nevertheless, a long-term high methionine intake can lead to a saturation of the transsulfuration pathway. This saturation is associated with an inhibition of remethylation that leads to a cell homocysteine efflux. Studies have shown that methionine-enriched diets or acute methionine loads cause homocysteine to be exported from cells, leading to increased plasma homocysteine levels (Ueland and Refsum, 1989).

The intracellular pool of folates also is involved in the regulation of homocysteine metabolism by supplying 5-methylTHF. Indeed, 5-methylTHF is necessary for the cellular remethylation of homocysteine into methionine (Fig. 1, reaction 7). 5-methylTHF thus maintains optimum tissue levels of SAM when there is a restricted exogenous methyl group supply from methionine and choline. Moreover, under conditions of high folate intake, 5-methylTHF reduces the alternative transmethylation pathway by inhibiting glycine methyltransferase (Fig. 1, reaction 3). The resulting increase in tissue concentrations of SAM limits intracellular folate pool mobilization in the homocysteine remethylation route (Fig. 1, reaction 10). Conversely, a decrease in the bioavailability of 5-methylTHF generates a reduction of homocysteine remethylation (Fig. 1, reaction 7) and an acceleration of the alternative transmethylation (Fig. 1, reaction 3). Decreases in tissue concentrations of SAM, resulting from depletion of the folate stores, induce inhibition of the transsulfuration reactions (Fig. 1, reaction 5) (Durand et al, 1997a; McCully, 1983). Dietary folate deficiencies and drugs that interfere with folate metabolism, eg, methotrexate, an anti-carcinogenic agent that inhibits dihydrofolate reductase, and nitrous dioxide (N₂O), an anesthetic gas that inhibits methionine synthase activity, lead to accumulations and cellular effluxes of homocysteine, causing increases in circulating homocysteine levels (Ueland and Refsum, 1989).

Characterization of the metabolic interactions between folate and methionine in the regulation of SAM-dependent homocysteine levels, as assessed by Selhub and Miller (Miller et al, 1994a, 1994b; Selhub and Miller, 1992) and by our laboratory in animal models (Durand et al, 1997a), has facilitated the understanding of mechanisms leading to hyperhomocysteinemia. These data help to explain why an excess of methionine does not lead to a higher elevation of homocysteine in patients with an alteration of the remethylation pathway (caused by folate or cobalamin deficiency and/or by a partial congenital MTHFR defect). The reduction in SAM tissue concentrations resulting from the altered homocysteine remethylation might be compensated for by the increase in SAM synthesis

with the methionine supply (Miller et al, 1994a). In these patients, the excess of dietary methionine might even reduce their hyperhomocysteinemia by favoring homocysteine catabolism (Loehrer et al, 1996). In contrast, when tissue folate or cobalamin stores are intact (Silberberg et al, 1997) or in the case of an alteration of the transsulfuration pathway dependent on vitamin B₆ deficiency or on heterozygosity for a CBS defect (Bostom et al, 1995a), an excessive methionine supply might lead to an important elevation of plasma homocysteine.

Moreover, recent studies have revealed the oxidative sensitivities of purified mammalian methionine synthase and CBS, suggesting a redox regulation of the remethylation and transsulfuration pathway. CBS, a heme protein, is active in the oxidized form, whereas methionine synthase is inactivated by oxidation and requires reductive methylation for reactivity (Chen and Banerjee, 1998; Taoka et al, 1998). In accordance with this regulatory mechanism, which may be physiologically significant, homocysteine metabolism is challenged toward the production of cysteine and glutathione through the transsulfuration pathway in human liver cells exposed to oxidative conditions (Mosharov et al, 2000). It is therefore possible that oxidant stress reduces remethylation and enhances transsulfuration to maintain, via an autocorrective process, the intracellular glutathione pool, which would be essential for the redox-regulating capacity of cells.

Abnormalities of Homocysteine Metabolism

Dysfunctions related to congenital defects in enzymes involved in homocysteine metabolism, to deficiencies in the supply, absorption, or metabolism of folates, vitamins B₁₂ and B₆, and to dietary methionine excess all contribute to increasing plasma homocysteine levels (Table 1). It is noteworthy that gene-nutrient interactions, especially between the C677T mutation of the MTHFR gene and folate status and perhaps between heterozygosity for CBS defect and folate status (Lentz et al, 2000), could be involved in the onset of hyperhomocysteinemia. Besides an increased homocysteine export from tissues into plasma that may be regulated by a "reduced-homocysteine carrier" (Blom, 2000), hyperhomocysteinemia can result from reduced renal function, which may have a central role in plasma homocysteine clearance, and from chronic hepatic insufficiency. Additionally, although the thiolactone form has not been unequivocally identified *in vivo*, homocysteine could undergo intracellular cyclization into homocysteine thiolactone, which could be a major by-product of impaired homocysteine metabolism (Jakubowski, 1997; Jakubowski, 2000a). Jakubowski and collaborators have recently reported that cells efficiently convert homocysteine into homocysteine thiolactone by methionyl-tRNA synthetase (Jakubowski et al, 2000b). In the circulation, the reduced form of homocysteine results from a direct cellular efflux (Blom, 2000) or from circulating thiolactone hydrolysis by homocysteine thiolactone hydrolase (Jakubowski, 2000b). Homocysteine can either

accept nitric oxide (NO) to form S-nitrosohomocysteine or can be oxidized to homocysteine sulfinic acid and homocysteic acid. Nevertheless, as soon as homocysteine is exported out of the cell, it is oxidized to a disulfide with any thiol-containing compound (Bourdon and Blache, 2001). Indeed, circulating homocysteine is bound mainly to plasma protein and nonprotein-bound homocysteine is mainly found in oxidized disulfide forms including homocystine and mixed disulfides.

Genetic Factors

Alteration of the Transsulfuration Pathway. One of the classic alterations of the homocysteine metabolism is represented by the homozygous deficiency of CBS (Mudd et al, 1995). This autosomal recessive defect results in homocystinuria associated with abnormal high plasma homocysteine and methionine concentrations. These patients represent a very small proportion of the population (approximately 1 of 200,000 individuals in the world) and have very severe hyperhomocysteinemia that can reach 400 $\mu\text{mol/L}$. In patients with residual CBS activity (between 0% and 2% of normal), approximately half of the cases of homozygous homocystinuria, pyridoxine treatment can decrease the hyperhomocysteinemia. Treatment by folates and betaine combined with a low-methionine diet is effective in vitamin B₆ nonresponders (Franken et al, 1994). However, betaine treatment may have some disadvantages because of an increase in methionine that is not found with folic acid treatment. Thirty-three point mutations of the CBS gene, which is located on chromosome 21, have been identified. Among these mutations, the G919A transition, leading to substitution of guanine by adenine in position 919, frequently encountered in the Irish population, is observed in subjects who are refractory to vitamin B₆ treatment. The T833C mutation, frequent in the German population, is observed in vitamin B₆ responders. People who are heterozygous for CBS deficiency (between 1 in 70 to 1 in 200 in the general population) have normal homocysteine levels in 30% to 50% of cases. An oral methionine-loading test (100 mg/kg of body weight) is currently used to identify heterozygous patients who have normal fasting homocysteine levels. The methionine loading results in an abnormal increase in homocysteinemia, revealing a defect in the transsulfuration pathway, which is attenuated by folic acid and/or pyridoxine supplementation. Because basal homocysteine levels may affect the increase in postmethionine-load homocysteine in patients with abnormal basal homocysteinemia, the net increase in postmethionine-load homocysteine above fasting values should be taken into account in the global assessment of CBS defects. Indeed, recent work with transgenic mice suggests that folate deficiency may predispose heterozygotes for the CBS defect to basal hyperhomocysteinemia (Lentz et al, 2000).

Alteration of the Remethylation Pathway. Rare forms of homocystinuria resulting from alterations of the

Table 1. Causes of Hyperhomocysteinemia

Genetic factors	Relationship
MTHFR defect (activity < 20%)	Homozygous thermostable mutations (rare), severe basal hyperhomocysteinemia under fasting conditions
MTHFR deficiency (activity < 50%)	Homozygous thermolabile C677T mutation (10%), moderate basal hyperhomocysteinemia
CBS defect (activity < 2%)	Homozygotes (rare), severe basal hyperhomocysteinemia
CBS deficiency	Heterozygotes (0.5–1.5%), abnormal postmethionine-load increase of homocysteine above fasting values
Functional methionine synthase deficiency	Altered cobalamin metabolism (rare mutations), intermediate to severe basal hyperhomocysteinemia
Nutritional factors	Relationship
Vitamin B ₆ deficiency	Abnormal postmethionine-load increment of homocysteinemia
Vitamin B ₁₂ deficiency	Moderate to intermediate basal hyperhomocysteinemia
Folate deficiency	Moderate to intermediate basal hyperhomocysteinemia
Excess of methionine-rich animal proteins	Moderate to intermediate basal hyperhomocysteinemia and postmethionine-load hyperhomocysteinemia?
Physiologic factors	Relationship
Aging	Plasma homocysteine levels increase with age
Male sex	Men have higher basal homocysteinemia than women
Menopause	Increased homocysteine levels in postmenopausal women that could be reduced by hormone replacement therapy
Race	Black South-Africans have lower homocysteine levels than the White population
Pathologic factors	Relationship
Tobacco smoking	Altered folate metabolism and vitamin B ₆ deficiency?
Alcoholism	Altered folate metabolism?
Decreased renal function	Reduced renal clearance of homocysteine
Psoriasis	Defect of intracellular homocysteine use?
Cancer	Defect of intracellular homocysteine use?
Cardiac or renal transplantations	Folate and vitamin B ₆ deficiency and reduced renal clearance of homocysteine
Iatrogenic factors	Relationship
Oral contraceptives	Altered folate metabolism and vitamin B ₆ deficiency?
Methotrexate	Dihydrofolate reductase inhibition
Nitrous oxide (N ₂ O)	Methionine synthase inhibition
Anticonvulsants	Altered folate metabolism
Sulfasalazine	Inhibition of folate absorption

remethylation pathway have been described. They can be secondary to a reduced MTHFR activity (< 20%) leading to severe hyperhomocysteinemia associated with a reduction of the plasma methionine concentration, or to a functional deficiency in methionine synthase caused by genetic impairment of cobalamin metabolism responsible for intermediate hyperhomocysteinemia combined with hypomethioninemia (Fenton and Rosenberg, 1995; Rosenblatt, 1995). Nine thermostable mutations of the MTHFR gene, located on chromosome 1, inherited as an autosomal recessive trait, could be responsible for its reduced activity. The two main mutations are the C599T and the G482A transitions. Hyperhomocysteinemia is only observed in the homozygous state. No vitamin treatment has

been effective in these patients, although their hyperhomocysteinemia is less severe than in CBS deficient homozygotes. However, treatment with methionine (sometimes with a supplement of betaine) and folic acid or 5-methylTHF is sometimes successful in reducing the observed hyperhomocysteinemia.

Five known mutations affect methylcobalamin synthesis, an essential cofactor of methionine synthase (Cbl E and G; Cbl C, D, and F) (Allen et al, 1993; Banerjee and Matthews, 1990; Fenton and Rosenberg, 1995; Rosenblatt, 1995). These mutations induce a functional deficiency in methionine synthase that leads to intermediate hyperhomocysteinemia and hypomethioninemia. The functional deficiency depends on mutations in the methionine synthase gene

(patients of the Cbl G complementation group) or in the gene encoding methionine synthase reductase, an enzyme involved in the reductive activation of methionine synthase (patients of the Cbl E group) (Leclerc et al, 1998). A large excretion of methylmalonic acid is observed when adenosinecobalamin synthesis is also affected (patients of the Cbl C, D, and F groups). Similar to the thermostable mutations of MTHFR, a treatment with methionine and/or betaine should be effective in patients bearing these genetic abnormalities.

The C677T mutation of the MTHFR gene, which leads to the synthesis of a thermolabile form of MTHFR that is responsible for 50% of the MTHFR activity, has a homozygosity prevalence, which may vary between 1.4% and 15% in the population, according to geographic area (D'Angelo and Selhub, 1997; Frosst et al, 1995; Kang et al, 1993; Kluijtmans et al, 1996). In homozygous individuals, this autosomal recessive mutation provokes a moderate hyperhomocysteinemia that can be treated with a folic acid supplement. Because no elevation of blood homocysteine has been observed when plasma folate concentrations are greater than 15.4 nmol/L (Jacques et al, 1996), folate deficiency could be partially responsible for the expression of the MTHFR thermolabile genotype. However, as discussed below (see "Genetic and/or Nutritional Factor-Mediated Hyperhomocysteinemia and Incidence of Cardiovascular Disease"), the hyperhomocysteinemia observed in homozygous patients for this mutation of the MTHFR gene might merely result from a greater folate need (Jacques et al, 1996; Molloy et al, 1997).

Nutritional Factors

Numerous studies conducted in humans and in animals have shown that dietary folate or cobalamin deficiencies induce a moderate or intermediate elevation of homocysteine under basal conditions (P. Durand, Ph.D. thesis, Université de Bourgogne, Dijon, France) (Durand et al, 1996b, 1997b; Kang et al, 1992; Miller et al, 1994a, 1994b; Ueland and Refsum, 1989). A restricted intake of methionine could accentuate the reduction of homocysteine catabolism dependent on these deficiencies by inducing a reduction in SAM tissue concentrations and, as a consequence, could result in a higher increase in plasma homocysteine. In accordance with this hypothesis, dietary protein intake is inversely related to serum homocysteine concentrations in an elderly population (mean age: 64 years), who might have had a suboptimal folate status (Stolzenberg-Solomon et al, 1999). However, only a severe and prolonged deficiency in vitamin B₆ could have affect basal homocysteinemia (Brattström, 1996). In contrast to deficiencies in cobalamin or folates, vitamin B₆ deficiency leads to an abnormal increment in plasma homocysteine after an oral methionine load (Miller et al, 1994a). A dietary excess of methionine, which decreases the mobilization of the intracellular folate pool in the homocysteine remethylation pathway, would therefore accentuate the vita-

min B₆ deficiency-dependent increment in post-methionine-load plasma homocysteine levels.

Additionally, dietary excess in methionine-rich animal protein leads to an elevation of circulating homocysteine (Brattström, 1996; Finkelstein, 1990; Ueland and Refsum, 1989). This hyperhomocysteinemia, which may result from a saturation of homocysteine catabolism, can be detected both in the fasting state and after an oral methionine load only when tissue folate and cobalamin stores are intact. Because this methionine-induced hyperhomocysteinemia is masked when the metabolism of folate or cobalamin is impaired, its occurrence has probably been underestimated.

Although a recent interventional study did not find evidence that short-term fluctuations in dietary methionine intake affect fasting plasma homocysteine levels in healthy men (Ward et al, 2000), a reduced folate intake could have a central role in hyperhomocysteinemia. The results of a randomized, controlled interventional study showed that modification of the dietary content in fruits, vegetables, dairy products, and saturated and total fats had significant effects on fasting homocysteine levels that were related to changes in the serum folate status but not to changes in the serum vitamins B₁₂ and B₆, after only 3 weeks (Appel et al, 2000).

Aging, Gender, Lifestyle Factors, and Other Causes

An interplay between inherited and/or acquired factors and iatrogenic factors and pathophysiologic conditions interfere with homocysteine metabolism. Under normal physiologic conditions, aging gradually increases homocysteine blood concentrations. Additionally, men have 25% higher plasma homocysteine levels than premenopausal women (Jacobsen et al, 1994; Lussier-Cacan et al, 1996). This might be partially explained by gender differences in the transsulfuration and remethylation of homocysteine, with a more efficient remethylation in women than in men (Fukagawa et al, 2000), who may have a more efficient transsulfuration (Silberberg et al, 1997). Hormone replacement therapy attenuates the increase in plasma homocysteine observed after menopause (Andersson et al, 1992; Mijatovic et al, 1998; van der Mooren et al, 1994). Black South Africans have lower homocysteine levels than white South Africans consuming the same diet, this resistance may be genetic in origin (Ubbink et al, 1995). Some pathologic situations can also be responsible for high circulating homocysteine levels. The increased plasma homocysteine observed in subjects with chronic renal failure is correlated with creatinine and may be related to reduced plasma homocysteine clearance (Chauveau et al, 1993; Guttormsen et al, 1997). Hyperhomocysteinemia has also been described in severe psoriasis and with different types of cancer, such as lymphoblastic leukemia (Ueland and Refsum, 1989). This latter hyperhomocysteinemia might be a result of the inability of germinal and malignant cells, auxotroph for methionine, to use

intracellular homocysteine. Indeed, proliferating cells export more homocysteine than quiescent cells.

Numerous drugs can generate hyperhomocysteinemia (Ueland and Refsum, 1989). Synthetic hormonal contraceptives; anti-carcinogenic agents such as methotrexate; sulfasalazine, used for treatment of intestinal inflammatory disorders; and anticonvulsants given for epilepsy, such as carbamazepine and phenytoin, can induce hyperhomocysteinemia secondary to folate metabolism impairment. In addition to the increase in folate requirement, the oral contraceptive-dependent hyperhomocysteinemia can result from an altered vitamin B₆ metabolism. Nitrous oxide (N₂O), an anesthetic gas that inhibits methionine synthase activity, also induces hyperhomocysteinemia (Ermens et al, 1991; Frontiera et al, 1994). Finally, cardiac and renal transplantation generate hyperhomocysteinemia that might depend on the onset of folate or vitamin B₆ deficiency combined with the use of immunosuppressive agents such as cyclosporine, which may interfere with the renal clearance of plasma homocysteine (Bostom et al, 1997).

Moreover, various studies indicate that unhealthy lifestyles are associated with hyperhomocysteinemia. Low folate intake, tobacco smoking, and alcohol excess lead to hyperhomocysteinemia, probably by altering folate metabolism and by depleting vitamin B₆ stores (Cravo et al, 1996; Nygård et al, 1995, 1998; Piyathilake et al, 1994). Additionally, heavy coffee consumption increases homocysteine levels whereas tea, vitamin supplements, vegetable and fruit intakes, and physical activity are inversely related to circulating homocysteine (Appel et al, 2000; Broekmans et al, 2000; Brouwer et al, 1999; Nygård et al, 1997b, 1998; Rasmussen et al, 2000).

Detection and Classification of Hyperhomocysteinemias

Assays of Plasma Homocysteine

Total plasma homocysteine concentration is approximately 10 $\mu\text{mol/L}$. Seventy percent to 80% of circulating homocysteine is bound to plasma protein, particularly to serum albumin, which has been correlated with total plasma homocysteine levels (Lussier-Cacan et al, 1996). Free homocysteine (nonprotein bound, 20% of total), is found mainly in the oxidized form and includes homocystine and mixed disulfides such as homocysteine-cysteine. A low proportion of homocysteine is present in its reduced form (2% to 5%).

Some precautions are necessary in assaying homocysteine. First, blood samples should be taken after a 12-hour fast. To avoid the production and the release of homocysteine by erythrocytes, blood samples should be placed on ice immediately after being drawn, and the plasma should be rapidly separated by cold centrifugation (less than 4 hours after sampling). Recent data indicate that acidic citrate can be conveniently used as an anticoagulant instead of EDTA to stabilize blood samples that

can then be kept at room temperature before plasma separation (O'Broin et al, 2000; Willems et al, 1998). To assay free homocysteine, plasma deproteinization should be performed before sample storage because it is rapidly bound to plasma proteins *ex vivo*. To skip this step, eg, in clinical and epidemiologic studies, one may measure total homocysteine. Sample storage is not problematic because plasma homocysteine is stable for years when frozen (-20°C), for a few weeks at 0°C to 2°C , and for 4 days at ambient temperature (Ueland et al, 1993). Assay methods include radioenzymology, gas chromatography coupled to mass spectrometry, capillary electrophoresis, and HPLC with electrochemical or fluorescence detection. Sensitive and reproducible measurements can be obtained by HPLC with the help of the rapidly eluted internal standard, acetylcysteine (Durand et al, 1996a). Chromatographic separation of cysteine, homocysteine, cysteinylglycine, glutathione, and acetylcysteine has been performed in as little as 11.4 minutes (Fig. 2). A commercial immunoenzym-

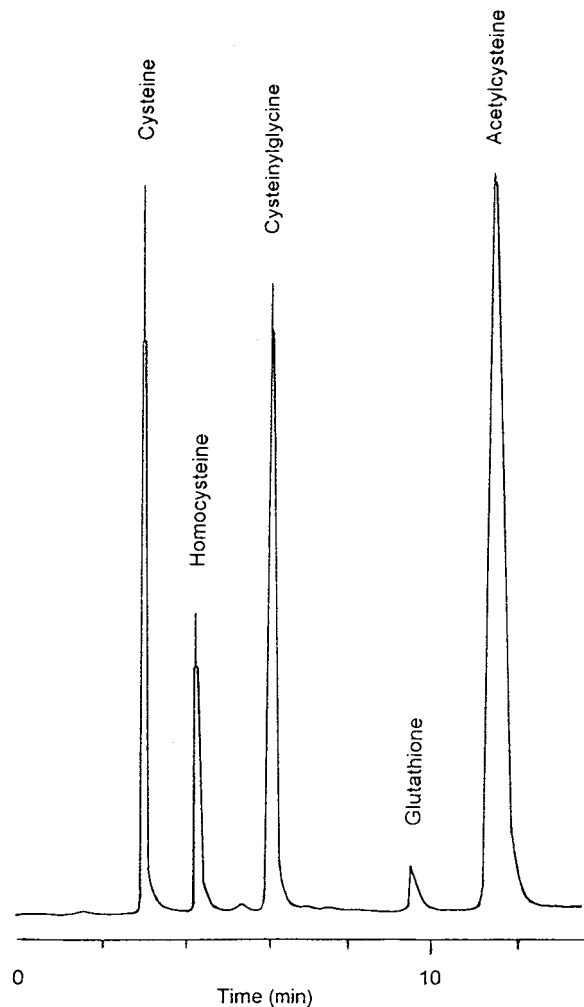


Figure 2.

Chromatogram of HPLC analysis of human plasma aminothiols (Durand et al, 1996a). Homocysteine was calculated as 32 $\mu\text{mol/L}$ with acetylcysteine as internal standard after an injection of 15 μl of the derivatized sample.

metric assay, which uses fluorescence polarization, recently has become available (IMX System; Abbott Diagnostic, Abbott Park, Illinois) (Shipchandler and Moore, 1995). This fully automated assay, which, similar to the Bio-Rad enzyme-linked immunoassay microtiter method, has a high correlation with HPLC results may be useful as a reference method for multicenter population studies (Donnelly and Pronovost, 2000; Yu et al, 2000).

Methionine-Loading Test

The methionine-loading test is used to reveal additional abnormalities of homocysteine metabolism not detected by the homocysteine concentration measurement obtained in the fasting state. In normal subjects, an oral administration of methionine (100 mg/kg of body weight) induces a transient increase in plasma homocysteine (up to 27 $\mu\text{mol/L}$), which peaks between 4 and 8 hours after administration. In normohomocysteinemic patients bearing transsulfuration defects, this overload results in an abnormal increase in circulating homocysteine ($>38 \mu\text{mol/L}$, according to Graham et al, 1997). Nevertheless, as discussed above, an abnormal postmethionine-load homocysteine elevation above fasting levels must be taken into account in the general identification of patients bearing transsulfuration defects, such as vitamin B₆-deficient subjects or heterozygotes for CBS deficiency.

Although the postmethionine-load increment is most sensitive to the transsulfuration pathway, the response might also be abnormal in subjects bearing remethylation defects (Cattaneo, 1999; De Jong et al, 1999a). Because folate deficiency may predispose to hyperhomocysteinemia in heterozygotes for the CBS defect, transsulfuration defects might be combined with remethylation defects in patients with basal hyperhomocysteinemia (Lentz et al, 2000). When tissue folate and cobalamin stores are intact, an abnormal increment of postmethionine-load homocysteinemia may also reveal a saturation of the transsulfuration pathway caused by dietary methionine excess that might be combined with transsulfuration defects (in cases with basal hyperhomocysteinemia) or with the homozygous thermolabile MTHFR defect (in cases with normal fasting homocysteine) (Cattaneo, 1999). The methionine overload would tend to decrease basal hyperhomocysteinemia rather than cause a further increase in basal hyperhomocysteinemia in patients bearing remethylation defects such as folate deficiency (associated or not with the C677T mutation of the MTHFR gene) (Durand et al, 1997a; Miller et al, 1994a, 1994b; Selhub and Miller, 1992). Conversely, higher folate and cobalamin statuses are associated with greater percentage rises in postload homocysteine after adjustments for age and sex (Silberberg et al, 1997). Population studies taking into account basal, absolute, and net increases of postmethionine-load homocysteine levels, in relation to dietary intake and methionine, folate, and status of vitamins B₆ and B₁₂ to confirm the involvement of methionine excess

in both basal and postload hyperhomocysteinemia. The postload sampling test does burden biomedical protocols, however, this test was shortened to 2 hours in a recent study (Bostom et al, 1995b).

Classification of Hyperhomocysteinemia

Kang and coworkers classified several types of hyperhomocysteinemia in relation to total plasma homocysteine concentrations (Kang et al, 1992). They defined hyperhomocysteinemia as severe for concentrations higher than 100 $\mu\text{mol/L}$, intermediate for concentrations between 30 and 100 $\mu\text{mol/L}$, and moderate for concentrations of 15 to 30 $\mu\text{mol/L}$, and a reference total plasma homocysteine range as 5 to 15 $\mu\text{mol/L}$ (mean, 10 $\mu\text{mol/L}$). Because of the influences of age, gender, and unhealthy lifestyle factors such as tobacco smoking, alcohol abuse, inadequate dietary habits, and lack of physical activity, there is no consensus on what levels constitute hyperhomocysteinemia at the present time. Some studies have tentatively determined the relationship between plasma homocysteine and the incidence of cardiovascular disease. A higher incidence is observed with concentrations above 16 $\mu\text{mol/L}$ (Selhub et al, 1993; Stampfer et al, 1992; Ubbink, 1994). However, more recent data show that fasting hyperhomocysteinemia defined with a lower cut-off value (12 $\mu\text{mol/L}$) is associated with increased odds ratios for atherosclerotic vascular diseases (see "Hyperhomocysteinemia and Vascular Pathologies") (Graham et al, 1997). Using the methionine-loading test, a postload homocysteine level above 38 $\mu\text{mol/L}$ and a postload increment above 27 $\mu\text{mol/L}$ increases the cardiovascular risk (Graham et al, 1997). Additionally, a recent study showed a low cardiovascular risk for postload concentrations less than 23 $\mu\text{mol/L}$ and an increased risk above 34 $\mu\text{mol/L}$ (Reis et al, 1995). The decreased cardiovascular risk might be explained by a reduced dietary methionine supply in subjects with intact vitamin stores.

However, based on the coordinated regulation of homocysteine metabolism by SAM, three main types of moderate to intermediate hyperhomocysteinemias can be proposed. They may result from: 1) an inhibition of the homocysteine remethylation pathway that is a consequence of folate or vitamin B₁₂ deficiencies, or the presence of the thermolabile MTHFR mutation or a functional deficiency in methionine synthase; 2) an inhibition of the homocysteine transsulfuration pathway caused by vitamin B₆ deficiency or a partial congenital CBS defect; and 3) a saturation of the transsulfuration pathway related to a dietary excess in methionine in cases of adequate folate and cobalamin intake. Nutritional folate deficiency combined more or less with the C677T MTHFR mutation may play an important role in basal hyperhomocysteinemia, whereas vitamin B₆ deficiency and heterozygosity for a CBS defect may only contribute to abnormal increment of postmethionine-load homocysteine levels (Ubbink et al, 1996). However, the true contribution of di-

etary methionine excess, which hasn't been specifically investigated in large-scale epidemiologic studies, may represent an alternative cause of moderate hyperhomocysteinemia.

Hyperhomocysteinemia and Vascular Pathologies

Hyperhomocysteinemia: An Expected Independent Risk Factor for Cardiovascular Disease. Background and Summary of Retrospective Studies

Homocystinuria caused by congenital CBS and MTHFR defects or by genetic alteration of cobalamin metabolism is characterized by clinical problems including frequent arterial and venous thromboembolism and premature atherosclerosis (McCully, 1983; Mudd et al, 1995). Based on this observation, it is proposed that severe hyperhomocysteinemia plays a role in the etiology of cardiovascular disease. The pioneer work of Wilcken and Wilcken (1976) showed an abnormal increase in homocysteine after an oral methionine load in patients with coronary artery disease. A prevalence of partial CBS and MTHFR inherited defects is observed in patients suffering from cardiovascular disease (Clarke et al, 1991; Kang et al, 1993). Numerous retrospective case-controlled (Brattström et al, 1992; Mansoor et al, 1995; Pancharuniti et al, 1994; Verhoef et al, 1994) and some prospective nested case-controlled studies (Arnesen et al, 1995; den Heijer et al, 1996; Stampfer et al, 1992) established the link between hyperhomocysteinemia and occlusive coronary artery (Arnesen et al, 1995; Pancharuniti et al, 1994; Stampfer et al, 1992), cerebrovascular (Brattström et al, 1992; Verhoef et al, 1994), and peripheral vascular (Mansoor et al, 1995) diseases, as well as venous thrombosis (den Heijer et al, 1996) (also reviewed in Refsum et al, 1998). Because this relationship persists after statistical adjustments for conventional risk factors, hyperhomocysteinemia seems to be an independent risk factor for cardiovascular disease.

Using the data from 19 case-controlled studies, 5 cross-sectional studies, and 3 prospective studies published before 1995, the meta-analysis of Boushey and colleagues (Boushey et al, 1995) relating basal hyperhomocysteinemia to atherosclerotic vascular disease indicates that, above 10 $\mu\text{mol/L}$, a 5 $\mu\text{mol/L}$ increment in circulating homocysteine is associated with a significant increase in the risk of coronary artery disease (60% in men and 80% in women). This increment was also associated with a 50% and 6.8-fold increase in the risk of cerebrovascular and peripheral arterial diseases, respectively. This study clearly documented that a 5 $\mu\text{mol/L}$ increment in homocysteine is equivalent to a 0.5 mmol/L increment (20 mg/dL) in plasma cholesterol for the increase in the odds for myocardial infarction.

The importance of moderate hyperhomocysteinemia, and of other conventional risk factors such as age and sex, on the incidence of vascular pathologies are stressed in a recent case-controlled study conducted

in nine European centers (Graham et al, 1997). From these data, based on low cut-off levels to define hyperhomocysteinemia, it was estimated that the cardiovascular risk associated with hyperhomocysteinemia detected both under basal conditions ($>12 \mu\text{mol/L}$) and after a methionine load (absolute increase greater than 38 $\mu\text{mol/L}$ and net increase greater than 27 $\mu\text{mol/L}$) is similar to that of hyperlipidemia or smoking, although lower than that of hypertension. This study also confirmed the usefulness of the methionine-loading test to identify all of the subjects bearing an hyperhomocysteinemia-dependent cardiovascular risk. Furthermore, because fasting values affect the increase in postmethionine-load homocysteine, the percent rise of homocysteine above fasting should be taken into account when assessing the cardiovascular risk.

Hyperhomocysteinemia: An Expected Causal Risk Factor for Atherothrombosis. Overview of Prospective and Experimental Studies

Despite the strong and independent relationship between homocysteine and cardiovascular disease found in the great number of retrospective case-controlled studies mentioned above, prospective cohort studies, which generally provide the most rigorous evidence, have yielded inconclusive results. Whereas several prospective studies provided evidence that elevated blood homocysteine concentration is a major independent causative risk factor for atherothrombotic disease (Arnesen et al, 1995; Boston et al, 1997; Moustapha et al, 1998; Nygård et al, 1997a; Perry et al, 1995; Petri et al, 1996; Stampfer et al, 1992), some prospective studies did not find a statistically significant association between elevated homocysteine levels and the incidence of cardiovascular disease, after adjustment for conventional risk factors (Alfthan et al, 1994; Evans et al, 1997; Folsom et al, 1998; Kuller and Evans, 1998; Verhoef et al, 1994). These equivocal results might be explained by the sample size, the ethnic background, and the lifestyle of studied subjects; or by the selection of the population-based cohort, especially the choice of exclusion criteria. There may also have been problems with sample storage over long periods, especially for plasma homocysteine and vitamin analyses. Asymptomatic control and case subjects and multivitamin users should be excluded on study entry. Moreover, reference methods for homocysteine and vitamin assays should be employed in these population studies.

Unfortunately, the Atherosclerosis Risk in Communities negative prospective study did not provide detailed information about a potential selection bias caused by vitamin supplement use (Folsom et al, 1998; Kuller and Evans, 1998). Nevertheless, because of the large population heterogeneity among the few published prospective studies, the reason for the apparent variation in results is unknown. The following arguments weigh in favor of the positive studies. First, as mentioned above, many retrospective case-controlled studies show a clear-cut association be-

tween elevated plasma homocysteine and atherothrombotic disease. Moreover, although one can expect that elevated homocysteine levels result from the vascular disease per se (Kuller and Evans, 1998), the cardiovascular risk is gradually related to plasma homocysteine in many studies (Hopkins et al, 1995; Nygård et al, 1997a; Pancharuniti et al, 1994; Stampfer et al, 1992). Recently, Wald et al (1998) estimated the risk of ischemic heart disease (angina pectoris or myocardial infarction) from eight retrospective studies and from their own positive prospective study, the British United Provident Association study, as a function of homocysteine level. Regarding the results of all of these studies, they found a dose-response relationship between serum homocysteine and the odds ratio of ischemic heart disease, supporting the idea that homocysteine is a causal risk factor for cardiovascular disease. Second, several studies reported an association between elevated circulating homocysteine and mortality in patients with coronary heart disease independent of conventional risk factors (Alfthan et al, 1997; Anderson et al, 2000; Christen et al, 2000; Hoogeveen et al, 2000; Nygård et al, 1997a; Cleophas et al, 2000; Stehouwer et al, 1999). This suggests that increased homocysteine levels predict mortality by contributing to acute thromboembolic events leading to death. The results of cross-sectional studies of subclinical vascular disease show that higher homocysteine concentrations are associated with greater carotid artery intimal-medial wall thickness (Malinow et al, 1993; Selhub et al, 1995; Willinek et al, 2000). It has also been reported that homocysteine level is related to the extent of atherosclerosis in coronary and peripheral arteries (Chao et al, 1999b; van den Berg et al, 1996; Verhoef et al, 1997b; von Eckardstein et al, 1994). Third, as we will detail below (see "Potential Pathogenic Mechanisms of Hyperhomocysteinemia"), our own work (Durand et al, 1997a) and the work of Lentz et al (1996) and Bellamy et al (1998) give experimental evidence that moderate hyperhomocysteinemia, which leads to vascular dysfunction, might increase the cardiovascular risk independently of other risk factors.

However, observational studies cannot exclude the possibility that elevated homocysteine levels may be associated with confounding variables such as unhealthy lifestyles and other established risk factors, rather than being causally related to vascular disease. Recent investigations indicate that, rather than homocysteine, unhealthy lifestyle factors might be responsible for the increased risk of cardiovascular disease; most positive observational studies have not been adjusted for lifestyle factors. These investigations also suggest that the role of homocysteine might be overcome by other risk factors (eg, hypertension, diabetes, and dyslipidemia) and/or by various protective mechanisms (such as antioxidant intakes and intrinsic antioxidant systems). Thus, homocysteine might be less important in the long-term progression and development of coronary artery disease (Cleophas et al, 2000; Omland et al, 2000).

Nevertheless, results from recent interventional studies favor a causal role of homocysteine in the onset of cardiovascular disease. Indeed, vitamin therapy (folic acid, pyridoxine plus cyanocobalamin) reportedly retards the progression of carotid plaques in patients with plasma homocysteine above and below 14 $\mu\text{mol/L}$ (Hackam et al, 2000). Additionally, data from a prospective cohort study of 273 patients with premature peripheral arterial occlusive disease showed the same incidence of events in the normohomocysteinemic and vitamin-treated (folic acid plus pyridoxine) hyperhomocysteinemic groups (De Jong et al, 1999b). Consistent with a protective effect of vitamin therapy, higher plasma homocysteine levels were associated with an increased risk of new cardiovascular events in normohomocysteinemic but not in vitamin-treated hyperhomocysteinemic subjects. Another nonrandomized study also suggests that a homocysteine-lowering treatment would decrease the risk of atherothrombotic disease (Peterson and Spence, 1998). Furthermore, a 2-year, double-blind, randomized, placebo-controlled therapeutic trial in 158 healthy siblings of hyperhomocysteinemic patients with premature atherothrombotic disease folic acid plus vitamin B₆ treatment was associated with a decreased occurrence of abnormal exercise electrocardiography tests, which is consistent with a decreased risk of atherosclerotic events (Vermeulen et al, 2000). Additional experimental and large-scale clinical designs with optimal dose and duration of homocysteine-lowering treatment are needed to definitively prove that homocysteine is responsible for the increased risk of cardiovascular disease, mostly of atherosclerosis.

Hyperhomocysteinemia: An Independent Risk Factor for Thrombosis

Recent studies underline the major role of moderate hyperhomocysteinemia on the odds of developing both arterial and venous thromboembolisms (Boers, 1997a; Cattaneo, 1999; D'Angelo and Selhub, 1997). This agrees with interventional (De Jong et al, 1999b; Peterson and Spence, 1998; Vermeulen et al, 2000) and experimental (Bellamy et al, 1998; Durand et al, 1996b, 1997a; Lentz et al, 1996) investigations that suggest a causal relationship between elevated homocysteine levels and vascular spasm and thrombosis and with cross-sectional studies, which suggest that elevated homocysteine levels contributed to acute fatal thromboembolic events (Alfthan et al, 1997; Nygård et al, 1997a). Hyperhomocysteinemia, when detected under basal conditions, was found to be related to recurrent (den Heijer et al, 1995) and/or premature (Falcon et al, 1994) venous thrombosis in subjects less than 40 years old. Hyperhomocysteinemia was also associated with both arterial and venous premature thromboembolic events in subjects less than 45 years old (D'Angelo and Selhub, 1997; Fermo et al, 1995). The prevalence of moderate hyperhomocysteinemia in patients with early onset venous and arterial occlusive disease was almost twice as high

when hyperhomocysteinemia was detected by the methionine-loading test as when detected in the fasting state (Fermo et al, 1995). Additionally, high plasma homocysteine levels are a risk for deep venous thrombosis in the general population (den Heijer et al, 1996). This relationship is stronger in women than in men (den Heijer et al, 1996). Although postload homocysteine measurements were not carried out, the stronger relationship in women might be partially caused by a more efficient transsulfuration in men, who present a lower response to the methionine overload than women at age-specific ranges (Silberberg et al, 1997).

Recently, despite a lack of significant association between hyperhomocysteinemia and the risk of venous thromboembolism, in a prospective study of patients with systemic lupus erythematosus, Petri et al (1996) report that hyperhomocysteinemia, defined as total plasma homocysteine concentrations higher than 14.1 $\mu\text{mol/L}$, is significantly associated with arterial thrombotic events (odds ratio 3.74), and stroke (odds ratio 2.24). Additionally, they point out that hyperhomocysteinemia remains an independent risk factor for thrombosis and stroke after adjustment for established risk factors. Indeed, the increased thrombotic risk caused by hyperhomocysteinemia, recently reviewed by D'Angelo and Selhub (1997), seems to be independent of the conventional risk factors including protein C, protein S, and antithrombin III deficiencies; and independent of the mutation in the gene encoding for factor V. This mutation results in resistance to activation by protein C (factor V Leiden), which, in turn, potentiates the thrombotic risk (D'Angelo and Selhub, 1997; Ridker et al, 1997).

Genetic and/or Nutritional Factor-Mediated Hyperhomocysteinemia and Incidence of Cardiovascular Disease

In keeping with the occurrence of three major mechanisms leading to moderate hyperhomocysteinemia, Graham et al (1997) distinguish three main types of hyperhomocysteinemia using the methionine-loading test. First, hyperhomocysteinemia detected only in the fasting state (approximately 23% of patients), which might mainly result from an inhibition of the homocysteine remethylation pathway (folate, cobalamin, or functional methionine synthase deficiencies or thermolabile MTHFR mutation), and which increases the cardiovascular risk 1.6-fold. Second, hyperhomocysteinemia detected only by the oral methionine-loading test (approximately 27% of patients), which might mainly result from an inhibition of the transsulfuration pathway (vitamin B₆ deficiency or heterozygous CBS defect), and which increases the risk 1.5-fold. Third, hyperhomocysteinemia detected both under basal conditions and after a methionine challenge (approximately 50% of patients), which might be partially explained by a saturation of the transsulfuration pathway caused by dietary methionine excess. This last type is associated with an odds ratio for atherosclerotic vascular disease of 2.5. Both animal and human data on dietary methionine and cardiovascular dis-

ease risk are too sparse to establish a methionine-excess involvement in atherothrombotic disease. Nevertheless, methionine-loading experiments suggest that methionine excess induced vasospasms and thrombosis (Bellamy et al, 1998; Durand et al, 1997a; Lentz et al, 1996) (also see additional references cited in Durand et al, 1997a).

Although the contribution of dietary methionine excess (which increases folate, vitamin B₆, and vitamin B₁₂ requirements) has not been clinically assessed, folate deficiency (associated or not with the thermolabile MTHFR mutation) and vitamin B₆ deficiency (associated or not with heterozygosity for CBS defects) could largely account for this increased risk of hyperhomocysteinemia-related cardiovascular disease. A large prospective study reported that folate and vitamin B₆ intakes are correlated negatively with homocysteine levels and with the incidence of coronary artery disease (The Nurses' Health Study) (Rimm et al, 1998). A great number of epidemiologic studies also show that low plasma and erythrocyte folate levels (Morrison et al, 1996; Pancharuniti et al, 1994; Robinson et al, 1998; Selhub et al, 1993; Verhoef et al, 1996) and low plasma vitamin B₆ concentrations (Robinson et al, 1995, 1998; Selhub et al, 1993; Verhoef et al, 1996) increase the cardiovascular risk.

In particular, a reduced folate intake would be an important determinant of carotid stenosis related to hyperhomocysteinemia (Selhub et al, 1995), and a decrease in plasma folate concentration below 13.6 nmol/L would be associated with a gradual increase in the risk of coronary artery disease (Morrison et al, 1996; Pancharuniti et al, 1994) and of cerebrovascular disease (Giles et al, 1995). Moreover, folate deficiency might contribute to the increased coronary risk associated with the thermolabile MTHFR mutation (Hopkins et al, 1995; Jacques et al, 1996), and an interaction between vitamin B₆ deficiency and heterozygous CBS defects might be expected. Indeed, the thermolabile MTHFR defect and heterozygosity for CBS deficiency might increase vascular disease risk by respectively increasing the amounts of dietary folate and vitamin B₆ that are needed to prevent hyperhomocysteinemia. This may partially explain why no significant associations have been found between homozygosity for the MTHFR thermolabile defect or heterozygosity for mutations in the CBS gene and cardiovascular risk (Folsom et al, 1998; Ma et al, 1996; Verhoef et al, 1997a). Indeed, the C677T mutation of the MTHFR gene is not unequivocally associated with increased cardiovascular risk. Some studies support the concept that C677T polymorphism in the MTHFR gene increases the risk (Gallagher et al, 1996; Kluijtmans et al, 1996; Ou et al, 1998) but others do not (Adams et al, 1996; Ma et al, 1996; Schmitz et al, 1996; Verhoef et al, 1997a; Wilcken et al, 1996). Nevertheless, the homozygote mutant genotype leads to hyperhomocysteinemia only when plasma folate status is low in healthy subjects and in patients with cardiovascular disease, thus subjects with this MTHFR mutation may be sensitive to low folate intake (Christensen et al, 1997; Hopkins et al, 1995; Jacques et al, 1996; Ma et

al, 1996; Verhoef et al, 1997a). Therefore, with a well selected population that would diminish the 95% confidence interval of relative risk of cardiovascular disease, we suggest that there might be an increased cardiovascular risk in the MTHFR TT mutants when folate status is low. In this hypothesis, the homozygous thermolabile MTHFR mutation would predispose to folate deficiency, which is likely to increase the cardiovascular disease risk by elevating homocysteine levels. Thus, the plasma homocysteine level would be stronger than that of the frequency of this genetic defect to predict the incidence of atherothrombotic disease.

Early studies in vascular patients concluded that heterozygosity for the mutations T833C and G919A of the CBS gene was associated with an abnormal response to a methionine load, which was caused by the heterozygosity (Boers, 1997b; Boers et al, 1985; Clarke et al, 1991; Wilcken and Wilcken, 1976). However, heterozygotes for CBS defects do not seem to have an increased risk for cardiovascular disease, because occurrence of these mutations is scarce in patients with vascular disease (Kluijtmans et al, 1996). Nevertheless, further specific studies are needed to clearly assess the role of CBS polymorphism in atherosclerotic disease, particularly in its interactions with folate deficiency, vitamin B₆ deficiency, and methionine intake. Moreover, other genetic traits, such as the functional deficiencies in cobalamin-dependent methionine synthase described above, could contribute to an increased relative risk of cardiovascular disease, but their respective contributions are not yet determined. Thus, to clarify the interaction among these various inherited and nutritional factors and their relative involvement in cardiovascular disease, complementary prospective trials and experimental studies in transgenic animals should be performed in the future.

Interactions between Hyperhomocysteinemia and the Usual Risk Factors for Cardiovascular Disease

In the Hordaland Homocysteine Study conducted in Norway (16,176 subjects), significant correlations between total plasma homocysteine and plasma cholesterol, blood pressure, tobacco smoking, and lack of physical activity were established (Nygård et al, 1995). Indeed, the European multicenter study coordinated by Graham reports that, whereas basal hyperhomocysteinemia only shows an additive effect with hypercholesterolemia, it has a synergistic effect with both hypertension and smoking on the incidence of atherosclerotic vascular disease (Graham et al, 1997). These interactions were further strengthened when postmethionine-load homocysteine levels were taken into account. Moreover, the hyperhomocysteinemia associated with an increased cardiovascular risk is stronger in non-insulin-dependent diabetes mellitus than in normal subjects (Hoogeveen et al, 1998). Thus, there are pronounced interactive effects with classic risk factors, suggesting that homocysteine might fur-

ther increase the cardiovascular risk in patients bearing these risk factors.

However, circulating homocysteine levels are related to plasma creatinine and uric acid concentrations and to sex and age (Brattström et al, 1994; Lussier-Cacan et al, 1996). The gender differences might be reversed when taking into account data obtained during the oral methionine-loading test (Silberberg et al, 1997). After adjustments for age, creatinine, and serum vitamins (folates and cobalamin), the percentage rise in homocysteine after the methionine load was greater in women than in men. Additionally, a decreased response to oral methionine has been observed in men older than 45 years (Silberberg et al, 1997). Although homocysteine levels increase with age, a reduction in the cardiovascular risk associated with hyperhomocysteinemia also has been reported in subjects older than 45 years (Reis et al, 1995). This negative interaction between age and hyperhomocysteinemia for cardiovascular risk may suggest that moderate hyperhomocysteinemia plays a major role in premature death from vascular pathologies. This negative interaction might be related to the hyperhomocysteinemia-dependent increased thrombotic risk in subjects younger than 46 years and to the low response to the methionine load in older subjects. Therefore, in combination with vitamin status and methionine intake, this negative interaction between age and homocysteinemia might be taken into account in future epidemiologic studies, which should use age- and gender-specific reference ranges. Because the association between homocysteine levels and cardiovascular disease is stronger at younger ages, age differences at the end of a study could partially explain the discrepancy found in prospective studies but not in retrospective studies. Indeed, the stronger relationship between elevated circulating homocysteine levels and atherothrombotic disease observed in retrospective case-controlled studies than in prospective studies might be explained by the younger average age of subjects sustaining a coronary event in retrospective studies (in the positive study by Wald et al (1998), see the age comparison between retrospective and prospective studies).

Potential Pathogenic Mechanisms of Hyperhomocysteinemia

Despite the large amount of epidemiologic data in favor of a relationship between hyperhomocysteinemia and increased risk for cardiovascular disease, the true mechanisms remain hypothetical because of a scarcity of experimental data. However, in agreement with a causal role of moderately elevated circulating homocysteine levels in cardiovascular disease, some putative mechanisms have been identified that may explain the atherothrombogenicity of hyperhomocysteinemia.

Alterations of Vascular Thromboresistance

The pioneer work of Harker et al (1976) suggests that endothelial cell injury associated with reduced platelet

survival is involved in the initiation of vascular disorders induced by experimental hyperhomocysteinemia. Because typical early atherosclerotic lesion formation observed in hyperhomocysteinemic animals can be prevented by an antiplatelet therapy with dipyridamole, the authors concluded that platelet-mediated proliferation of smooth muscle cells is a potential cellular mechanism involved in the atherogenicity of hyperhomocysteinemia. The reported reduced platelet survival has not been confirmed by others in homocystinuric patients, and *in vitro* results of platelet function abnormalities have been controversial (reviewed in Malinow, 1994; Stamler and Slivka, 1996). However, favoring the theory of hyperhomocysteinemia-induced platelet activation, *in vitro* studies have proposed that, besides its direct cytotoxicity that damages endothelial cells (Dudman et al, 1991; Starkebaum and Harlan, 1986; Wall et al, 1980), homocysteine alters endothelium antithrombotic properties (Harpel et al, 1996). Although data concerning the prostacyclin (PGI₂) biosynthesis by endothelial cells incubated with homocysteine are controversial (Graeber et al, 1982; Panganamala et al, 1986; Wang et al, 1993), homocysteine could impair the ability of these cells to inhibit platelet aggregation, probably by reducing nitric oxide (NO) bioavailability (Stamler et al, 1993). Additionally, exposure of monocytes to homocysteine induces tissue factor (TF) expression (Khajuria and Houston, 2000), and exposure of endothelial cells to homocysteine increases TF expression (Fryer et al, 1993), and the activation of factor V to factor Va (Rodgers and Conn, 1990), thus favoring thrombin generation through initiation and propagation of the coagulation cascade. By increasing TF activity, homocysteine would also enhance GPIIb-IIIa/fibrinogen-dependent platelet adhesion to endothelial cells (Dardik et al, 2000). Moreover, homocysteine alters anticoagulant effectors such as the thrombomodulin/protein C system and antithrombin III. Homocysteine may inhibit thrombomodulin expression by altering the thiol redox-regulated intracellular transport, and may inhibit thrombomodulin activity by altering the disulfide-bond-rich motif inside the protein, protein C activity (Lentz, 1997; Lentz and Sadler, 1991, 1993), and thrombin activation of the anticoagulant thrombomodulin/protein C process (Rodgers and Conn, 1990). Additionally, homocysteine would specifically inhibit inactivation of factor Va by activated protein C through homocysteinylation of the cofactor (Undas et al, 2001). The binding of antithrombin III to the endothelial surface is also reduced by homocysteine through inhibition of heparin sulfate expression (Nishinaga et al, 1993). As regards the fibrinolytic process, an impairment of the binding and action of tissue-type plasminogen activator (t-PA) at the endothelial cell surface has been reported in the presence of homocysteine, which would thus inhibit endothelial-dependent fibrinolysis (Hajjar, 1993). It is noteworthy that the disulfide linkage between homocysteine and the cysteine-rich domain of annexin II is responsible for the homocysteine-specific inhibition of t-PA binding to annexin at the cell surface (Hajjar et al, 1998). In

contrast to the secretion and mRNA expression of t-PA, the secretion and gene expression of type 1 plasminogen activation inhibitor in human endothelial and smooth muscle cells is enhanced by homocysteine (Midorikawa et al, 2000). Finally, by increasing the affinity of lipoprotein (a) for fibrin, homocysteine would inhibit the conversion of plasminogen into plasmin on the fibrin surface (Harpel et al, 1992; Jacobsen, 1993).

Taken together, these observations suggest that hyperhomocysteinemia favors thrombogenesis. However, data obtained from most experiments dealing with *in vitro* cell exposure to homocysteine are disappointing because the concentrations required are generally high and the effects generally have not been specific to homocysteine (Jacobsen, 1993). The effects also occurred with cysteine, and thus, could be related to the nonspecific reactivity of the sulfhydryl group of homocysteine (Fryer et al, 1993; Nishinaga et al, 1993; Stamler et al, 1993). To confirm the onset of a prethrombotic state, clinical studies have been performed in hyperhomocysteinemic patients. Some abnormal plasma elevations of hemostatic parameters, including factor VIII (Freyburger et al, 1997), von Willebrand factor (van den Berg et al, 1995), fibrinogen (von Eckardstein et al, 1994), thrombomodulin (van den Berg et al, 1995), thrombin/antithrombin III complex and activated protein C (Cattaneo, 1999; Cattaneo et al, 1998), and type 1 plasminogen activation inhibitor (Freyburger et al, 1997) have been reported. However, the increase of the anticoagulant effectors is inconsistent with a prothrombotic environment. Additionally, contrasting with clinical results, homocysteine would inhibit both von Willebrand factor and thrombomodulin expression in cultured endothelial cells by affecting the redox-thiol status-regulated transport of newly synthesized glycoproteins in the endoplasmic reticulum. Data interpretation is difficult because the observed modifications in hyperhomocysteinemic subjects may merely result from the occurrence of cardiovascular disorders.

More encouraging results derive from recent *in vivo* experiments that provide evidence that hyperhomocysteinemia is responsible for an alteration of vascular thromboresistance. In keeping with the reduced capacity of homocysteine-exposed endothelial cells to inhibit platelet aggregation, Lentz and collaborators demonstrated, using experimental hyperhomocysteinemia (induced by a diet depleted of folate, high in methionine, and choline-free) that the endothelial-dependent vasodilation is impaired in hyperhomocysteinemic primates. Collagen-induced vasoconstriction also increased, probably through a decrease in the vasodilator responsiveness to platelet-generated ADP (Lentz, 1997; Lentz et al, 1996). This alteration of the endothelium-mediated vasorelaxation is also observed in healthy subjects affected with moderate hyperhomocysteinemia (Tawakol et al, 1997; Woo et al, 1997). The crossover study of an oral methionine load by Bellamy et al (1998) also supports the view that elevated homocysteine levels may promote vascular disease by impairing flow-mediated endothelium

dependent vasodilation. Moreover, the decreased thrombomodulin-dependent activation of protein C observed in hyperhomocysteinemic primates suggests that a moderate increase in circulating homocysteine alters endothelial antithrombotic properties (Lentz et al, 1996). Furthermore, recent studies in both laboratory animals and humans increasingly suggest that moderate hyperhomocysteinemia impairs endothelial-dependent vasodilation (Bellamy et al, 1999; Chambers et al, 1999a, 1999b; Kanani et al, 1999; Nappo et al, 1999; Ungvari et al, 1999; Woo et al, 1999). Despite normalization of plasma homocysteine concentration, administration of folic acid, vitamin B₆, and vitamin B₁₂ for 6 months was insufficient to restore normal vascular function in monkeys maintained for 17 months on an atherosclerotic diet that produced both hypercholesterolemia and hyperhomocysteinemia (Lentz et al, 1997). Nevertheless, two placebo-controlled crossover studies demonstrated that 2 months of folate supplementation improves endothelial function in hyperhomocysteinemic subjects by decreasing homocysteine levels (Bellamy et al, 1999; Chambers et al, 1999a, 1999b). Accordingly, administration of folic acid in combination with vitamins B₆ and B₁₂ improved endothelial dysfunction subsequent to hyperhomocysteinemia induced by methionine loading in healthy humans (Chao et al, 1999a).

Supporting the hypothesis of the impact of a moderate elevation of circulating homocysteine on the onset of a hypercoagulant state, we demonstrated that prolonged dietary folate deficiency in rats induced a prothrombotic state characterized by platelet hyperactivity combined with a large increase in macrophage TF activity (Durand et al, 1996b). Additionally, our results suggested that the prothrombotic effects of folate deficiency depend on an increase in circulating homocysteine. Methionine-loading experiments in control and folate-deficient animals indicated that homocysteine plasma concentration correlates with platelet aggregation and the biosynthesis of the powerful proaggregant thromboxane A₂ (Durand et al, 1997a). In agreement with this latter finding, an increase in thromboxane synthesis was described in homocystinuric patients (Di Minno et al, 1993). Along with a possible reduction of the antiplatelet effects of NO, this increase in thromboxane biosynthesis might contribute to platelet activation in moderate hyperhomocysteinemia (Durand et al, 1997a). Studies of the von Willebrand factor and fibrinogen (important vascular determinants of increased platelet aggregation and secretion that may be involved in the development of thrombosis) also favor the theory of platelet hyperactivity. Indeed, plasma homocysteine concentration was correlated with plasma fibrinogen in hyperhomocysteinemic patients with coronary heart disease (von Eckardstein et al, 1994). Furthermore, an increased plasma von Willebrand factor is found in hyperhomocysteinemic subjects bearing premature arterial disease (Freyburger et al, 1997) and, of major relevance, acute methionine loading induced moderate hyperho-

mocysteinemia results in a significant increase in von Willebrand factor (Constant et al, 1999).

These observations suggest that the loss of endothelial cell antithrombotic function combined with the induction of a procoagulant state may contribute to the vascular disorders linked to moderate hyperhomocysteinemia. Furthermore, our own work, together with that of others, emphasizes that a moderate elevation of circulating homocysteine related to impaired homocysteine metabolism (resulting from folate deficiency or dietary methionine excess) might trigger vascular disorders by promoting vascular spasm and thrombosis (Bellamy et al, 1998; Chambers et al, 1999a, 1999b; Durand et al, 1996b, 1997a; Kanani et al, 1999; Lentz et al, 1996; Nappo et al, 1999; Ungvari et al, 1999). Although the molecular role that reduced homocysteine plays in vascular disorders is unclear, experimental evidence indicates that the association between moderately elevated homocysteine levels and atherothrombotic disease is likely to be causal. Therefore, the uncertainty resulting from some prospective studies is partially resolved, because elevated circulating homocysteine is a major independent causative risk factor for cardiovascular disease.

Hypothetical Molecular Mechanisms Underlying Hyperhomocysteinemia-Mediated Vascular Disorders

Hyperhomocysteinemia-Mediated Oxidant Stress through Imbalanced Thiol Redox Status. Numerous studies indicate that oxidant stress plays a role in the development of occlusive atherosclerotic lesions that are observed primarily in homozygotes for the CBS defect (McCully, 1983) and later in experimental hyperhomocysteinemia. Because cellular damage of endothelial cells exposed to homocysteine may be prevented by pretreatment of cells with catalase, which allows the detoxification of hydrogen peroxide that freely crosses the cell membrane (Starkebaum and Harlan, 1986; Wall et al, 1980), an increased oxidant stress may be a main mechanism of homocysteine-induced endothelial cell injury. Although cytotoxicity was not specific to homocysteine, endothelial cell detachment is also observed with cysteine (Dudman et al, 1991), an increase in the hydrogen peroxide-sensitive cellular fluorescent probe, 2',7'-dichlorofluorescein, was observed in cultured endothelial cells exposed to homocysteine (Toborek and Hennig, 1996). Besides the initiation of lipid peroxidation at the cell surface, homocysteine auto-oxidation with trace metal ions, generating reactive oxygen species such as superoxide anion, hydrogen peroxide, hydroxyl, and thiol free radicals (Munday, 1989; Schöneich et al, 1989), would directly oxidize low-density lipoprotein (LDL) (Heinecke et al, 1987; Hirano et al, 1994; Wood and Graham, 1995). Homocysteine would also contribute to the LDL oxidative modifications mediated by endothelial cells, macrophages, and smooth muscle cells (Heinecke et al, 1987; Sparrow and Olszewski, 1993; Wood and Graham, 1995), which are deeply involved in the initial steps of atherogenesis. In agreement with these observations, an increase in the

reduced form of plasma homocysteine associated with an elevation of the oxidation rate of homocysteine and cysteine (expressed as a decreased ratio of plasma reduced to total aminothiols) was reported in experimental hyperhomocysteinemia (Guttormsen et al, 1993; Mansoor et al, 1992, 1993a) and in hyperhomocysteinemic patients (Andersson et al, 1995; Mansoor et al, 1993b, 1994, 1995). This may partially explain why cysteine is reported as a risk factor for cardiovascular disease in hyperlipidemic patients (Jacob et al, 1999), and why cysteine is related to several factors that take part in the cardiovascular risk profile (El Khairy et al, 1999). Indeed, high levels of oxidized and bound cysteine in cardiovascular patients may create an oxidative environment that results in enhanced susceptibility to vascular damage (Mills et al, 2000). Also supporting the hypothesis of involvement of an oxidant stress in hyperhomocysteinemia-mediated vascular disorders, an increase in plasma and aortic thiobarbituric acid reactive substances was related to the occurrence of atherosclerotic lesions in animals fed a methionine-enriched diet (Matthias et al, 1996; Toborek et al, 1995; Toborek and Hennig, 1996). Furthermore, a therapy with dipyrindamole, which is now recognized as a potent antioxidant, can prevent the development of early atherosclerotic intimal lesions (which are composed of proliferating smooth muscle cells surrounded by large amounts of collagen, elastic fibers, and glycosaminoglycans) that are observed in hyperhomocyst(e)inemic baboons (Harker et al, 1976). The increase in circulating homocysteine related to impaired homocysteine metabolism may favor the accretion of a pro-oxidant environment by increasing concentrations of free radicals, by maintaining metal ions in their reduced state suitable for the Fenton reaction, and by changing the redox status of intra- and extracellular thiol groups and, hence, the intracellular redox potential (Mansoor et al, 1993a, 1994, 1995). Indeed, unlike other thiols, homocysteine incubation with endothelial cells induces an increased expression of the stress protein GRP78 and a decreased expression of glutathione peroxidase, a major intracellular antioxidant enzyme, through a mechanism that depends on an alteration of the intracellular redox potential (Kokame et al, 1996; Outinen et al, 1998). In addition to increasing the generation of superoxide anions, hydrogen peroxide, and other peroxides, homocysteine may thus decrease the cellular ability to detoxify peroxides, especially hydrogen peroxide. In light of these intracellular observations (Kokame et al, 1996), we hypothesize that changes in the intracellular redox state associated with elevated homocysteine levels can alter the expression of multiple genes involved in other specific cellular functions that could be partially responsible for vascular disorders associated with moderate hyperhomocysteinemia.

There is abundant evidence that oxidant stress may play an important role in the modification of vascular thromboresistance in moderate hyperhomocysteinemia. Endothelial dysfunction, including decreased endothelial cell-dependent vasorelaxation, is increas-

ingly proposed to be triggered by free radicals generated during auto-oxidation of homocysteine (Loscalzo, 1996). According to the work of Stamler et al, (1993), homocysteine auto-oxidation might prevent thiol nitrosation that, under normal physiologic conditions, sustains the antiplatelet and vasorelaxing activities of NO by increasing the stability of NO (Simon et al, 1993; Stamler et al, 1989). Conversely, this auto-oxidation, which is detrimental to the nitrosothiol formation, contributes to the production of highly reactive peroxy-nitrite resulting from the reaction between superoxide anions and NO. Furthermore, homocysteine-induced inhibition of endothelium-dependent relaxation may also be caused by increased endothelial superoxide anion production (Lang et al, 2000). Moreover, recent work indicates that, unlike cysteine, homocysteine inhibits endothelial and plasma glutathione peroxidase (Chen et al, 2000; Upchurch et al, 1997). In preventing hydrogen peroxide and hydroperoxide detoxification by glutathione peroxidase, homocysteine indirectly contributes to oxygenated reactive species-mediated inactivation of NO. In accordance with this inhibition of vasorelaxation by reactive oxygen species, moderate hyperhomocysteinemia-induced endothelial dysfunction in mice heterozygous for a CBS defect was associated recently with an increased oxidant stress (Eberhardt et al, 2000). Additionally, we demonstrated that the prethrombotic state induced by moderate hyperhomocysteinemia occurs during oxidant stress in rats (Durand et al, 1996b, 1997a). The imbalance of the redox status mediated by folate deficiency or methionine load has been characterized by various indices of oxidation such as increases in end-products of lipid oxidation (conjugated dienes, hydroperoxides, and thiobarbituric acid reactive substances), changes in the fatty acid composition with decreased polyunsaturated fatty acids (mostly n-3 fatty acids), and a lower antioxidant defense, as demonstrated using the free radical-mediated erythrocyte hemolysis test described in Durand and Blache (1996) and in Prost (1992). Additionally, the significant correlations obtained between homocysteine levels and lipid peroxidation parameters suggest that, under our experimental conditions, the observed pro-oxidant effects truly depend on the elevation of circulating homocysteine (Durand et al, 1997a). Moreover, elevated homocysteine levels have been associated with enhanced *in vivo* lipid and protein oxidation in humans, as suggested by the correlation obtained between plasma homocysteine and F₂-isoprostanes, relevant biomarkers of nonenzymatic peroxidation (Ventura et al, 2000; Voutilainen et al, 1999). Supporting a major role of oxidant stress in the thrombogenicity of moderate hyperhomocysteinemia, pretreatment by antioxidants prevents acute methionine load-induced endothelial and platelet dysfunctions in healthy subjects (Chambers et al, 1999; Kanani et al, 1999; Nappo et al, 1999). Impaired homocysteine metabolism-mediated moderate hyperhomocysteinemia (caused by folate deficiency, vitamin B₆ deficiency, or by methionine excess) might thus have primarily pro-oxidant effects

resulting from an altered extra- and intracellular redox potential that can affect essential vascular functions.

Hyperhomocysteinemia-Mediated Homocysteinylation. Inconsistent with the hypothesis of an increased oxidant stress, a decrease in LDL susceptibility to oxidation and in plasma peroxidation indicators was reported in homozygous patients for CBS deficiency (Blom et al, 1992, 1995; Dudman et al, 1993; Jones et al, 1994). Although a low concentration of homocysteine in its reduced form behaves as a pro-oxidant, in high concentration, homocysteine, like other thiols, presents a free-radical scavenger activity (Munday, 1989). This bivalent effect of aminothiols (pro-oxidant and antioxidant), confirmed for in vitro LDL oxidation (Halvorsen et al, 1996), might explain the discrepancies observed between moderate and severe hyperhomocysteinemia. Contrasting with a moderate elevation, a large increase in circulating homocysteine leads to an elevation of the plasma ratio of reduced aminothiols to total aminothiols for homocysteine and cysteine, which favors the occurrence of an antioxidant status (Andersson et al, 1995; Bourdon and Blache, 2001; Ueland et al, 1996). However, Ferguson et al (1999) reported that the reaction between homocysteine thiolactone and LDL generates an LDL adduct that is more resistant to oxidative modification than native LDL. This finding might also explain the decreased LDL susceptibility to oxidation observed in patients with homocystinuria. Although homocysteine thiolactone has not been unequivocally identified in vivo, it might be a major by-product of deregulated homocysteine metabolism. In human cell culture, Jakubowski (1997) showed that the thiolactone produced by an altered homocysteine metabolism may be incorporated into cellular and extracellular protein through lysine acylation by its activated carboxyl group. Therefore, we hypothesize that deregulated homocysteine metabolism in homocystinuria with severe hyperhomocysteinemia will lead to the cyclization of homocysteine into homocysteine thiolactone, which may render LDL less susceptible to oxidation. Indeed, an independent mechanism of the oxidant stress might be involved in the formation of the fibrous atherosclerotic lesion observed in hyperhomocysteinemia. Vascular damages reported in homocystinuria might result from the alternative oxidative metabolism of homocysteine to homocysteine thiolactone (Jakubowski, 1997, 2000a; McCully, 1983, 1996; Olszewski and McCully, 1993). This metabolism, which is oriented toward the synthesis of phosphoadenosine phosphosulfates from homocysteine thiolactone sulfur atoms, might lead to an excessive sulfation of extracellular matrix proteoaminoglycans. Thus, it may impair the basal membrane of the vascular wall and may contribute to LDL sequestration (McCully, 1983; Olszewski and McCully, 1993). Moreover, macrophage-driven foam cell formation might be increased by the interaction between homocysteine thiolactone and LDL (McCully, 1996; Olszewski and McCully, 1993). Along with the direct activation of matrix metalloproteinase-2 (Bescond et al, 1999), vascular damage might also result from an impairment of

the tertiary structure of connective tissue protein, because of the reduced degree of cross-linkage between collagen and fibrin monomers (Lubec et al, 1996a; McCully, 1996; Rolland et al, 1995). A defect in collagen polymerization was reported in homocystinuric patients with severe hyperhomocysteinemia (Lubec et al, 1996a).

In addition to this attack, which might be caused by homocysteinylation of the vascular wall proteins by homocysteine and/or its oxidized products (homocysteic acid, homocysteine sulfinic acid, and homocysteine thiolactone), in vitro and in vivo work suggests that reduced homocysteine can inhibit endothelial cell turn-over (Wang et al, 1997) and promote smooth muscle cell proliferation (Lubec et al, 1996b; Majors et al, 1997; Tsai et al, 1994, 1996). Wang et al (1997) reported that low concentrations of homocysteine (10 to 50 $\mu\text{mol/L}$) specifically inhibit the growth of endothelial cells. Tsai et al (1994, 1996) reported that homocysteine is mitogenic to both human and rat aortic smooth muscle cells by stimulating the induction of cyclin A protein expression. Because other thiols such as cysteine and glutathione could replace homocysteine, the stimulation of smooth muscle cell proliferation probably was caused by nonspecific reactivity of the sulfhydryl group of homocysteine (Lubec et al, 1996b; Tsai et al, 1994). Nevertheless, this latter in vivo study demonstrates that homocysteine ingestion in rat stimulates, in a dose-dependent manner, the transcription of aortic cyclin-dependent kinase, which is required for the mitosis and subsequent proliferation of vascular smooth muscle cells. Because cysteine did not influence cyclin-dependent kinase production in this case, these vascular changes would be specific to homocysteine. The in vivo stimulation of cyclin-dependent kinase activity by homocysteine was confirmed in humans with severe hyperhomocysteinemia. Indeed, plasma cyclin-dependent kinase levels strongly correlated with homocysteine in homocystinuric patients (Fritzer-Szekeres et al, 1998). Recently, an in vitro study by Tyagi (1998) demonstrated that homocysteine (10 $\mu\text{mol/L}$) induces human vascular smooth muscle cell proliferation and collagen expression. These effects were reversed by the addition of N-acetylcysteine and reduced glutathione, reducing agents that can disrupt the binding to the homocysteine redox-sensitive receptor involved in signal transduction. These results suggest that reduced homocysteine causes protein thiol homocysteinylation that may induce smooth muscle cell proliferation by binding to a redox receptor and that may exacerbate atherosclerotic lesion formation by inducing collagen expression (Tyagi, 1998). Furthermore, the results suggest that the homocysteinylation might be regulated by the thiol redox status. In agreement with these latter results, the work of Majors et al (1997) showed that a relatively low concentration of homocysteine (50 to 300 $\mu\text{mol/L}$) stimulates collagen production in rabbit aortic smooth muscle cells that may favor extracellular matrix component accumulation in intimal lesions.

Thus, by promoting intimal hyperplasia, these vascular disorders (which are related to smooth muscle cell proliferation, collagen accumulation, and the extracellular matrix modification that may result from homocysteinylation of the vascular wall structure leading to connective tissue deterioration) might contribute to the atherogenic tendency of hyperhomocysteinemia. Nevertheless, although a specific action of homocysteine seems to occur at low concentrations, experimental trials have been conducted only with homocysteine administration, which would reflect a severe hyperhomocysteinemia rather than a more physiologic moderate elevation of circulating homocysteine, and clinical observations have only come from homocystinuric patients with severe hyperhomocysteinemia. Therefore, whether the occurrence of a pro-oxidant state caused by an altered intracellular redox status, which might increase homocysteinylation, is one of the molecular bases of the potential atherogenicity of moderate hyperhomocysteinemia remains to be determined. Nevertheless, emerging data allow us to propose that severe hyperhomocysteinemia might primarily have homocysteinylation effects related to homocysteine and/or its by-products (as homocysteine thiolactone) that would affect the vascular wall structure through a mechanism independent of an oxidant stress.

Alternative Role of Hypomethylation. An alternative explanation for hyperhomocysteinemia-induced vascular disease is that elevated homocysteine levels indicate an inefficiency of essential intracellular methylation reactions, which may predispose to vascular disease. Inefficient intracellular methylations may lead to the chronic damage of numerous proteins, which might in turn be involved in atherothrombogenesis. Additionally, elevated homocysteine levels may indicate inefficient biosynthesis of essential proteins, caused by folate metabolism abnormalities. However, our own experimental work and that of others on methionine load-induced hyperhomocysteinemia are inconsistent with these hypotheses. We found that, as with folate deficiency (which may effectively alter methylation reactions and deoxyribonucleotide biosynthesis), methionine loading (which maintains an efficient transmethylation pathway) promotes the occurrence of an oxidant stress-mediated prethrombotic state that is correlated with total plasma homocysteine concentration (Durand et al, 1997a; Durand and Blache, 1996). Several studies report that by altering flow-mediated endothelium-dependent vasodilation (Bellamy et al, 1998; Chambers et al, 1999a, 1999b; Kanani et al, 1999; Ungvari et al, 1999; Woo et al, 1999) and by affecting the blood coagulation system (Constant et al, 1999; Nappo et al, 1999), methionine load-induced hyperhomocysteinemia favors the occurrence of vascular spasm and thrombosis, and that endothelial dysfunction can be prevented by an anti-oxidant treatment (Chambers et al, 1999a; Kanani et al, 1999). These findings suggest that impaired methylation reactions and/or protein synthesis may not be the main mechanisms underlying moderate hyperhomocysteinemia-induced vascular disease.

Nevertheless, the involvement of an hypomethylation cannot be ruled out, because in vitro studies suggest that a low concentration of homocysteine specifically inhibits endothelial cell regeneration and directly activates quiescent vascular smooth muscle cells through a mechanism involving decreased carboxymethylation of p21^{ras} (Lee and Wang, 1999; Wang et al, 1997). The involvement of an hypomethylation cannot be ruled out. Indeed, recent data suggest that a chronic elevation of homocysteine level might indirectly reduce methylation of DNA, RNA, proteins, and phospholipids through a concomitant increase in SAH level secondary to homocysteine-mediated reversal of the SAH hydrolase reaction (Yi et al, 2000). High plasma levels were effectively associated with increased plasma and lymphocyte SAH and a decreased SAM to SAH ratio that could negatively affect cellular methylation potential (Fu et al, 2000; Melnyk et al, 2000; Yi et al, 2000). Therefore, further investigations are needed to assess potential vascular effects of hyperhomocysteinemia-associated changes in the SAM to SAH ratio, which regulates SAM-dependent methyltransferases and hence, methylation reactions.

These findings strengthen the hypothesis of a causal role for increased homocysteine levels in cardiovascular disease. They also suggest that the elevation of homocysteine may have multifactorial effects, interacting with both the vascular wall structure and the blood coagulation system, with different outcomes depending on the tissue and on the consequent degree of hyperhomocysteinemia. Although additional studies are required to clarify the molecular mechanisms responsible for hyperhomocysteinemia-induced vascular disorders, there is evidence that the direct cytotoxicity of markedly elevated homocysteine and/or of its oxidized by-products (homocysteine thiolactone, homocysteine sulfinic acid, and homocysteic acid) damages endothelial cells (Dudman et al, 1991; Starkebaum and Harlan, 1986; Wall et al, 1980). This direct cytotoxicity may also cause alteration in the tertiary structure of proteins in connective tissue and hence affect the vascular wall structure (Lubec et al, 1996a; McCully, 1996; Rolland et al, 1995). Specific disulfide linkage between homocysteine and the sulfhydryl group of an intracellular protein may be an alternative molecular mechanism underlying hyperhomocysteinemia-induced vascular dysfunction. Along with connective tissue proteins, many proteins contain cysteine-rich epidermal growth factor-like domains that may be preferential sites of homocysteinylation (Krumdieck and Prince, 2000). For instance, convincing in vitro work indicates that homocysteine may cause a specific impairment of the binding capacity of annexin II for t-PA that may alter the endothelial-dependent fibrinolytic potential (Hajjar et al, 1998). Homocysteine also may induce smooth muscle cell proliferation and collagen expression through binding to an homocysteine redox receptor involved in signal transduction (Tyagi, 1998). Nevertheless, experimental data suggest that the altered intracellular redox status associated with hyperhomocysteinemia-induced oxidant stress is the major

molecular mechanism responsible for the atherothrombogenicity of moderately elevated circulating homocysteine. An altered intracellular redox potential, which may be governed by the intracellular glutathione status (Dröge et al, 1994; Mihm et al, 1999), is involved in the oxidant stress-mediated regulation of numerous gene expression, particularly at the transcriptional level. By enhancing intracellular hydroperoxides, especially hydrogen peroxide, decreasing glutathione concentration, increasing cysteine oxidation, and activating protein kinases, oxidant stress can enhance the activity of transcription factors, such as the nuclear factor kappa B and activator protein-1, which are involved in the expression of cell surface adhesion molecules, growth factors, immunoreceptors, and cytokines; well known inflammatory mediators with chemotactic properties (Blache et al, 1999; Sen and Packer, 1996). Changes in the intracellular redox potential could thus modulate specific cellular functions of vascular cells (Blache et al, 1999; Flohé et al, 1997; Lander, 1997; Sen and Packer, 1996). Interestingly, exposure of endothelial cells to homocysteine would accelerate the rate of cellular senescence and increase the amount of telomere length lost per population doubling, through a redox-dependent pathway (Xu et al, 2000). Additionally, the degree of endothelial senescence positively correlated with the level of expression of two surface molecules linked to vascular disease, the intracellular adhesion molecule-1 and type 1 plasminogen activation inhibitor (Xu et al, 2000). Further experimental studies and clinical trials are now necessary to document the relative importance of the oxidant stress caused by an altered redox potential associated with an elevation of

homocysteine level, in comparison with alternative molecular mechanisms.

Vitamin Treatment of Hyperhomocysteinemia

Hyperhomocysteinemia can be reduced significantly or reversed by vitamin treatment of affected patients, except those bearing severe functional defects of methionine synthase or a thermostable mutation of MTHFR. In these latter cases, a betaine and/or methionine supplement should be given in combination with folinic acid or 5-methylTHF (Table 2).

A great number of studies investigated the beneficial effect of vitamin supplements on homocysteine levels (Brattström, 1996). Data show that folic acid supplementation reduces both fasting and postmethionine-load hyperhomocysteinemias, including those in patients with partial congenital defects of CBS and MTHFR. The data also indicate that pyridoxine supplementation only reduces the hyperhomocysteinemia detected after oral methionine administration in vitamin B₆-deficient patients or in heterozygotes for CBS deficiency. Treatment with cyanocobalamin would be efficient only in cases of vitamin B₁₂ deficiency.

The main role of folates in decreasing plasma levels of homocysteine might be explained merely by the fact that, in contrast to 5-methylTHF, cobalamin and pyridoxal-5'-phosphate only act as cofactors and are not consumed during homocysteine processing (Durand et al, 1998a, 1998b). Dietary folate intake or folate plasma concentration was negatively correlated to homocysteine level, not only in diseased (Mansoor et al, 1995; Selhub et al, 1993; Stampfer et al, 1992;

Table 2. Treatment of Hyperhomocysteinemia

Drug	Use
Folates and cobalamin	
Folic acid	In all patients with basal and postmethionine-load hyperhomocysteinemia. Nutritional folate deficiency, thermolabile MTHFR mutation, homozygotes for the CBS deficiency who do not respond to pyridoxine, heterozygotes for the CBS deficiency. 400 μ g to 1 mg per day in acquired deficiency, 5 mg per day in congenital deficiency.
Folinic acid or N ⁵ -methyltetrahydrofolate	Instead of folic acid in patients with altered folate metabolism or thermostable MTHFR mutations.
Cyanocobalamin	Correction of acquired vitamin B ₁₂ deficiency before folate therapy. 400 μ g to 2 mg per day according to the degree of deficiency.
Pyridoxine	In patients with postmethionine-load hyperhomocysteinemia who do not respond to folate treatment. Nutritional deficiency of vitamin B ₆ , dietary excess of methionine-rich animal proteins, 3 to 15 mg per day. Homozygosity and heterozygosity for CBS deficiency, 30 to 100 mg per day.
Betaine and/or methionine	
Betaine	Functional methionine synthase deficiency, thermostable MTHFR mutations.
Methionine	Functional methionine synthase deficiency, thermostable MTHFR mutations.

Ubbink, 1994) but also in healthy subjects (Lussier-Cacan et al, 1996; Mansoor et al, 1995; Selhub et al, 1993; Ubbink, 1994). Folic acid supplementation is the only treatment to effectively reduce plasma homocysteine levels in healthy subjects (Brattström, 1996). Moreover, in contrast to vitamin B₆ deficiency, the increased atherosclerotic vascular risk associated with folate deficiency is mediated through elevation of plasma homocysteine (Robinson et al, 1998).

Taken together, these observations suggest that except in vitamin B₁₂ deficiency (which only responds to cyanocobalamin treatment because of the inactivation of methionine synthase), folate treatment should be the most adequate therapy to reverse hyperhomocysteinemia detected both under basal conditions and after an oral methionine load. The treatment with folic acid should be combined with pyridoxine in vitamin B₆-deficient patients who are responders to an oral methionine load. Additionally, it is important to correct cobalamin deficiency before starting folic acid treatment, or provide cyanocobalamin together with folic acid. A folic acid supplement, particularly at high dosage (>5 mg per day) could mask the hematologic manifestations of megaloblastic anemia induced by cobalamin deficiency and could thus exacerbate the associated neurologic disorders.

The optimal doses of therapeutic vitamins remain to be clearly defined. To date, folic acid doses of 5 to 10 mg per day are currently used. Nevertheless, some data now suggest that a daily administration of folic acid at a dose lower than 1 mg may be adequate to reverse hyperhomocysteinemia (Boushey et al, 1995; Brattström et al, 1998; den Heijer et al, 1998; Rimm et al, 1998). In a recent study, cereal fortification, providing 499 and 665 μ g of folic acid daily, increased plasma folic acid by 64.8% and 105.7%, respectively, and decreased plasma homocysteine by 11% and 14%, respectively (Malinow et al, 1998). Moreover, the meta-analysis of Boushey et al (1995) indicates that a daily folate supplement at doses as low as 400 μ g may induce a 6 μ mol/L decrease in plasma homocysteine and prevent 28,000 deaths per year by myocardial infarction. Furthermore, recent studies indicate that folic acid therapy in a daily dosage of 0.5 mg is the lowest effective therapy for reducing both fasting and postmethionine-load homocysteine concentrations in hyperhomocysteinemic subjects, with similar effects in a daily dosage of 5 mg (Clarke and Armitage, 2000; van der Griend et al, 2000). Additionally, pyridoxine therapy alone does not reduce fasting homocysteine and the reduction in postmethionine-load homocysteine levels was smaller for pyridoxine than for the combined therapy using folic acid and pyridoxine (van der Griend et al, 2000). The large doses of pyridoxine often used (30 to 100 mg per day) may be required only for inherited defects of CBS or severe vitamin B₆ deficiency. A daily dose of 3 to 15 mg combined with folic acid would be sufficient for patients who do not achieve an efficient lowering of plasma homocysteine levels with folic acid therapy. Finally, a cyanocobalamin treatment may be necessary only in situations of vitamin B₁₂ deficiency, at a dosage of 0.4 to 2 mg per

day, depending on the severity of the deficiency. It is noteworthy that an excessive dietary intake of methionine-rich animal protein is an additional cause of hyperhomocysteinemia detected both in the fasting state and after an oral methionine load, especially when tissue folate and cobalamin stores are intact. In this case, a dietary reduction of methionine in conjunction with pyridoxine treatment should be instituted to correct hyperhomocysteinemia. Moreover, some studies show that a folate supplement improves endothelial function in hyperhomocysteinemic subjects (Bellamy et al, 1999; Woo et al, 1999). Furthermore, encouraging data from recent interventional studies favor the benefit of homocysteine-lowering therapy in the prevention and correction of vascular disorders related to elevated homocysteine levels. As described above, folic acid plus pyridoxine treatment might effectively contribute to the reduction of the atherothrombotic risk (De Jong et al, 1999b; Hackam et al, 2000; Peterson and Spence, 1998; Vermeulen et al, 2000; Yap et al, 2000). However, further prospective cohort studies with clinical endpoints in healthy subjects, and results from on-going randomized, placebo-controlled interventional trials are awaited to truly define the efficiency of homocysteine-lowering therapy in the prevention and correction of vascular disorders among patients with occlusive coronary artery, cerebrovascular and peripheral vascular diseases, and thrombosis related to hyperhomocysteinemia.

Conclusion

Mild to moderate hyperhomocysteinemia seems to be an independent and causal risk factor for atherosclerotic vascular disease. It also increases the risk for arterial and venous thrombosis and may play an important role in premature deaths from these vascular pathologies. Three main mechanisms leading to hyperhomocysteinemia are these: (1) an inhibition of the homocysteine remethylation pathway dependent on folate or cobalamin deficiency, on the C677T mutation of MTHFR, or on the functional deficiency in methionine synthase; (2) an inhibition of the transsulfuration pathway related to vitamin B₆ deficiency or heterozygosity for the CBS defect; and (3) a saturation of the transsulfuration pathway that may be caused by dietary methionine excess in cases of adequate folate and cobalamin intakes. Although the contribution of an excessive intake of methionine has not been clinically proven, folate deficiency (associated or not with the thermolabile mutation of MTHFR) and vitamin B₆ deficiency (perhaps associated with heterozygosity for CBS deficiency or with methionine excess) would be strongly involved in the incidence of cardiovascular disease associated with moderate hyperhomocysteinemia. The relative involvement of these various inherited and nutritional factors in the atherothrombotic risk and the interaction between genetic and environmental factors should be clearly assessed in the future. As regards the pathogenic mechanisms, the occurrence of an oxidant stress (resulting from an impaired homocysteine metabolism, which modifies

the redox status of extra- and intracellular thiols and thus alters the intracellular redox potential) might play a central role in moderate hyperhomocysteinemia-mediated vascular disorders. However, extracellular matrix modification and vascular wall structure damage might be principally involved in the atherothrombogenicity of severe hyperhomocysteinemia. Further experimental and clinical trials are now needed to confirm this hypothesis and to clarify the molecular mechanisms underlying hyperhomocysteinemia-induced vascular disease. Because vitamin (especially folate) supplementation can efficiently reduce plasma homocysteine levels, further prospective cohort studies and ongoing, randomized, placebo-controlled trials will determine the efficiency of folate supplementation, possibly in combination with vitamins B₁₂ and B₆, in the prevention and correction of vascular disorders related to moderate hyperhomocysteinemia.

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