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# Homocysteine Determinants and the Evidence to What Extent Homocysteine Determines the Risk of Coronary Heart Disease

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*Abstract*——**Cardiovascular diseases (CVD), especially coronary heart disease (CHD), are the most important causes of death in industrialized countries. Increased concentrations of total plasma homocysteine (tHcy) have been associated with an increased risk of CHD. Assuming that this relation is causal, a lower tHcy concentration will reduce the occurrence and recurrence of CHD. Therefore, it is important to know which factors determine the tHcy concentration. In the general population, the most important modifiable determinants of tHcy are folate intake and coffee consumption. Smoking and alcohol consumption are also associated with the tHcy concentration, but more research is necessary to elucidate whether these relations are not originating from residual confounding due to other lifestyle factors. The most important nonmodifiable determinant is the 677 C>T polymorphism in the gene that encodes methylenetetrahydrofolate reductase (MTHFR), a regulating enzyme in homocysteine metabolism. Especially subjects with the homozygous form of this polymorphism (i.e., 677TT genotype) and a low folate status have elevated tHcy concentrations. Specific clinical conditions like the use of antiepileptic drugs or methotrexate, renal failure, cancer, rheumatoid arthritis, and hypothyroidism may lead to elevated tHcy concentrations. The available epidemiological evidence indicates that an increased tHcy concentration is not an important risk factor for CHD in healthy subjects. However, prospective studies, which included subjects at high risk of CHD, and secondary prevention trials with intermediary endpoints consistently show that elevations in the tHcy concentration may be an important risk factor in these subjects for a (recurrent) CHD event. The induction of vascular endothelial dysfunction by homocysteine may underlie this increased risk. Ongoing intervention trials will indicate whether homocysteine-lowering through vitamin supplementation, prevents CHD in the treatment groups.**

## I. Introduction

Cardiovascular diseases (CVD) are a major public health problem in affluent countries. In 1999 about 36% (or ~50,000) of all deaths in The Netherlands were due to CVD. In comparison, about 27% were due to cancer, indicating that vascular diseases are the most important cause of mortality in The Netherlands (Netherlands Heart Foundation, 2001a).

CVD can roughly be classified into three categories: 1) coronary heart disease (CHD), 2) cerebrovascular accidents (CVA), and 3) other vascular diseases. In CHD, the coronary arteries that supply blood to the heart are blocked, and in CVA, the arteries that supply blood to the brains are obstructed. The category "other vascular diseases" comprises occlusions of peripheral arteries or veins, and congenital, infectious, and rheumatoid heart diseases. Of all vascular diseases, CHD is the most prevalent one (Netherlands Heart Foundation, 2001a).

Blockage of the coronary arteries often begins with atherosclerosis. This is characterized by the deposition of cholesterol, cellular waste products, calcium, and other substances in the inner layer of the arterial wall, together with the formation of connective fibrous tissue. This is called an atherosclerotic plaque. If plaques grow to a great extent they significantly reduce or obstruct the blood flow through an artery. They can also become fragile and rupture, which induces the formation of blood clots (thrombosis). These clots may locally block the blood flow or break off and travel to other parts of the body where they may occlude other arteries or veins (Fuster, 1994).

A high blood pressure, an unfavorable lipid profile (i.e., high total or low-density lipoprotein and low high-density lipoprotein cholesterol levels, high triglyceride levels) and smoking explain the majority of all CHD cases. However, the search for other risk factors remains, because not all CHD cases can be explained by these established risk factors. One factor that has been associated with CHD is an elevated plasma homocysteine concentration.

## II. History of Homocysteine As a Risk Factor for Vascular Diseases

The process of identifying homocysteine as a possible risk factor for vascular disease already started in 1964. By that time, Mudd and coworkers (Mudd, 1964) showed that the accumulation of homocysteine in blood, and consequently in urine leading to homocystinuria, was due to deficiency of the enzyme cystathionine $\beta$-synthase (CBS). After this discovery, McCully (1969) observed that a patient with CBS deficiency had arterial damage comparable to another patient with a different enzymatic abnormality that also led to homocystinuria. Since both abnormalities only shared the accumulation of homocysteine, McCully postulated that homocysteine itself, or one of its derivatives, was responsible for the

[1] Abbreviations: CVD, cardiovascular disease; CHD, coronary heart disease; CBS, cystathionine $\beta$-synthase; tHcy, total plasma homocysteine; THF, tetrahydrofolate; MTHFR, methylenetetrahydrofolate reductase; RR, relative risk; CI, confidence interval; CVA, cerebrovascular accidents; eNOS, endothelial nitric-oxide synthase; NO, nitric oxide; ADMA, asymmetric dimethylarginine.

arterial damage (McCully, 1969). This formed the basis for the hypothesis that moderate elevations of homocysteine in blood may be a risk factor for atherosclerosis in the general population (McCully and Wilson, 1975). The first to test this hypothesis were Wilcken and Wilcken in 1976 (Wilcken and Wilcken, 1976), who showed that patients with coronary artery disease suffered more often from an abnormal homocysteine metabolism than controls.

## III. Homocysteine Metabolism

Homocysteine is a sulfur-containing amino acid that is not used for the synthesis of proteins. Foods only contain traces of homocysteine. Homocysteine is formed when cells metabolize the essential amino acid methionine. The intracellular homocysteine concentration is precisely regulated and any excess is transported to plasma. In plasma, approximately 99% is oxidized to disulfides. The vast majority (~70%) of homocysteine is bound to proteins. Nonprotein bound homocysteine consists of homocystine (the disulfide of homocysteine), and mixed disulfides of homocysteine with e.g., cysteine. Only about 1% of all homocysteine moieties is reduced "free" homocysteine. The term total plasma homocysteine (conventionally abbreviated with tHcy) refers to all these forms of homocysteine in plasma (Mudd et al., 2000). Roughly, moderate elevations in the tHcy concentration refer to fasting plasma concentrations >15 to 30 $\mu$M, intermediate hyperhomocysteinemia refers to concentrations between 30 and 100 $\mu$M, and severe hyperhomocysteinemia refers to concentrations >100 $\mu$M (Kang et al., 1992).

Intracellular homocysteine can be irreversibly degraded to cysteine through the transsulfuration pathway, which is mainly limited to cells of the liver and kidneys. The enzymes in this pathway, CBS and $\gamma$-cystathionase, are both dependent on pyridoxal-5′-phosphate, a biologically active form of vitamin $B_6$, as cofactor. Homocysteine can also be remethylated to methionine by the enzyme methionine synthase (MS). This enzyme uses methylcobalamin (a biologically active form of vitamin $B_{12}$) as cofactor. The methyl group for the latter reaction is donated by 5-methyl-tetrahydrofolate (5-methyl-THF). This form of folate is produced by the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR). MTHFR in turn uses flavin adenine dinucleotide (a biologically active form of vitamin $B_2$) as cofactor (Finkelstein, 1990; Guenther et al., 1999). In an alternative remethylation route, which is also mainly restricted to the liver and kidney, betaine is used as the methyl donor by the enzyme betaine-homocysteine methyltransferase (Finkelstein, 1990). A simplified overview of the homocysteine metabolism is presented in Fig. 1.

Disturbances in intracellular homocysteine metabolism lead in most cases to elevated tHcy concentrations.

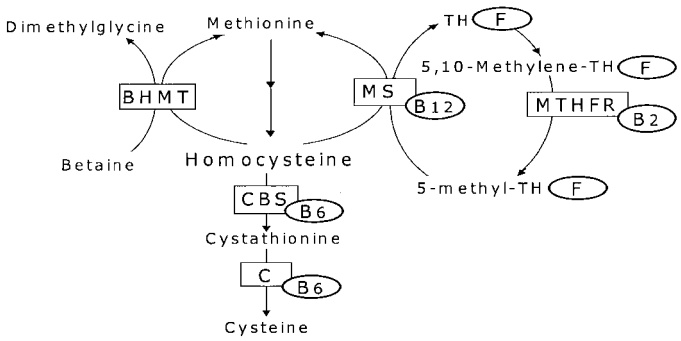


FIG 1. Simplified intracellular homocysteine metabolism. BHMT, betaine-homocysteine methyltransferase; C, $\gamma$-cystathionase; MS, methionine synthase; TH, tetrahydro; F, folate; B2, vitamin $B_2$; B6, vitamin $B_6$; B12, vitamin $B_{12}$.

Genetically determined functional deficiencies of enzymes in homocysteine metabolism, like deficiency of CBS, have an extremely large impact on the tHcy concentration. In the general population, these inborn errors of homocysteine metabolism are not important contributors of elevated tHcy concentrations, because they are too rare; homozygous CBS deficiency, which is the most common inborn error in homocysteine metabolism, has an estimated prevalence of about 1:335,000 (Mudd et al., 1989; Rosenblatt, 1989). Possible tHcy determinants on a population scale are evaluated in the next section.

## IV. Determinants of Homocysteine Concentration in the General Population

### *A. Age and Sex*

Increasing age and male sex are associated with a higher tHcy concentration (Andersson et al., 1992a; Lussier Cacan et al., 1996; de Bree et al., 2001e). The difference between the sexes could be due to larger muscle mass in men, since the formation of muscles is associated with the simultaneous formation of homocysteine in connection with creatine/creatinine synthesis (Norlund et al., 1998). It could also be due to the influence of sex hormones (Andersson et al., 1992a), which was confirmed in a study with transsexual males and females (Giltay et al., 1998). Part of the relationship with age in women might be explained by menopause, since the tHcy concentration was found to be higher in post-menopausal women compared with premenopausal women (Andersson et al., 1992a; Wouters et al., 1995).

### *B. Supplemental and Dietary B Vitamin Intake*

Several intervention studies have provided evidence for the importance of B vitamins in homocysteine metabolism. Especially supplements with folic acid (synthetic form of folate) and combinations of folic acid, vitamin $B_2$, $B_6$, and $B_{12}$ effectively reduced the tHcy concentration in subjects with normal (Ward et al., 1997; Brouwer et al., 1999b) and elevated baseline levels (Brattstrom et al., 1988;Olszewski et al., 1989; Ubbink

et al., 1993, 1994; Wald et al., 2001). A meta-analyses of 12 randomized trials showed that folic acid supplementation reduced tHcy concentrations by 25% with similar effects in a daily dosage of 500 to 5000 $\mu$g. This reduction in tHcy concentration was based on an average pretreatment level of 12 $\mu$M; however, higher pretreatment concentrations result in even a larger reduction of tHcy in response to folic acid treatment (Clarke and Armitage, 2000). Vitamin $B_{12}$ in an average dose of 500 $\mu$g produced an additional reduction in tHcy of 7%. Vitamin $B_6$ did not appear to have a significant effect on the tHcy concentration on top of the other B vitamins. However, the summarized trials in this meta-analyses did not assess the effects on the tHcy concentration after a methionine load, which are influenced by vitamin $B_6$ (Bostom et al., 1995).

A higher dietary folate intake is associated with a lower tHcy level in adults, independent of other dietary and lifestyle factors (Rasmussen et al., 2000; de Bree et al., 2001c; Jacques et al., 2001). These results complement those found in observational studies on dietary folate intake and the tHcy concentration in middle-aged (Shimakawa et al., 1997; Ubbink et al., 1998; Saw et al., 2001) and elderly subjects (Selhub et al., 1993; Bates et al., 1997; Koehler et al., 2001; Saw et al., 2001). The relation between intake of vitamin $B_2$ and tHcy concentration is scarcely investigated (Shimakawa et al., 1997; Bates et al., 1997; Jacques et al., 2001; de Bree et al., 2001c), and the weak inverse associations found could well be due to inadequate corrections for the intake of other dietary components like methionine and alcohol consumption (de Bree et al., 2001c). The latter may also be true for observed inverse relations between vitamin $B_6$ and tHcy concentrations (Selhub et al., 1993; Shimakawa et al., 1997; Bates et al., 1997; Ubbink et al., 1998; Rasmussen et al., 2000; de Bree et al., 2001c; Jacques et al., 2001; Koehler et al., 2001; Saw et al., 2001). Data on the effect of vitamin $B_{12}$ intake on the tHcy concentrations were absent in only one of the above-mentioned studies (Bates et al., 1997). A lower tHcy concentration at higher $B_{12}$ intakes was only observed in study samples with elderly and middle-aged subjects (Shimakawa et al., 1997; Ubbink et al., 1998; Saw et al., 2001). This is not surprising because the intake level of vitamin $B_{12}$ is generally higher than the recommended level in developed countries. Furthermore, in the elderly, malabsorption of vitamin $B_{12}$ from the diet is more common due to atrophic gastritis (van Asselt et al., 1998).

The fact that folate is the most important dietary determinant of tHcy concentrations is in line with its metabolic role. Folate is used as a substrate; it donates the methyl group for the conversion of homocysteine to methionine (Fig. 1). On the contrary, vitamins $B_2$, $B_6$, and $B_{12}$ are not utilized when homocysteine is metabolized; they function as cofactors of enzymes involved in homocysteine metabolism.

### C. Lifestyle factors

*1. Coffee.* Coffee consumption is positively associated with the tHcy concentration in both men and women in most (Nygard et al., 1997b; Oshaug et al., 1998; Stolzenberg Solomon et al., 1999; de Bree et al., 2001d; Jacques et al., 2001; Koehler et al., 2001), but not all (Nieto et al., 1997; Rasmussen et al., 2000; Saw et al., 2001) observational studies. Recent intervention trials have shown that this effect of coffee is causal (Grubben et al., 2000; Urgert et al., 2000; Christensen et al., 2001). Caffeine might be the factor that elevates the tHcy concentration (Nygard et al., 1997b; Grubben et al., 2000; Jacques et al., 2001), because it may inhibit the conversion of homocysteine to cysteine by acting as a vitamin $B_6$ antagonist (Grubben et al., 2000). Additionally, recent evidence showed that chlorogenic acid, a polyphenol that is present in coffee in the same amount as caffeine, may also partly be responsible for the increase in the tHcy concentration. When polyphenols are metabolized, methyl groups from methionine are necessary, which results in a higher production of homocysteine (Olthof et al., 2001). Both caffeine and chlorogenic acid are also present in tea, although in smaller doses, which explains the absence of a clear association between tHcy and tea consumption.

*2. Smoking.* Smoking is positively associated with the tHcy concentration (Nygard et al., 1995; Giles et al., 1999; Rasmussen et al., 2000; de Bree et al., 2001d; Jacques et al., 2001; Koehler et al., 2001). The fact that the smoking effect remains after correction for coffee consumption and folate intake (Nygard et al., 1998; Rasmussen et al., 2000; de Bree et al., 2001d) excludes important confounding. However, smokers generally consume a less healthy diet (Dallongeville et al., 1998), thus residual confounding of, for example, B vitamin intake cannot fully be excluded. Indeed, in one study the effect of smoking disappeared after correction for plasma folate (Saw et al., 2001). The exact mechanism behind the increase in the tHcy concentration is unidentified, but smoking may induce local effects in cells exposed to cigarette smoke (Piyathilake et al., 1992), influence the tHcy concentration by changing the plasma thiol redox status (Pryor and Stone, 1993; Mansoor et al., 1995; Bergmark et al., 1997), or inhibit enzymes such as methionine synthase (Blom, 1998).

*3. Alcohol.* Alcohol consumption is probably associated with the tHcy concentration in a J-shaped fashion (Halsted, 2001); moderate alcohol consumers have a lower tHcy concentration compared with nondrinkers, whereas alcoholics have elevated tHcy concentrations (Cravo et al., 1996b; de Bree et al., 2001b; Koehler et al., 2001). An inverse relationship between alcohol consumption in the moderate consumption range was observed in men only (Ubbink et al., 1998; de Bree et al., 2001b), and in men and women combined (Verhoef et al., 1997b; Vollset et al., 1997; Mayer et al., 2001). However,

in American studies also weak positive associations have been observed (Verhoef et al., 1996; Folsom et al., 1998; Giles et al., 1999; Jacques et al., 2001). Because extremely high alcohol consumption is associated with elevations in the tHcy concentration (Cravo et al., 1996a; Bleich et al., 2000), alcoholics might have drawn the positive association between alcohol and the tHcy concentration. Finally, there are also studies in which no association between alcohol and tHcy were observed (Lussier Cacan et al., 1996; Gudnason et al., 1998). In summary, the overall results indicate that the relationship between alcohol consumption and the tHcy concentration is complex. However, as some studies accounted for the most important lifestyle confounders (folate intake, smoking, coffee drinking) (Vollset et al., 1997; Ubbink et al., 1998; de Bree et al., 2001b) and similar results were observed after exclusion of ex-drinkers (de Bree et al., 2001b), this suggests that moderate alcohol consumption is associated with a beneficial lower tHcy concentration compared with nondrinking.

Considering the type of alcoholic beverage, beer might be responsible for the inverse (or the absence) of an association between alcohol consumption and the tHcy concentration as opposed to a positive association (de Bree et al., 2001b; Jacques et al., 2001; Mayer et al., 2001). The results from two intervention trials with beer, wine, and spirits were inconsistent; one 3-week randomized crossover trial showed no association with the tHcy concentration after intervention with four glasses of beer per day, compared with an elevation of the tHcy concentration with four glasses of wine or spirits per day (Gaag et al., 2000). Another trial, with a marginal 6-week nonrandomized design in which participants could drink the alcoholic beverage of their own preference (Bleich et al., 2001), showed elevations in the tHcy concentration for all three alcoholic beverage groups after daily consumption of three glasses. Hence, the results for types of alcoholic beverages are not clear. Intervention studies with lower doses of alcoholic beverages or with ethanol-water solutions may provide more insight in this issue.

*4. Physical Activity.* Physical activity is probably not (Lussier Cacan et al., 1996; Gudnason et al., 1998; Saw et al., 2001) or weakly inversely (Nygard et al., 1995) associated with the tHcy concentration. One intervention study showed that acute exercise does not affect the tHcy concentration (Wright et al., 1998). Because an active lifestyle is generally associated with a more healthy lifestyle, and a more healthy lifestyle with a lower tHcy concentration, our results of a higher tHcy concentration (in the multivariate models of women only) is likely a chance finding, because residual confounding of, e.g., smoking is expected to result in an inverse association (de Bree et al., 2001d).

### *D. Genetics*

Besides the rare inborn errors that lead to a severely diminished activity of the enzymes involved in homocysteine metabolism, there are genetic mutations (or genetic variations) that have a relatively small effect on the enzymes' activity. The 677 C>T polymorphism in the gene that encodes MTHFR has been investigated most extensively in relation to its effect on the tHcy concentration. The prevalence of this polymorphism is relatively high in the general population; the prevalence of homozygosity (677TT) is 5 to 15% in most Caucasian populations (Brattstrom et al., 1998b)

MTHFR catalyzes the formation of 5-methyl-THF out of 5,10-methylene-THF; the former folate derivative is necessary for the remethylation of homocysteine to methionine (see Fig. 1). The 677 C>T mutation results in a reduced specific MTHFR activity in isolated lymphocytes (~34% residual activity in 677TT, ~71% residual activity in 677CT relative to 677CC) (van der Put et al., 1996), which leads to higher tHcy concentrations (Frosst et al., 1995).

The higher tHcy concentrations are most pronounced in 677TT subjects with a marginal folate status (Jacques et al., 1996; Brattstrom et al., 1998b; McQuillan et al., 1999; de Bree, 2001), or a suboptimal folate intake (McQuillan et al., 1999; de Bree, 2001). The fact that 677TT subjects do not have elevated tHcy concentrations when their folate status is optimal (adequate) is elegantly explained by a recent study of Guenther et al. (1999). In *Escherichia coli* bacteria they showed that a mutation homologous to the human MTHFR 677 C>T mutation was associated with an enhanced dissociation of FAD (i.e., the cofactor form of vitamin $B_2$). An optimal folate supply prevented the loss of FAD binding and in this way suppressed the inactivation of the enzyme (Guenther et al., 1999).

### *E. Biological Coronary Heart Disease Risk Factors*

In general, the associations of total cholesterol, high-density lipoprotein cholesterol, systolic and diastolic blood pressure, and measures of body fat and the tHcy concentration are not very strong (Nygard et al., 1995; Refsum et al., 1998; de Bree et al., 2001a). Nevertheless, because they are associated with the tHcy concentration in the direction of an increased risk of CHD, adjustments for these factors while studying the relation between the tHcy concentration and the risk of CHD is necessary.

## V. Drugs and Diseases As Determinants of Homocysteine Concentrations

Besides factors that affect the tHcy concentration in the general population, there are also factors like drugs and diseases that determine the tHcy concentration in specific groups of the population. Their effect on the tHcy concentration is described briefly in the following

section as several elegant reviews have already been dedicated to this topic (Ueland and Refsum, 1989; Refsum and Ueland, 1990; Schneede et al., 2000; Blom, 2001).

### A. Drugs Influencing the Homocysteine Concentration

*1. Hormones.* The effect of sex steroid hormones on the tHcy concentration is indicated by gender differences and the observation of lower tHcy concentrations in premenopausal women (Andersson et al., 1992a; Wouters et al., 1995) and during pregnancy (Kang et al., 1986; Andersson et al., 1992b).

Earlier studies with small samples sizes indicated that oral contraceptives may increase the tHcy concentration (Brattstrom et al., 1992), especially in the low hormonal phase (Steegers-Theunissen et al., 1992). However, more recent evidence indicated no difference in the tHcy concentration between pill users and non-pill users (Green et al., 1998; Morris et al., 2000), which could also be the results of improvements of the pill itself over time. Postmenopausal hormone replacement therapy with estrogen-like hormones decreases the tHcy concentration (Blom, 2001). Interestingly, tamoxifen, an antiestrogen used to treat women with breast cancer, reduces the tHcy concentration (Anker et al., 1995; Cattaneo et al., 1998).

Conflicting results have been described for the effect of insulin in populations varying from patients to apparently healthy subjects (Blom, 2001). Nevertheless, recent evidence showed direct suppressing effects of insulin on MTHFR and CBS enzyme activity in liver cells (Dicker-Brown et al., 2001). Before drawing a conclusion on the effect of insulin on the tHcy concentration, more research is warranted.

*2. Antiepileptic Drugs.* Although there are not many human studies on the effects of antiepileptic drugs on the tHcy concentration, it is suspected that these drugs interfere with folate absorption, catabolism, and inhibition of enzymes involved in folate metabolism (Lambie and Johnson, 1985). Phenytoin is the drug most often associated with folate deficiency, but drugs like phenobarbital, carbamazepine, primidone, and valproate may also interfere with the remethylation of homocysteine (Ueland and Refsum, 1989). Besides this, the transsulfuration route of homocysteine may be compromised because of a diminished vitamin $B_6$ status (Schwaninger et al., 1999).

*3. Methotrexate.* Antifolates like methotrexate deplete cells of reduced folates because they inhibit the conversion of dihydrofolates to tetrahydrofolates. This decreases the synthesis of DNA and RNA nucleotides, which are necessary for cell function and reproduction. Because methotrexate inhibits cell reproduction, it is given as a treatment for cancer, psoriasis, or rheumatoid arthritis (Refsum and Ueland, 1990). Other antifolates like sulfasalazine, raltritrexed, trimetrexate, and trimethoprim will most likely have a similar effect on the tHcy concentration (Haagsma et al., 1999).

*4. Nitrous Oxide.* Plasma tHcy concentrations increased rapidly in patients that were given nitrous oxide as an anesthetic. The mechanism behind this increase is the inactivation of methionine synthase, the enzyme that remethylates homocysteine to methionine (Ermens et al., 1991). Preoperative treatment with betaine, which remethylates homocysteine independently of methionine synthase, may prevent a steep increase in tHcy concentrations. Theoretically, this may reduce the risk of postoperative complications like atherothrombotic events.

*5. Other Drugs That Have an Effect on the Homocysteine Concentration.* Lipid-lowering drugs like statins, niacin, cholestyramine, and fibrates may, besides their effect on blood lipids, have an elevating effect on the tHcy concentration, as has been shown with niacin in rats (Basu and Mann, 1997). Fenofibrate is a fibrate that increases the tHcy concentration in particular (de Lorgeril et al., 1999; Dierkes et al., 1999; Westphal et al., 2001). Nevertheless, the statin simvastatin did not influence the tHcy concentration in dyslipidemic patients (de Lorgeril et al., 1999).

Metformin, a drug used in diabetes, influences the insulin level and decreases B vitamin concentrations (Yeromenko et al., 2001). The expected effect of administration of vitamin $B_6$ antagonists is an increase in tHcy concentration. The drug theophylline, used to treat asthmatic patients, is an example of a vitamin $B_6$ antagonist that increases the tHcy concentration (Ubbink et al., 1996). Other drugs that may also act as B6 antagonists are isoniazid, cycloserine, hydralazine, phenelzine, and procarbazine (Blom, 2001).

Administration of drugs that need methylation before they are activated or detoxified may cause elevation in the tHcy concentration because they increase the formation of homocysteine. Examples of such drugs are the Parkinson's disease drug L-dopa (Blandini et al., 2001) and the anti-cancer drug 6-mercaptopurine (Blom, 2001).

Drugs with a free thiol group, such as *N*-acetylcysteine, D-penicillamine or cysteamine can react with the disulfide forms of homocysteine (99% of the plasma tHcy concentration). When this reaction takes places, free homocysteine remains, which is available for cellular uptake, metabolic conversion, and (possibly) excretion. Administration of these types of drugs may decrease tHcy concentrations in patients with renal failure that are not responding to B vitamin or betaine therapy.

The effect of gastric proton pumps on the tHcy concentration is unknown, although they are expected to interfere with vitamin $B_{12}$ absorption through their influence on the intestinal pH (Blom, 2001). Finally, immunosuppressive drugs like cyclosporine may augment the tHcy concentration (Schneede et al., 2000).

*B. Diseases That Influence the Homocysteine Concentration*

*1. Kidney Dysfunction.* The most frequent clinical cause of hyperhomocysteinemia, next to nutritional deficiencies in folate and vitamin $B_{12}$, is renal failure. The basis of hyperhomocysteinemia in renal failure is not completely clear, although several processes may explain the close correlation between kidney function and the tHcy concentration; the kidney may influence or regulate homocysteine metabolism in other tissues and it may convert a major amount of the homocysteine present in blood. Renal reabsorption of homocysteine in the tubular cells only occurs for the nonprotein-bound disulfide forms (about 30% of the plasma tHcy concentration). The redox status of the tubular cells allows a reduction of the disulfides, which makes homocysteine available for conversion via the transsulfuration or remethylation pathway (Arnadottir and Hultberg, 2001; Blom, 2001).

*2. Proliferating Diseases.* Diseases like cancer and psoriasis are associated with higher tHcy concentrations (Refsum and Ueland, 1990). These conditions are accompanied by rapidly dividing cells, which have a high demand for methyl groups to methylate vital cell components, including proteins. When methionine donates its methyl group, homocysteine remains. Another process that may lead to higher tHcy concentrations in these diseases is that one-carbon units from THF are preferentially used for the synthesis of DNA and RNA, at the expense of homocysteine remethylation.

*3. Rheumatoid Arthritis.* Hyperhomocysteinemia is commonly observed in rheumatoid arthritis and not necessarily dependent on methotrexate use (Schneede et al., 2000). The origin of hyperhomocysteinemia in these patients is not clear, as a combination of drug use, vitamin deficiencies, MTHFR 677C>T genotype and gastrointestinal dysfunction all may play a role (van Ede et al., 2001).

*4. Endocrine Disorders.* Type I, i.e., insulin-dependent, diabetes is accompanied by high tHcy concentrations only at advanced stages of the disease. In this stage, the creatinine levels are also increased and patients have macroalbuminuria. Thus, in these patients hyperhomocysteinemia may also be due to an impaired kidney function (Schneede et al., 2000). Compared with apparently healthy subjects, subjects with type I and II diabetes had lower tHcy concentrations, possibly due to hyperfiltration (Wollesen et al., 1999). However, a lower tHcy concentration may also be the effect of insulin, as was reviewed by Schneede et al. (2000). Mild elevations in the tHcy concentration are observed in type II diabetes treated with metformin (Hoogeveen et al., 1997).

Concentrations of tHcy were higher in hypothyroidism and lower in hyperthyroidism (Nedrebo et al., 1998; Diekman et al., 2001). This finding could be related to the influence of the thyroid function on metabolic turnover; however, other factors like B vitamin status and kidney function may have been involved (Schneede et al., 2000).

*5. Intestinal Diseases.* Several gastrointestinal disorders may lead to a deficiency of folate or vitamin $B_{12}$ or both, which in turn will result in higher tHcy concentrations. Intestinal diseases associated with higher tHcy levels are ulcerative colitis, Crohn's disease, celiac disease, and inflammatory bowel disease. Treatment of patients with these kinds of diseases often involves gastrointestinal surgery, which may further elevate tHcy levels. In addition, bacterial overgrowth, pelvic and abdominal radiotherapy, and an increased gastric pH may lead to a diminished B vitamin uptake (Schneede et al., 2000).

## VI. Homocysteine and the Risk of Coronary Heart Disease

*A. Inborn Errors*

The first and strongest evidence for elevated tHcy concentrations as a causal risk factor for atherothrombotic disease came from patients with inborn errors of homocysteine metabolism. When patients with a genetically determined CBS deficiency are untreated, about 50% will have a vascular event before the age of 30 (Mudd et al., 1985). Patients with other inherited defects of homocysteine metabolism, like MTHFR deficiency (Rosenblatt, 1989) and defects in cobalamin (vitamin $B_{12}$) metabolism (McCully, 1969), also suffer from vascular diseases at a very young age. The common denominator in these different metabolic defects is a severely elevated tHcy concentration (i.e., >100 $\mu$M). Treating patients with inherited CBS deficiency with tHcy-lowering nutrients (e.g., folic acid, vitamin $B_{12}$, vitamin $B_6$, and betaine) prevents vascular events (Kluijtmans et al., 1999;Yap et al., 2000). Despite treatment, the tHcy concentrations of these patients are well above the normal range (i.e., >30 $\mu$M) (Yap et al., 2000). This could mean that the relative risk of these patients to develop premature CHD (<60 years) is still higher compared with those with normal tHcy concentrations.

*B. Retrospective, Cross-Sectional, and Prospective Epidemiological Studies*

Earlier retrospective and cross-sectional studies have consistently shown a stronger relationship with the tHcy concentration than the more recent prospective studies (Boushey et al., 1995; Danesh and Lewington, 1998; Christen et al., 2000; Ueland et al., 2000; Ford et al., 2002). The *meta*-analysis of Boushey et al. (1995) summarized 11 retrospective and cross-sectional studies that all showed a significantly increased risk of CHD for each 5 $\mu$M increase in the tHcy concentration (odds ratios all ≥1.5). Of the two prospective studies available at that time, one found a positive association (Stampfer et al., 1992) and one did not find such an association

(Alfthan et al., 1994). In 1998, this *meta*-analysis was updated by Refsum et al. (1998). Of the additional 16 retrospective and cross-sectional studies that evaluated CHD as one of the endpoints of interest only three did not find a positive association between the tHcy concentration and CHD risk. One additional population-based prospective study (Arnesen et al., 1995) showed a significant positive relationship between the tHcy concentration and the risk of CHD.

In 1998, another *meta*-analysis was performed that calculated the relative risk (RR) for prospective and retrospective studies separately (Danesh and Lewington, 1998). The summary RR of CHD from all available retrospective studies with population-based controls was 1.6 [95% confidence interval (CI) = 1.4–1.7]. For prospective studies this RR was lower: 1.3 (95% CI = 1.1–1.5). In 2000, the totality of evidence was evaluated qualitatively (Christen et al., 2000), and the results from prospective studies were in general weaker (or absent) in comparison with those of retrospective and cross-sectional studies. Nevertheless, recent *meta*-analyses based on prospective studies for which, except for one (Evans et al., 1997), subjects were not selected for their increased risk of CHD, calculated that each 5 $\mu$M increase in the tHcy concentration was associated with a 20% increase in risk of CHD [odds ratio: 1.2, 95% CI = 1.1–1.3 (Ueland et al., 2000); 1.2, 95% CI = 1.1–1.4 (Ford et al., 2002)]. Table 1 gives a detailed overview of all the prospective studies included in both *meta*-analyses and is updated with the most recent studies.

There are several reasons for the different results between cross-sectional and retrospective studies on the one hand and prospective studies on the other hand. These will be evaluated in the following paragraphs. Furthermore, explanations for different results between prospective studies will be discussed.

First, the issue of the chronological sequence between collecting information on study subjects (including blood drawing) and the occurrence of the disease. In retrospective studies, data collection takes place after the CHD event. This means that the event might have distorted the recall of certain lifestyle (such as smoking) and dietary habits. Furthermore, it implicates that medical treatment, like lipid-lowering drugs, anti-hypertensive therapy, or smoking cessation, may have altered the levels of traditional CHD risk factors. Moreover, the disease could have influenced blood levels of tHcy and of risk factors like blood pressure and cholesterol level (Evans et al., 1997). In cross-sectional studies, tHcy concentrations and the extent of the disease are assessed at the same time. As cases are typically persons with early signs of vascular disease (e.g., angiographically confirmed stenosis), the effect of the disease on CHD risk factors like blood pressure and cholesterol level cannot be excluded (Christen et al., 2000). Prospective studies have the major advantage that data and blood are collected before the event, thus there is no influence of the disease on lifestyle and dietary habits and on blood parameters, if subjects with vascular diseases at baseline are excluded.

In summary, the fact that the risk estimates of retrospective and cross-sectional studies are generally higher compared with prospective studies can on the one hand be explained by higher tHcy concentrations after a vascular event (Landgren et al., 1995) (see Table 2 for further discussion of this issue). On the other hand, if the disease and/or medical treatment modify the levels of CHD risk factors, then retrospective and cross-sectional studies might also show higher risk estimates. However, adequate control of the confounding effect of these risk factors on the association between the tHcy concentration and the risk of CHD is not possible due to the masking of "true" levels of these risk factors (Evans et al., 1997).

Second, there is the issue of the simultaneous presence of higher tHcy concentrations and CHD, irrespective of whether these higher levels are the cause or the consequence of CHD. Due to this issue one can assume that prospective studies that included subjects with preexisting CHD, in theory, should show a more consistent statistically significant positive association between the tHcy concentration and the risk of CHD. Of the studies that are summarized in Table 1, four studies offer support for this assumption. Stehouwer et al. (1998) showed that the tHcy concentration was more strongly associated with the recurrence of an event rather than with a first episode of myocardial infarction. Vollset et al. (2001) observed that after stratification for high and low baseline risk (high risk indicated a history of myocardial infarction, stroke, angina pectoris, diabetes, or hypertension), the tHcy concentration was only a significant risk factor for cardiovascular mortality in high-risk persons. Furthermore, besides the results mentioned in Table 1, two studies reported on subjects with prevalent CHD or CVA at baseline (de Bree, 2001; Knekt et al., 2001). In these subjects, a higher tHcy concentration was either significantly associated with the risk of CHD mortality and morbidity (Knekt et al., 2001), or the estimated RR of CHD mortality was larger than the estimate for men and women free of CVD at baseline (1.6, 95% CI = 0.8–3.1 versus 1.03, 95% CI = 0.8–1.3, for each 5 $\mu$M increase in the tHcy concentration) (de Bree, 2001).

Finally, in elderly populations the number of subjects with silent preclinical CHD will be larger than in adult populations. Of the five prospective studies with the elderly (average age >60 years) (Stehouwer et al., 1998; Bostom et al., 1999; Bots et al., 1999; Kark et al., 1999a; Vollset et al., 2001), only one (Stehouwer et al., 1998) was not in favor of a significant association between the tHcy concentration and the risk of CHD.

Prospective studies performed in selected high-risk populations consistently showed that the tHcy concentration is a strong predictor of cardiovascular mortality

TABLE 1

*Prospective studies on plasma tHcy concentrations and risk of (non-) fatal CHD in populations not selected on the basis of risk of CHD,[a] adapted from Ueland et al., (2000)*

| Reference | Study | Follow-up Years | Age | Sex | Outcome | At Baseline Exclusion of: | RR for Each 5-μM Increase in tHcy[a] |
|---|---|---|---|---|---|---|---|
| | | | *yr* | | | | |
| Stampfer et al., 1992 | Physician's Health Study, United States | 5 | 40–84 | M | Fatal and non-fatal MI and CHD | Past history of MI, stroke, or TIA | 1.29 (1.01–1.64) |
| Alfthan et al., 1994 | North Karelia Project, Finland | 9 | 40–64 | M, F | Fatal and non-fatal MI | Past history of CVD | 1.03 (0.66–1.53) |
| Arnesen et al., 1995 | Tromsø Health Study, Norway | 4 | 34–61 | M, F | Fatal and non-fatal CHD | Past history of MI | 1.41 (1.06–1.88) |
| Evans et al., 1997 | Multiple Risk Factor Intervention Trial, United States[b] | 11 + | 35–57 | M | Non-fatal MI, fatal CHD | Past history of morbidity (not explained) | 0.98 (0.83–1.15) |
| A'Brook et al., 1998 | Scottish part of MONICA (MONItor trends in CArdiovascular diseases) | 7.6 | 35–64 | M, F | CHD, unclear whether fatal or non-fatal | ? | 1.50 (1.28–1.78) |
| Ubbink et al., 1998 | Caerphilly Cohort, United Kingdom | 5 | 50–64 | M | Fatal and non-fatal IHD | No, adjustments for prevalent CHD | 1.22 (0.88–1.64) |
| Folsom et al., 1998 | Atherosclerosis Risk In Community Study, United States | 3.3 | 45–64 | M, F | Fatal and non-fatal CHD | Past history of CHD, stroke, or TIA | 1.15 (0.68–1.92) |
| Wald et al., 1998 | British United Provident Association, United Kingdom | 8.7 | 35–64 | M | Fatal IHD | Past history of CHD | 1.41 (1.20–1.65) |
| Stehouwer et al., 1998 | Zutphen Elderly Study, The Netherlands | 10 | 64–84 | M | Fatal and non-fatal CHD | No, adjustments for prevalent CHD | 1.05 (0.97–1.15) |
| Bostom et al., 1999 | Framingham Study, United States | 10 | 59–91 | M, F | Fatal CHD | ? | 1.42 (1.13–1.77) |
| Bots et al., 1999 | Rotterdam Elderly Study, The Netherlands | 2.7 | >55 | M, F | Fatal and non-fatal MI | No, adjustments for prevalent CHD | 1.28 (1.05–1.76) |
| Ridker et al., 1999 | Women's Health Study, United States | 3 | Mean 59 | F | Fatal and non-fatal CHD | Past history of CVD | 1.74 (1.13–2.64) |
| Whincup et al., 1999 | British Regional Heart Study, United Kingdom | 12.8 | 40–59 | M | Fatal and non-fatal MI | No, adjustments for prevalent CHD | 1.13 (0.99–1.29) |
| Kark et al., 1999a | Jerusalem Study, Israel | 9–11 | >50 | M, F | Fatal CHD | No, association remained after exclusion of history of CVD | 1.34 (1.05–1.62) |
| Voutilainen et al., 2000 | Kuopio Ischaemic Heart Disease Risk Factor Study, Finland | 8, 9 | 42–60 | M | Fatal and non-fatal CHD | History of CHD | 0.88 (0.44–1.76) in highest tHcy quartile vs. lowest quartile |
| Vollset et al., 2001 | Hordaland Elderly, Norway | 4.1 | 65–67 | M, F | Fatal CHD | No, adjustments for prevalent CHD | 1.46 (1.14–1.87) |
| Knekt et al., 2001 | Mobile Clinical Health Examination Study, Finland | 13 | 45–64 | M | Fatal and non-fatal CHD | History of CHD | 0.90 (0.51–1.60) in highest tHcy quintile vs. lowest quintile |
| de Bree, 2001 | Monitoring Project on Chronic Disease Risk Factors, The Netherlands | 10.3 | 20–59 | M, F | Fatal CHD | History of CVD | 1.03 (0.83–1.29) |

TIA, transient ischemic attack; MI, myocardial infarction; IHD, ischemic heart disease; ?, unclear whether subjects with CVD at baseline were excluded.

[a] Unless indicated otherwise.

[b] These men were selected for a baseline moderate risk of CHD, based on a combination of their total cholesterol level, diastolic blood pressure, and smoking habits.

and morbidity (including CHD) in subjects with CHD (Nygard et al., 1997a), diabetes (Kark et al., 1999b; Stehouwer et al., 1999), renal insufficiency (Bostom et al., 1997; Moustapha et al., 1998), peripheral artery disease (Taylor et al., 1991), and systemic lupus erythematosus (Petri et al., 1996).

The third issue is the aspect of the duration of follow-up. In two prospective studies, the association between the tHcy concentration and the risk of CHD (Stehouwer et al., 1998) or total cardiovascular mortality (Kark et al., 1999a) was most strong in the first few years of follow-up. In addition, the studies in Table 1 with a short follow-up period (<5 years) showed in general more often a statistically significant association between the tHcy concentration and the risk of CHD. These results indicate that the tHcy concentration might be a short-term risk factor for CHD. Because subjects with preclinical disease will, in general, decease earlier than those without preclinical disease and apparently the subjects that died during the first few years of follow-up had the highest tHcy concentrations.

The short-term risk factor aspect of tHcy is confirmed by data of the Physicians' Health Study. After 5 years of follow-up a significant increased risk of CHD was found in men with elevated tHcy levels (Stampfer et al., 1992), however, extending the follow-up to 7.5 years yielded a nonsignificant RR (Chasan Taber et al., 1996). Furthermore, in the same study population, no significant association was observed between the tHcy concentration and the risk of angina pectoris after 9 years of follow-up (Verhoef et al., 1997a).

An additional feature of prospective studies with a longer follow-up is that a reduction in the risk estimate may occur as a result of changes in diet, lifestyle, or medical treatment during follow-up. These factors may alter the tHcy concentration in such a way that the baseline tHcy concentration is no longer representative of the concentration at the time of the event. In addition, due to a combined effect of measurement errors and intraindividual variation, the "usual" level of tHcy that is related to the risk of CHD might be difficult to approach with a single tHcy measurement. The result of this so-called regression dilution bias is an attenuated association between the tHcy concentration and the risk of CHD. This bias can be estimated and corrected for by taking more blood samples over the period of follow-up and using the data of replicate tHcy measurements (Clarke et al., 2001).

In conclusion, the differences in strength of the association between prospective studies and retrospective and cross-sectional studies can be ascribed to the fact that in prospective studies data are collected before the event. Prospective studies with subjects not selected for their risk of CHD show in general a weak association between the tHcy concentration and the risk of CHD. Yet, ordering prospective studies into studies with and without subjects with pre-existing CHD, with younger and older subjects, and with a long and short follow-up shows that associations are more consistently found in studies including subjects with CHD, elderly, and a short follow-up. Prospective studies in high-risk populations consistently show a strong relation between the tHcy concentration and CHD. These results could either mean that the tHcy concentration is a short-term risk factor in subjects with a high risk of CHD, as suggested by some researchers (Evans et al., 1997; Refsum and Ueland, 1998), or it could mean that elevations in the tHcy concentration are merely a marker of the degree of the underlying vascular disease.

Other studies that give insight into whether the tHcy concentration is a marker of disease or causally related to the development of CHD are evaluated in the next paragraphs.

### *C. Homocysteine and Thrombosis*

As will be mentioned more extensively in the section on mechanisms, an elevated tHcy level might interfere with normal coagulation and fibrinolysis. There is considerable epidemiological evidence that the tHcy concentration is a risk factor for venous thrombosis (den Heijer et al., 1998; Ray, 1998). The three prospective studies on the relationship between the tHcy concentration and venous thrombosis all show a significant positive association in subjects healthy at baseline (Ridker et al., 1997), subjects with systemic lupus erythematosus (Petri et al., 1996), and in subjects with a history of venous thrombosis (Eichinger et al., 1998). In addition, thrombotic disease is responsible for 50% of the vascular events in patients with CBS deficiency (Mudd et al., 1985). If the tHcy concentration is more a thrombogenic factor than an atherogenic factor, part of the weak association between the tHcy concentration and the risk of CHD could theoretically be due to the fact that CHD comprises heart diseases with both a thrombogenic and an atherogenic etiology.

A primary thrombogenic effect of the tHcy concentration might explain why it is consistently associated with an increased risk of CHD in high-risk subjects. If these subjects already have a certain degree of atherosclerosis, tHcy-induced thrombosis might be the crucial factor triggering the vascular occlusion.

### *D. Methylenetetrahydrofolate Reductase 677C>T Genotype and Coronary Heart Disease*

A person's genotype of the 677C>T variant in the MTHFR gene is present from birth onwards and will not change over the years. The 677TT variant of this genotype leads to an approximately 25% higher tHcy concentration compared with 677CC subjects (Brattstrom et al., 1998b). Nevertheless, this genotype has not consistently been associated with CHD (Kluijtmans et al., 1997; Brattstrom et al., 1998b; Brattstrom and Wilcken, 2000). These inconsistencies might be attributable to a power problem (Fletcher and Kessling, 1998; Blom and

TABLE 2
*The influence of vascular disease on tHcy concentrations*

| |
|---|
| Several studies have measured tHcy concentrations on the day of a CHD event and compared it with the levels measured on days after the event (up to 180 days after the event) (Landgren et al., 1995; Egerton et al., 1996; Verhoef et al., 1996). Typically they observed lower tHcy concentrations on the day of the event compared with the concentrations on the days after the event. As no study measured tHcy concentrations just before the event, it cannot be excluded that tHcy concentrations were lower during the event and, over time, return to the normal level from before the event. In addition, a rise in tHcy concentration may be due to the fact that a patient is going from an active life to bedridden; i.e., the tHcy concentration in blood collected in supine position is higher than that collected sitting (maximally 30% higher) (Thirup and Ekelund, 1999). |
| A metabolic reason for an increase in the tHcy concentration after the event is provided by Dudman (1999). He suggests that the increase in the tHcy concentration is the result of tissue damage and repair. Tissue repair involves synthesis of DNA, RNA, and proteins that require methyl groups. Most methyl groups originate from methionine, and when methionine donates its methyl group, homocysteine is generated (see Fig. 1). Dudman (1999) postulates that a higher tHcy concentration attracts leukocytes to the vascular endothelium, where they play a role in tissue repair and remodeling. However, to our knowledge no studies have been done to test whether induced vascular damage increases tHcy concentrations. |
| Another mechanism that could explain a higher tHcy level in subjects with CHD is proposed by Brattstrom and Wilcken (2000). They reviewed currently available evidence and propose that atherosclerosis caused by traditional cardiovascular risk factors (such as high blood pressure and smoking) impairs the renal function. Because the kidneys are quantitatively important for the catabolism of homocysteine, this might lead to higher tHcy concentrations. |

Verhoef, 2000). The average difference in tHcy concentration between 677TT and 677CC subjects is ≈2.6 $\mu$M (Brattstrom et al., 1998b). In accordance with a pooled RR estimate based on prospective studies, this difference in tHcy concentration might produce a RR of 1.1 to 1.2 (Ueland et al., 2000). The calculated point estimate of the *meta*-analysis (1.1, 95% CI = 0.9–1.4) performed by Brattstrom et al. (1998b), although not statistically significant, is in line with an effect of this magnitude. To identify a statistically significant RR of this size with a statistical power of 80%, one needs between 7,800–16,300 cases and an equal number of controls (Ueland et al., 2000). Currently the largest *meta*-analysis with ~12,000 cases and ~12,000 controls is being performed and the outcome will give a more definite answer to the question whether this genotype is related to an increased risk of CHD (Klerk et al., 2001). A positive answer will also be in favor of a causal relationship between the tHcy concentration and the risk of CHD.

### *E. Mechanism by Which Homocysteine Increases the Risk of Coronary Heart Disease*

The observation of McCully in 1969, already referred to under *Section II.,* led to the hypothesis that homocysteine per se was responsible for the arterial damage (McCully, 1969). Although many in vitro and in vivo studies have addressed this important issue, the mechanisms by which hyperhomocysteinemia favors the development and progression of vascular disease have not been fully elucidated. In the following paragraphs, we will describe current knowledge on this topic.

*1. In Vitro Studies.* Studies performed in vitro showed that elevated tHcy concentrations affect the endothelial cell at multiple levels. Endothelial cell injury, platelet activation, deleterious effects on thrombomodulin expression, protein C activation, tissue factor activity, and increased oxidizability of low-density lipoproteins have been described as a few possible mechanisms by which homocysteine provokes arteriosclerosis and thrombosis (Starkebaum and Harlan, 1986; Rodgers and Conn, 1990; Lentz and Sadler, 1991; Fryer et al., 1993; Cobbaert et al., 1997; Vychytil et al., 1998). In addition, homocysteine has also been reported to have adverse effects on smooth muscle cells by induction of cyclin A gene expression (Tsai et al., 1994, 1996; Schachinger et al., 1999) and increased transcription of cyclin-dependent kinase, a regulatory protein in mitosis (Lubec et al., 1996). Both these actions may lead to enhanced smooth muscle cell proliferation.

Several recent studies have used genetic techniques, such as differential display and gene expression arrays, to assess potentially pathogenic changes in gene expression upon exposure of cultured endothelial cells to (very) high tHcy concentrations. Among the changes reported are (*i*) the induction of the stress protein GRP78/BiP (Kokame et al., 1996); (*ii*) the induction of the translational elongation factors EF-1$\delta$, EF-1$\alpha$, and EF-1$\beta$ (Chacko et al., 1998); and (*iii*) the induction of several mitochondrial genes (Austin et al., 1998).

The diversity in effects mentioned above may reflect a true variety, but may also be due to the difficulty to mimic atherosclerotic processes in short term in vitro studies, or to account for the in vivo interrelations between aminothiols, such as homocysteine and cysteine (Refsum et al., 1998). Furthermore, the in vitro studies are severely hampered by the use of super physiological concentrations, often up to 5 to 10 mM, and commercial preparations that also contain the D-homocysteine enantiomer next to the physiologically relevant L-homocysteine form. Finally, most studies were performed with the reduced form of homocysteine, whereas in vivo ~99% of the homocysteine moieties are present in oxidized forms in blood. The experimental concentrations used, sometimes approached levels that were a few orders of magnitude higher than observed in vivo. There-

fore, the relevance of these studies for our understanding of the pathophysiology of hyperhomocysteinemia is debatable.

*2. Studies in Patients with Homocystinuria.* Studies in patients with severe hyperhomocysteinemia have shed some light on potentially relevant mechanisms. Di Minno et al. (1993) studied 11 homocystinuria patients with a homozygous cystathionine β-synthase deficiency and observed an increased excretion of thromboxane metabolites, a parameter of platelet activation. Whether this activation reflected an etiologic factor or a phenomenon secondary to other mechanisms remained unsolved. One potential mechanism that could have led to enhanced platelet activation involves the increased oxidation of low-density lipoproteins. However, Blom et al. (1995) did, for example, not find evidence for increased lipid peroxidation in 10 homocystinuria patients compared with 10 healthy subjects.

Celemajer and coworkers (1993) were one of the first to investigate whether endothelial dysfunction, which is assumed to be an early sign of vascular damage, was present in subjects with hyperhomocysteinemia. They studied endothelium-dependent and endothelium-independent vasodilatation in nine young children with homocystinuria and in 14 obligate heterozygous parents and showed that the endothelium-dependent vasodilatation was impaired in the children, but preserved in the heterozygous adults. In contrast, endothelium-independent vasodilatation (as assessed by sublingual administration of nitroglycerin) was similar in both groups, indicating that the impaired vasodilatation in the children was secondary to endothelial dysfunction (Celermajer et al., 1993).

*3. Studies on Homocysteine and Endothelial Function.* Endothelial cells play a crucial role in regulating and maintaining vascular health. In addition, these cells are essential to hemostatic processes of cell adhesion and migration, coagulation, and fibrinolysis (Brown and Hu, 2001). A key regulatory system of endothelial cells involves nitric-oxide synthase (eNOS), which synthesizes nitric oxide (NO) and citrulline from L-arginine. Endothelium-derived NO regulates vessel tone, inhibits platelet activation, adhesion and aggregation, limits smooth muscle proliferation, and modulates endothelial-leukocyte interaction (Thambyrajah and Townend, 2000).

Recently, prospective epidemiological evidence became available on the assumption that endothelial dysfunction indeed precedes the development of atherosclerosis. After following 157 patients with mild coronary artery disease for a mean period of 28 months, a significantly higher proportion of cardiac events occurred in patients with severe endothelial dysfunction ($n = 42$) compared with those with no or mild endothelial dysfunction (Suwaidi et al., 2000).

The endothelial dysfunction observed in subjects with hyperhomocysteinemia may be due to a reduced bioavailability of endothelium-derived NO. Several hypotheses have been raised to explain the reduced availability of NO. First, at normal tHcy concentrations, homocysteine may react with NO to form *S*-nitroso-homocysteine, which has some of the properties of NO: it inhibits platelet aggregation, it is a vasodilator, and it prevents the formation of reactive oxygen species. However, when endothelial cells are continuously exposed to higher tHcy concentrations they are no longer able to reduce the toxicity of homocysteine (Stamler et al., 1993; Upchurch, Jr. et al., 1996). Second, homocysteine may also reduce NO availability by the formation of reactive oxygen species (Starkebaum and Harlan, 1986; Jia and Furchgott, 1993; Loscalzo, 1996) and by inhibition of glutathione peroxidase (Loscalzo, 1996; Upchurch et al., 1997), which is an important enzyme in the protection of the endothelial cell against oxidative stress.

Recent insights from studies with monkeys (Boger et al., 2000), cell cultures (Stuhlinger et al., 2001) and humans (Boger et al., 2001) indicate that the reduced bioavailability of NO seen in hyperhomocysteinemia might (also) be the result of the generation of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of eNOS. ADMA is formed when L-arginine is methylated. In other words, formation of ADMA does not only result in the inhibition of eNOS, but it also leads to a lower availability of L-arginine for the formation of NO.

Methionine may serve as the methyl group donor for the methylation of L-arginine and when methionine is demethylated, homocysteine remains (Boger et al., 2001). This suggests that increased tHcy concentrations may be a marker of elevated ADMA levels. ADMA concentrations are associated with endothelial dysfunction (Boger et al., 1998), atherosclerosis (Boger et al., 1997) and, independently of tHcy concentrations, with stroke (Yoo and Lee, 2001). Yet, exposure of endothelial cells to DL-homocysteine increased the levels of ADMA in a dose-response fashion (Stuhlinger et al., 2001), which points to a causal role of homocysteine instead of a marker role. More research on how homocysteine may affect NO bioavailability is warranted before definitive conclusions on this mechanism can be drawn.

*4. Studies on Homocysteine and Endothelium-Derived Nitric Oxide.* Given that NO regulates the vessel tone and that homocysteine might decrease the amount of NO, many studies have focused on the relationship between endothelium-dependent vasodilatation and homocysteine.

Impaired endothelium-dependent flow-mediated vasodilatation has been documented in subjects with mildly elevated tHcy concentrations. Tawakol et al. (1997) compared two groups of elderly people, i.e., subjects with tHcy >16 μM versus subjects with tHcy <11 μM, and demonstrated an impaired flow-mediated dilatation in those with a high tHcy concentration. Similar results were reported in a small group ($n = 14$) of Chinese middle-aged subjects with a mean tHcy concentra-

tion of 34.8 $\mu$M (Woo et al., 1997). Furthermore, endothelium-dependent vasodilatation was impaired in healthy volunteers subjected to a standardized methionine loading test (Bellamy et al., 1998; Chambers et al., 1998, 1999a; Nappo et al., 1999). Moreover, a low dose of oral methionine or dietary animal protein induced an impaired flow-mediated dilatation in 18 healthy volunteers (Chambers et al., 1999b).

As mentioned before, the effect of homocysteine on endothelial function might be mediated by oxidative stress (Starkebaum and Harlan, 1986; Jia and Furchgott, 1993; Loscalzo, 1996). This hypothesis has been tested by the administration of antioxidant vitamins. In a blinded randomized crossover study with 20 healthy staff volunteers, Nappo et al. (1999) demonstrated that pretreatment with vitamin E and vitamin C prevented the impairment of endothelial functions following acute hyperhomocysteinemia due to methionine loading. These results agree with those of Chambers et al. (1999a) who demonstrated that a rapid onset of endothelial dysfunction, after a methionine load-induced hyperhomocysteinemia, was reversible with vitamin C therapy.

Note that some of the above studies can formally not exclude the possibility that the endothelial dysfunction was an effect of methionine (or another metabolite) rather than of homocysteine. This can be excluded by using folic acid, a compound that lowers the tHcy concentration (Clarke and Armitage, 2000) but does not affect methionine levels (Brouwer et al., 1999a). The following section will evaluate the effect of folic acid supplementation on endothelial function and other endpoints.

### *F. Intervention Trials*

Because of the tHcy-lowering effect of folic acid, several studies investigated the effect of folic acid supplementation on intermediate endpoints of vascular damage. Endothelial dysfunction is such an intermediate endpoint. Brown and Hu (2001) recently reviewed trials that considered the effect of folic acid supplementation on endothelial function. The general picture that emerges from these studies is that folic acid (5 to 10 mg/day) improves or restores endothelium-dependent vasodilatation and may decrease the chance of thrombosis by reducing levels of coagulation factors in healthy subjects and in patients with high tHcy concentrations (Brown and Hu, 2001). The observed benefit is probably largely explained by the lowering of tHcy concentrations. However, in one study, infusion of 5-methyl-THF, the natural form of folic acid, improved endothelial function without an effect on the tHcy concentration (Verhaar et al., 1998). An independent beneficial effect of 5-methyl-tetrahydrofolate on the endothelial function in diabetic rats has recently also been shown (De Vriese et al., 2002). Potential mechanisms to explain the beneficial effect of folates on the endothelial function independent of the tHcy concentration need to be established but may involve an antioxidant effect (Verhaar et al., 1998), the regeneration of the cofactor for eNOS, i.e., tetrahydrobiopterin ($BH_4$) (Verhaar et al., 1999), or stimulation of eNOS (Stroes et al., 2000).

In light of these results, it is interesting to mention that Chambers et al. (2000) suggest that the lack of a strong association between lower tHcy concentrations in response to folic acid therapy, and improved endothelial function is due to the measurement of *total* homocysteine as the only index of the homocysteine status. Table 3 describes this more extensively.

Currently the results of three noncontrolled trials are available. Two Dutch trials showed that supplementation with folic acid (5 mg) and vitamin $B_6$ (250 mg) reduced the risk of cardiovascular events (coronary, peripheral, and cerebral) in patients with high tHcy concentrations and existing CVD to the level of patients with existing CVD but with normal tHcy concentrations (de Jong et al., 1999; Vermeulen et al., 2000a). The other trial investigated the effect of folic acid (2.5 mg), vitamin $B_6$ (25 mg), and vitamin $B_{12}$ (250 $\mu$g) on the regression of carotid plagues. Vitamin supplementation resulted in a decreased rate of progression of the plague growth in 101 patients with vascular disease with normal and elevated tHcy concentrations (Hackam et al., 2000).

TABLE 3
*Nonprotein-bound and reduced free homocysteine as an index of the biological activity of homocysteine*

| |
|---|
| Chambers et al. (2000) showed in a randomized placebo-controlled intervention study in 89 male CHD patients, that endothelium-dependent flow-mediated dilatation improved after 8 weeks of supplementation with folic acid and vitamin $B_{12}$. This improvement correlated significantly with a reduction in nonprotein-bound homocysteine ($\sim$30% of the tHcy concentration) but was independent of changes in protein-bound homocysteine and plasma folate and vitamin $B_{12}$. Furthermore, in another study of their group with 14 healthy volunteers (10 men, 4 women) (Chambers et al., 2001), they found that the reduction in flow-mediated dilatation maximally correlated with concentrations of free reduced homocysteine ($\sim$1% of the tHcy concentration) but not with the other forms of homocysteine. |
| The correlation between the tHcy concentration and reduced free homocysteine is small (0.4, $p < 0.05$) (Mansoor et al., 1995), which excludes that the tHcy concentration can be used as a proxy for the free reduced homocysteine concentration. Measuring free reduced homocysteine is a complicated procedure, requiring immediate blood sampling processing due to rapid changes in redox status of free aminothiols ex vivo and stored plasma samples cannot be used (Mansoor et al., 1992). It therefore seems unlikely that the hypothesis that free reduced homocysteine is responsible for atherothrombosis (Chambers et al., 2001) can be confirmed in large scale prospective epidemiological studies. |

Besides the noncontrolled trials, the results of two double-blind randomized placebo-controlled trials are also available (Vermeulen et al., 2000b; Schnyder et al., 2001). Both trials used intermediate endpoints. Vermeulen et al. (2000b) observed fewer abnormal exercise electrocardiography tests after 2 years of supplementation with 5 mg of folate and 250 mg of vitamin $B_6$ ($n$ = 78) compared with the placebo group ($n$ = 80). However, the internal validity of the exercise electrocardiography tests is questioned (Bostom and Garber, 2001). Moreover, an effect of treatment on other surrogate endpoint measures (ankle-brachial pressure index and duplex scanning of the carotid and peripheral arteries) was not observed (Vermeulen et al., 2000b). The intervention study of Schnyder et al. (2001) investigated the effect of a daily combination of folic acid (1 mg), vitamin $B_{12}$ (400 $\mu$g), and pyridoxine (10 mg) on the rate of the restenosis after angioplasty. After 6 months of supplementation, the intervention group ($n$ = 105) showed a significant reduction of the rate of restenosis compared with the placebo group ($n$ = 100) as assessed by quantitative coronary angiography (Schnyder et al., 2001).

The results of the above-mentioned intervention trials with intermediate endpoints favor the hypothesis that lower tHcy concentrations are causally associated with a decreased risk of vascular disease in patients with CVD (de Jong et al., 1999; Hackam et al., 2000; Vermeullen et al., 2000a; Schnyder et al., 2001) and in healthy subjects (Vermeulen et al., 2000b). But, in all intervention trials, folic acid was used. As this vitamin may have a favorable effect on e.g., endothelial function (Verhaar et al., 1998; Stroes et al., 2000) independently of the tHcy concentration, it is not clear whether the observed effect is due to a direct folic acid effect or to a decrease in tHcy concentration.

Ongoing intervention trials will answer the question whether a lower tHcy concentration through vitamin supplementation (folic acid, vitamin $B_6$, and $B_{12}$) has an effect on "hard" endpoints, like CHD mortality (Clarke and Collins, 1998; Clarke and Armitage, 2000). The results of these trials will become available within 2 to 4 years. All these trials used pharmacological doses of the B vitamins, which, in case of a positive outcome, precludes lowering the tHcy concentration in high-risk subjects by means of low doses of supplemental folic acid, fortification, or dietary measures. This is unfortunate because it is not quite clear what the effects may be of long-term supplementation with folic acid (Kelly et al., 1997). Therefore, by the end of 2002, we will start a double-blind randomized placebo-controlled secondary intervention trial with supranutritional doses of B vitamins in France (SU.FOL.OM3 study).

If the above-mentioned trials show a reduction in CHD (or other vascular endpoints), this does not answer the question whether the tHcy concentration is a causal risk factor in healthy subjects, because all trials included high-risk populations to increase the statistical power. Note, that it is questionable whether the trials performed in the United States are able to show a reduction in CHD, because they will likely suffer from a lack of power due to the mandatory folic acid fortification since 1998 (Bostom et al., 2001).

A question that also will not be answered by the ongoing trials is whether a lower tHcy concentration or a higher B vitamin intake (or both) are causes of a reduction in CHD. This answer might be provided by trials using other tHcy-lowering compounds, like betaine (Brouwer et al., 2000). However, it is not inconceivable that extra betaine will affect the availability of folates, by influencing methyl group metabolism, and therefore it may still not be possible to indicate a causal component.

### *G. Conclusion about the Relationship between Homocysteine and Coronary Heart Disease*

Table 4 weighs the available evidence to the extent that it offers support for the tHcy concentration as a causal risk factor for CHD. It is beyond reasonable doubt that the extremely disturbed homocysteine metabolism in patients with inborn errors of homocysteine metabolism cause CHD. However, what is the relevance of this causal relation for the relation between moderately elevated tHcy concentrations and CHD? Moderately elevated tHcy concentrations are associated with CHD in high-risk subjects, for instance with diabetes or with a history of CHD. In these patients an increased tHcy concentration might provoke the event, resulting in a short-term association with the risk of CHD. On the other hand, the tHcy concentration might just as well be a marker of the degree of vascular disease.

Current epidemiological evidence does not provide strong evidence that elevations in the tHcy concentration are harmful in healthy subjects, but lowering tHcy concentrations through administration of folic acid and vitamin $B_6$ favorably influenced the progress of atherosclerotic disease in healthy subjects measured with an exercise electrocardiography test (Vermeulen et al., 2000b). The results of another primary intervention trial with healthy subjects and well validated intermediate endpoints (carotid wall thickness and stiffness) will be available in 2004 (Durga et al., 2001).

The only study that can answer the question whether the tHcy concentration is a causal risk factor for CHD in healthy subjects is an intervention trial with healthy subjects, hard endpoints, and tHcy-lowering nutrients other than folic acid. This trial should be carried out in apparently healthy subjects (e.g., free of CVD and diabetes) with elevated tHcy concentrations to avoid a statistical power problem. Betaine may be used as a tHcy-lowering nutrient (Brouwer et al., 2000). It is, however, more likely that such a trial will be initiated after the results of the secondary intervention trials become available. If these trials do not show a reduced incidence

of CHD in treated high-risk subjects, this will likely exclude an intervention study in healthy subjects.

## VII. Directions for Future Research

Pending the results of the intervention trials, more epidemiological (prospective) studies on the tHcy concentration and CVD are not desirable, because they cannot provide the ultimate answer for the causality question. Future research should focus on experiments that elucidate which *form* of homocysteine [reduced, (non-) protein bound] might cause atherosclerosis and/or thrombosis and by which etiologic *pathway*. A promising pathway involves endothelial dysfunction. So far, endothelial function in response to high tHcy concentrations, or in response to folic acid administration, has only been investigated in small groups of patients or healthy subjects. Future studies should include larger groups of healthy subjects. In addition, to disentangle whether the increase in folic acid or the reduction in the tHcy concentration is beneficial for the endothelium, it would be interesting to explore the independent effects of betaine, vitamin $B_6$, vitamin $B_{12}$, or vitamin $B_2$ supplementation on endothelial function.

Irrespective of whether beneficial effects on endothelial function are due to a lower tHcy concentration or a higher folate concentration, a higher folate intake will improve endothelial function. Thus, research on the desired folate intake level to achieve beneficial endothelial responses is warranted. For example, endothelium-dependent vasodilatation and other responses associated with endothelial function (improved hemostatic balance) could be monitored in experiments in which participants are provided with folate-rich meals containing different doses of folate.

A research question that remains interesting to investigate in observational studies is to what extent the tHcy determinants in the general population are important in subjects with a high risk of vascular disease. For example, in the general population, drinking 6 cups of coffee per day is associated with an average increase in the tHcy concentration of 1.3 $\mu$M (de Bree et al., 2001d). It is not known how strong this relationship is in subjects with CVD or in other high-risk subjects.

Finally, effort should be taken to standardize the measurement of the tHcy concentration. If it turns out that the tHcy concentration causes vascular diseases, it will be of utmost importance to precisely and validly measure its concentration.

## VIII. Implications for Prevention and Treatment

Although definitive proof for a causal role of the tHcy concentration in the etiology of CHD is lacking, even a moderate effect of the tHcy concentration on the occurrence of CHD deserves attention, particularly since simple, safe, and inexpensive treatments exist that can lower the tHcy concentration. Because folate is the most important modifiable determinant of the tHcy concentration, it seems attractive to this vitamin as a means to reduce the tHcy concentration. In the United States, folic acid fortification for the prevention of neural tube defects has proven to effectively lower tHcy concentrations (Jacques et al., 1999; Lawrence et al., 1999). However, folic acid fortification has the negative side effect that it may mask a vitamin $B_{12}$ deficiency by correcting the hematological, but not the neurological, abnormalities of vitamin $B_{12}$ deficiency. Based on this masking effect of folic acid, the Health Council of The Netherlands decided that only those products specifically in-

TABLE 4
*Evidence for homocysteine being causally involved in the etiology of CHD*

| Type of Evidence | Extent to Which It Offers Support | Comment |
|---|---|---|
| Inborn errors | ++++ | Untreated, these patients suffer or even die of vascular disease; treatment prevents or delays vascular events |
| Retrospective and cross-sectional studies | + | Due to the fact that blood sampling occurs after the event, the effect of CHD on tHcy concentrations cannot be excluded |
| Prospective studies with healthy subjects | + | Evidence is stronger in older subjects and in studies with a short follow-up period |
| Prospective studies with high-risk subjects | ++ | In these types of studies it cannot be excluded that the increased levels of tHcy are a marker of the degree of vascular disease |
| Prospective studies with venous thrombosis | + | These studies indicate that the tHcy concentration may predominantly be a thrombogenic factor, although the number of prospective studies is small |
| MTHFR 677C>T genotype | + | Lack of evidence that the 677TT genotype is associated with CHD may be a power problem |
| Mechanism of action | ++ | Especially the relationship with endothelial function seems plausible |
| Intervention trials with intermediate endpoints | +++ | Beneficial effects could also be the result of folic acid |

+, indicates minor support; ++++, indicates strong support.

tended for women who want to become pregnant may be fortified with folic acid (Health Council of The Netherlands, 2000). It is possible that this recommendation will be reconsidered as soon as evidence from the secondary trials on the prevention of CVD (described above) becomes available. Note, however, that the ongoing discussion of the masking of a vitamin $B_{12}$ deficiency would become irrelevant when fortification with folic acid is accompanied by simultaneous fortification with vitamin $B_{12}$.

The increased attention on the tHcy concentration has created a demand for guidelines by general practitioners and specialists, like cardiologists. As a result of this, The Netherlands Heart Foundation has published a report that contains temporary guiding principles, pending the results of intervention trials. For the Dutch situation, a "screen-and-treat" scenario is suggested (Netherlands Heart Foundation, 2001b). In addition to avoiding negative side effects of the "treat-all" scenario by fortification, the screen-and-treat scenario is probably more cost-effective than a treat-all scenario (Nallamothu et al., 2000).

Persons that are eligible for screening, i.e., determined by measurement of their tHcy concentration, are those with a high risk of CHD, e.g., patients with diagnosed cardiovascular occlusions, thrombosis, diabetes, renal insufficiency, and an unfavorable history of CHD in the family. Subjects with an elevated tHcy concentration can be treated with a daily supplement of 500 $\mu$g/day folic acid because this was proven to be equally effective in lowering the tHcy concentration as supplements with higher doses (Brattstrom et al., 1998a; Clarke and Armitage, 2000). Depending on the laboratory, the definition of elevated tHcy concentrations may differ, but generally a level above $>15$ $\mu$M (Ueland et al., 1993) is considered elevated. Patients that are treated with folic acid should be followed up regularly to monitor their vitamin $B_{12}$ status and to see whether their tHcy concentration decreases. If the tHcy concentration does not decrease, a higher dose of folic acid can be chosen or additional ways to lower the tHcy concentration may be used, like administration of vitamin $B_6$ or betaine. The tHcy-lowering treatment should never interfere with the established treatments to prevent CHD, like cholesterol-lowering medication, antihypertensive treatment, and encouragement of a healthy lifestyle (Netherlands Heart Foundation, 2001b).

For subjects without a high risk of CHD, an optimal folate intake might be beneficial as well, since a low folate concentration or intake is not only inversely associated with the risk of CHD but also with colon cancer (Chen et al., 1999; Konings, 2001), pregnancy complications (van der Put et al., 2001), and dementia (Ebly et al., 1998; Hassing et al., 1999; Lindeman et al., 2000; Seshadri et al., 2002). Therefore, it seems worthwhile to initiate and maintain public health educational programs targeted at increasing the consumption of plant foods. Furthermore, stimulating a healthy lifestyle with moderate coffee and alcohol consumption, and no smoking, may contribute to a lower tHcy concentration. This will lead to a lower incidence of CHD, independent of the tHcy concentration.

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