

REVIEW

Total homocysteine and cardiovascular disease

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Abstract. Nygård O, Vollset SE, Refsum H, Brattström L, Ueland PM (University of Bergen, Norway; County Hospital, Kalmar, Sweden). Total homocysteine and cardiovascular disease (Review). *J Intern Med* 1999; **246**: 425–454.

Recent data have shown that an elevated plasma level of the amino acid homocysteine (Hcy) is a common, independent, easily modifiable and possibly causal risk factor for cardiovascular disease

(CVD) which may be of equal importance to hypercholesterolemia, hypertension and smoking. This paper reviews the biochemical, clinical, epidemiological and experimental data underlying this conclusion and is critically questioning whether elevated tHcy is a causal factor.

Keywords: homocysteine, atherosclerosis, cardiovascular disease, pathogenesis.

History

The sulphur amino acid homocysteine (Hcy) was first described by Butz and du Vigneaud in 1932 [1]. One year later, a case report in the *New England Journal of Medicine* described an 8-year-old mentally retarded boy who died from a massive infarction of the right cerebral hemisphere and atherothrombosis of the internal carotid arteries [2]. This is believed to be the first description of homocystinuria. More than 30 years later the brother and two nieces of the patient were then confirmed to have this rare metabolic error characterized by severely elevated Hcy levels [3].

The biochemical finding of homocystinuria was first described in mentally retarded children in 1962 [4, 5]. Only two years later, Mudd *et al.* reported that the metabolic defect was due to cystathionine β -synthase (CBS) deficiency. Later, it was recognized that defects in other enzymes, including methionine

synthase and methylenetetrahydrofolate reductase (MTHFR) also cause homocystinuria [6].

Clinical and pathological observations in patients with homocystinuria suggested a pathogenic role of elevated Hcy: First, the homocystinuria patients have a high incidence of premature cardiovascular episodes, in adolescence and even in childhood. Secondly, the vascular lesions occur independently of the site of the metabolic lesion, suggesting that Hcy itself and not a remote metabolic defect is responsible for the CVD [3, 6–10]. Based on these observations, McCully in 1969 presented his homocysteine theory of atherosclerosis [8, 11], suggesting that an elevated Hcy level may be a risk factor for CVD in the general population.

In 1976, the first clinical study supporting this theory was published by Wilcken and Wilcken who found that patients with angiographically verified coronary artery disease (CAD) had higher levels of Hcy-cysteine mixed disulphide after a methionine

load than the controls [12]. Since then, about 100 clinical and epidemiological studies on the relationship between Hcy and occlusive disease in the coronary, cerebral and peripheral arteries, and in the veins have been published. These studies demonstrate that an elevated Hcy level is a strong risk factor for CVD [13–16].

Biochemistry

Metabolism

The sulphur amino acid Hcy is not a dietary constituent and is not incorporated into proteins [6] but is exclusively formed as an intermediary product of methionine metabolism (Fig. 1). Through the action of methionine adenosyltransferase (E.C. 2.5.1.6), methionine is converted to S-adenosylmethionine, which is the major biological methyl donor required for numerous cellular processes, including the formation of creatinine and methylation of phospholipids [6, 17–20]. These reactions are catalysed by various methyltransferases that demethylate S-adenosylmethionine to S-adenosylhomocysteine, which is the immediate precursor of Hcy [6].

Once Hcy is formed, it may be salvaged to methionine by methylation, or degraded to cysteine by transsulphuration. Remethylation to methionine is in most tissues catalysed by the ubiquitous

enzyme, methionine synthase (E.C. 2.1.1.13). This enzyme uses vitamin B₁₂ as cofactor, and 5-methyltetrahydrofolate as methyl donor. 5-Methyltetrahydrofolate is formed by the vitamin B₂-dependent enzyme MTHFR (E.C.1.1.99.15). Hcy may also be converted to methionine by betaine-homocysteine methyltransferase (E.C. 2.1.1.5) using betaine as a methyl donor. This reaction is probably confined to the liver and possibly the kidney [6, 21].

Two vitamin B₆-dependent enzymes are involved in the transsulphuration pathway. The enzyme CBS (E.C.4.2.1.22) first condenses Hcy with serine to form cystathionine, which is then cleaved into cysteine and α-ketobutyrate by cystathionine γ-lyase (E.C.4.4.1.1) [6, 22]. Cysteine may be utilized in the synthesis of proteins or as a precursor of the antioxidant glutathione. The transsulphuration of Hcy to cysteine is irreversible, and therefore directs Hcy to catabolism via cysteine to sulphates as the final product.

Under normal metabolic circumstances, there is a strict balance between Hcy formation and elimination. Usually about 50% of the Hcy formed is remethylated to methionine. When protein or methionine intake is in excess, a larger proportion is catabolized by the transsulphuration pathway [19, 22]. If there is an increased formation of Hcy relative to its consumption, Hcy is excreted from the cells. This can be detected as an increased level of Hcy in plasma/serum or in the urine [23].

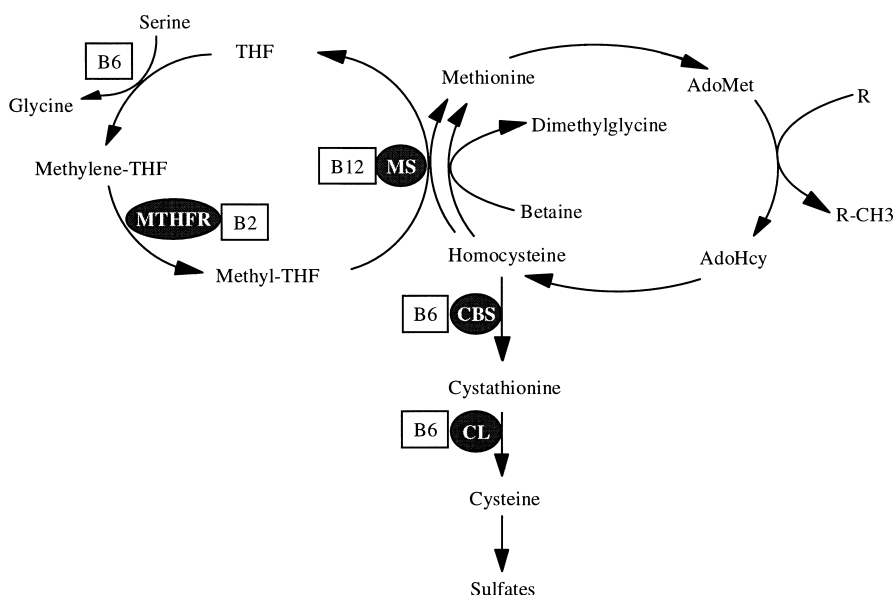


Fig. 1 Homocysteine metabolism: AdoMet; S-adenosyl methionine, AdoHcy; S-adenosyl homocysteine, THF; tetrahydrofolate, CBS; cystathionine β-synthase, CL; cystathionine γ-lyase, MS; methionine synthase, MTHFR; methylenetetrahydrofolate reductase, B6; vitamin B6, B12; vitamin B12, B2; vitamin B2.

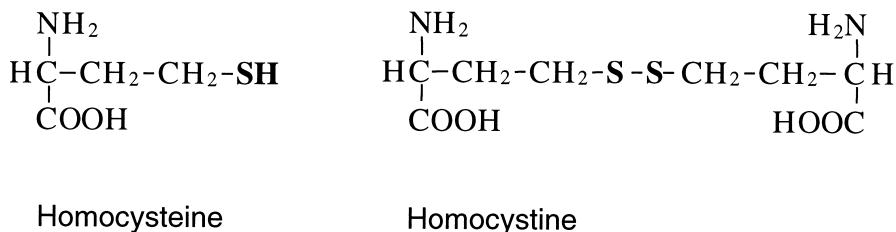


Fig. 2 Structural formulas of homocysteine and homocystine. Homocysteine is an amino acid with a free sulfhydryl (SH) group, whereas homocystine is the symmetric disulphide of two homocysteine molecules.

Homocysteine in blood

The Hcy concentration in plasma or serum is about 10 μmol L⁻¹ in adults. However, Hcy exists in various forms (Figs 2 and 3); only trace amounts (~1%) are in the reduced (sulfhydryl) form, the remaining part is oxidized and exists as various disulphides [24]. About 70% is bound to albumin (via a disulphide bond), whereas the remaining 30% exists as free disulphides, mostly as a Hcy-cysteine mixed disulphide [6]. After blood sampling, there is a rapid redistribution between the free and protein-bound fractions of Hcy [25]. This interconversion of the different Hcy species explains the analytical problems that were overcome by the introduction of new methods for total Hcy (tHcy) determination during the 1980s [23].

Terminology

Homocysteine refers to a specific chemical com-

ound; it is an amino acid with a free sulfhydryl group (Fig. 2). The homocysteine symmetric disulphide is termed homocystine, and the term homocystinuria (see below) denotes homocystine in urine and reflects the way in which this disease was originally identified. The other oxidized forms of homocysteine are referred to as homocysteine mixed disulphides (Fig. 3). The abbreviation Hcy is usually used for both homocysteine and its oxidized species. Total homocysteine, abbreviated tHcy, is a methodological term and refers to the concentration of homocysteine obtained after plasma/serum has been treated with a reductant which converts the free and bound disulphides into their respective sulfhydryl compounds.

Hyperhomocysteinemia refers to an elevated tHcy concentration in blood, and has been divided into mild (15–30 μmol L⁻¹), intermediate (30–100 μmol L⁻¹) and severe (> 100 μmol L⁻¹) hyperhomocysteinemia [26]. Homocystinuria is now a term exclusively used for the inborn errors of

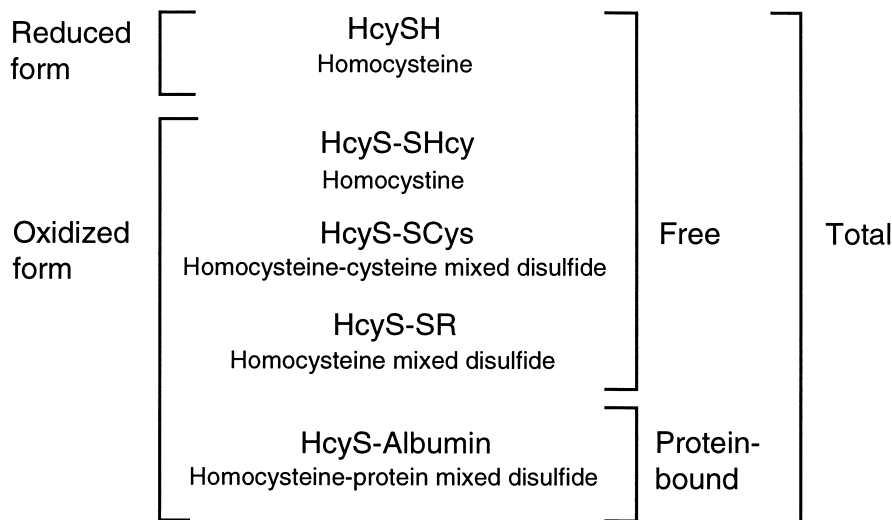


Fig. 3 The various forms of homocysteine (Hcy) in blood.

metabolism leading to severe hyperhomocysteinaemia. Because Hcy is always present in blood, the term homocysteinemia is not clinically useful and should be abandoned.

Homocysteine determination

Measurement. A comprehensive review of the history and principles underlying the various methods for Hcy or tHcy determination has been published [23]. The initial methods were based on qualitative tests, such as a positive nitroprusside reaction, or the use of amino acid analyses developed in the late 1950s [27]. In the mid-70s, these methods were replaced by second-generation amino acid analysers, which for the first time allowed the determination of the Hcy-cysteine level in plasma or serum from healthy subjects. Methods for protein bound Hcy [28] and tHcy [25] determination were introduced some few years later and greatly facilitated clinical research on tHcy. The common principle for all tHcy methods is the use of a reductant in order to cleave the disulphide bonds between Hcy and proteins or other thiols, thereby forming reduced homocysteine, which can be quantified directly or after derivatization. The majority of the assays are based on chromatographic techniques; high performance liquid chromatography (HPLC) with fluorescence detection is the method most commonly used [23]. The development of a rapid and fully automated HPLC method [29, 30] was a prerequisite for performance of the large-scale clinical and epidemiological Hcy studies in Norway [31, 32].

Factors that influence tHcy determination. The procedures for collection and processing of the blood sample are critical steps in the determination of tHcy [30, 33].

It is generally recommended that subjects should be fasting. In healthy subjects, the influence of food intake is, however, limited [33–36]; a protein-rich meal may increase the level by 10–15%, reaching a maximum 6–8 h after food intake [36]. The posture of the subject during blood collection should also be taken into account. Albumin, which binds the major portion of Hcy in plasma, is lower in the recumbent compared to the upright position [37]. The problem might be relevant in case-control studies where the patients may be in bed, whereas the controls are

usually called in, and the blood collected in the sitting position.

After blood sampling, there is a time- and temperature-dependent release of Hcy from blood cells [38]. This leads to an artificial increase in plasma/serum tHcy concentration which amounts to 15–20% per hour at room temperature [23]. This artifact can be prevented by keeping the whole blood on ice or by adding a stabilizer such as sodium fluoride [33] or acidic citrate [39]. Once separated from the blood cells, the plasma tHcy concentration is stable for at least 4 days at room temperature, for 2 weeks at 0–2 °C, and for months or years when frozen at –20 °C [23, 40–42].

The within-person or biological variability of tHcy has been tested in several studies, and the tHcy levels show minor variation during repetitive analyses for 24 h [35], weeks [43], months [35, 43–45] or a few years [43]. The intraindividual biological coefficient of variation (CV) has been estimated to be about 9% [44, 45]. In addition, the reliability coefficient, which reflects the ability to correctly classify a subject with respect to its short-term average concentration by a single measurement, is slightly higher for tHcy (0.88) than for total cholesterol (0.85) [45] and most other clinical chemistry analytes [46]. These data therefore suggest that tHcy concentration of an individual is relatively constant, and can be determined by a single measurement when the sample handling is standardized and the method has an acceptable analytical CV.

Methionine-loading test. The concentration of tHcy in plasma or serum is usually between 5 and 15 $\mu\text{mol L}^{-1}$ in adult populations [23]. However, tHcy is increased (2, 4 or 6 h) after the intake of a high dose of methionine (0.1 g kg^{-1} or 3.8 g m^{-2}), hence, stressing the metabolic pathways. This so-called methionine loading test was originally designed to detect heterozygosity for CBS deficiency [47–52], but later studies have demonstrated that an abnormal response to methionine is common in the general population, and probably cannot be fully explained by mutations in the CBS [53]. The fact that up to 40% of subjects with elevated postload tHcy have normal fasting levels [54–56], suggests that this test provides information beyond that obtained by determining fasting tHcy level. The physiological corollary of the methionine loading is

uncertain, but it may mimic the response to dietary methionine intake. The procedure is cumbersome and difficult to apply in large-scale epidemiological studies.

Determinants of total homocysteine concentration

The tHcy level is a function of a complex interaction between multiple genetic and environmental factors [6, 57].

Genetic determinants

Discovery of homocystinuria due to CBS deficiency in 1962 [4, 5] initiated the focus on Hcy as a possible pathogenic factor. These patients have extremely high levels of tHcy in plasma (300–400 $\mu\text{mol L}^{-1}$) and urine [58]. The clinical condition is characterized by ectopia lentis, osteoporosis, skeletal anomalies, mental retardation and a high incidence of premature vascular episodes [3–7, 9, 59, 60]. CBS deficiency, an autosomal recessive disorder [6, 61], is the most common cause of homocystinuria [6, 62, 63]. However, MTHFR deficiency [64, 65] and methionine synthase deficiency [66] also lead to severe hyperhomocysteinemia ($>100 \mu\text{mol L}^{-1}$) and thromboembolic vascular disease even in childhood [10].

The genes encoding for CBS [67], MTHFR [68], methionine synthase [66, 69], as well as betaine-homocysteine methyltransferase [70], have now been cloned. In patients with mutations of the CBS gene, more than 50 different pathogenic mutations that have been found [53], and these can partly explain ethnic variations and differences in phenotypic expression amongst patients with CBS deficiency [53, 63, 71, 72].

It was discovered early that obligate heterozygotes for CBS deficiency usually have a normal fasting level of tHcy [49] but frequently respond to the methionine loading test with an abnormal increase in the tHcy concentration [6, 73]. Interestingly, patients with Down syndrome have an additional copy of the CBS gene and generally low tHcy levels, and this condition has been regarded as an atheroma-free model [74, 75].

A common polymorphism in the MTHFR gene causes low activity and thermolability of the enzyme

and is associated with elevated tHcy. Thermolability of MTHFR was first described by Kang *et al.* in 1988, who also demonstrated that this enzyme variant was commonly occurring in CVD patients [76, 77]. In 1995 it was recognized that thermolability was due to a C to T substitution at position 677 of this gene. Several studies have demonstrated that the C677T polymorphism is associated with hyperhomocysteinemia in subjects with impaired folate status [77–83]. The C677T allele frequency is high (30–40%) in most populations, and about 10% of the Caucasian population is homozygous, and therefore at particularly high risk of developing hyperhomocysteinemia. However, there is substantial interethnic variation [84, 85]. In populations of African descent [84, 86], Asian Indians (Refsum, unpublished) or Canadian Inuit [87], the prevalence is 0–2%, whereas it may be about 20% in Asians [84] and even higher in Northern Italy [88]. This difference may partly explain variable tHcy levels according to ethnicity [23, 89–91].

Physiological determinants

Blood levels of tHcy are higher in men than in women and increase with age [31, 92–99]. The difference between the genders becomes apparent in puberty [100] and is believed to be related to hormonal factors, but also to lifestyle, diet and vitamin status.

The tHcy concentration is decreased in normal pregnancies [101] but higher levels are observed in pregnancies complicated by recurrent spontaneous abortions or abruptio placentae [102, 103]. The level of tHcy is strongly related to renal function [104]. Two different mechanisms may be involved. First, in healthy subjects, the main source of Hcy is the adenosylmethionine-dependent methylation of guanidoacetate to form creatine and its anhydride creatinine [6, 99]. Creatine/creatinine synthesis is related to muscle-mass, and this may partly explain the higher tHcy (and serum creatinine) levels in men compared to women. Secondly, renal function plays a central role for clearance of both creatinine and Hcy [105–107]. Because the urinary excretion of Hcy is low [25, 108], it has been suggested that an extensive metabolism of Hcy, probably through transsulphuration, takes place in the kidneys [105, 109, 110]. Although this has recently been contested [111], the normal physiological decline in

renal function by age may to some extent explain the increase in tHcy with age [99]. A recent study including the GFR-marker cystatin C supports this assumption, and that the sex difference in tHcy is at least in part due to differences in creatinine synthesis [112].

Nutritional determinants

The postprandial as well as long-term effect of methionine intake from proteins has been investigated, but probably only causes a marginal change in the tHcy level [33–36, 54, 113]. In contrast, dietary intakes [114–117] or plasma levels (Fig. 4) of folate and vitamin B₁₂ are inversely related to tHcy concentration. Deficiencies of these vitamins may cause a moderate or even severe hyperhomocysteinemia, and an increased tHcy level is a sensitive marker of disturbed function of both folate and vitamin B₁₂ [118]. Intake or plasma levels of vitamin B₆ may be weakly related to basal tHcy [115, 119]. However, most studies find that vitamin B₆ deficiency is associated with normal fasting from tHcy, but with a marked elevation in

tHcy after methionine loading [6, 26, 57, 120, 121]. Insufficient dietary intake of the vitamins involved in Hcy metabolism seems to be common in Western countries, especially amongst the elderly [115, 119, 122].

Lifestyle

The influence of various aspects of lifestyle on the tHcy levels has been studied in the Hordaland Homocysteine Study. Data from this large-scale population based study show that smoking and heavy coffee consumption are associated with elevated tHcy levels, whereas physical activity is associated with low tHcy [31, 117, 123]. A moderate alcohol consumption may be associated with reduced tHcy levels [124], whereas a chronic high alcohol consumption is associated with elevated tHcy [124–126], possibly via impaired folate or vitamin B₆ function [126–129].

Drugs

A number of drugs influence tHcy concentration by

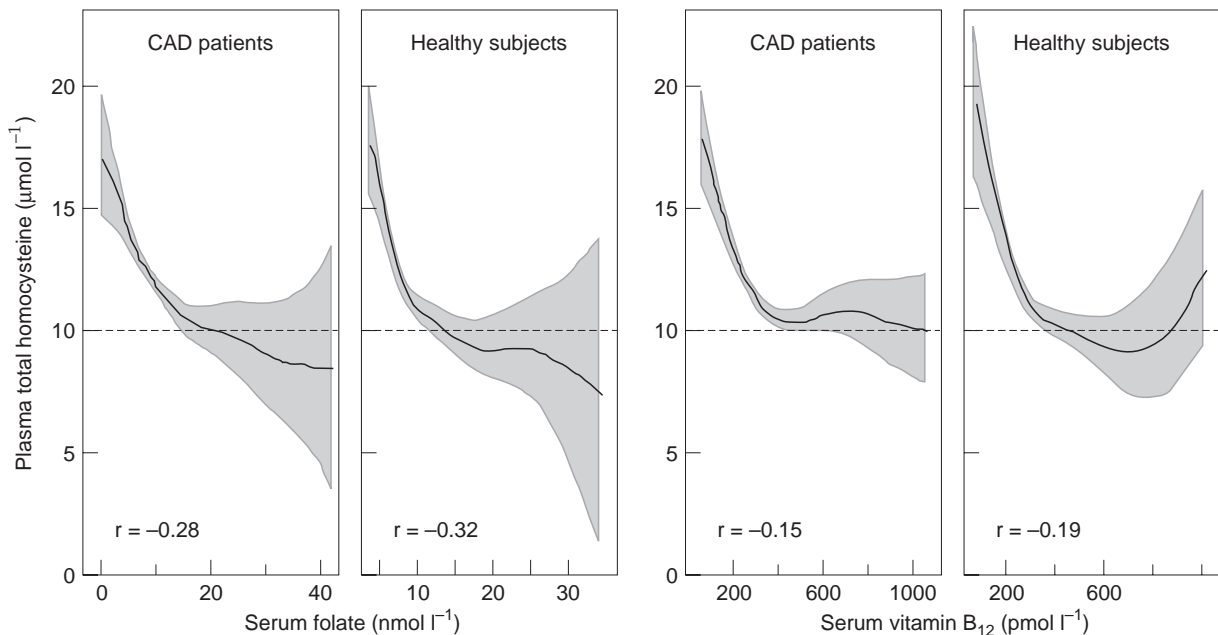


Fig. 4 Serum levels of folate and vitamin B₁₂ vs. plasma total homocysteine in 587 patients with coronary artery disease (CAD) [32] and 329 healthy subjects [117, 123]. The relationships between the vitamins and tHcy levels are similar in the two populations, although slightly stronger dose–response relationships are observed amongst the healthy individuals than amongst the patients. In both populations, folate levels show a substantially stronger relationship to tHcy than vitamin B₁₂, whereas folate demonstrates a dose–response relationship to tHcy even at high levels, the relationship to tHcy of vitamin B₁₂ is more uncertain at the highest vitamin B₁₂ levels. Crude correlations are presented.

interfering with the Hcy metabolism. Folate antagonists such as methotrexate and the vitamin B₁₂ antagonist nitrous oxide may lead to a marked tHcy elevation [130]. In renal transplant recipients, treatment with cyclosporin causes a moderate increase in tHcy [131]. The antidiabetic drug metformin may elevate tHcy by affecting folate as well as vitamin B₁₂ levels [132, 133], and tHcy is elevated in epileptics treated with anticonvulsant drugs probably because of interference with folate polyglutamation and retention [134].

Amongst hypolipidemic drugs, it has been known for several years that a combination of colestipol and niacin may elevate tHcy [135], and the association was primarily related to the folate-antagonistic effect of cholestyramine [135]. This was later confirmed in a study on hyperlipidemic children [136]. The C677T mutation in the MTHFR gene seems to predispose to this effect [137]. Niacin may also induce hyperhomocysteinemia, but by affecting vitamin B₆ status [138].

Theofyllin [139] and azaribine [140] increase tHcy by inhibiting vitamin B₆ function. Notably, azaribine, a drug previously used in the treatment of psoriasis, was related to an increased incidence of thromboembolism, and was therefore prohibited by the Food and Drug Administration in 1976 [140–142].

L-dopa becomes methylated by adenosylmethionine and may therefore elevate tHcy by enhancement of Hcy production [143, 144]. Several hormone related drugs may influence the tHcy level, but the mechanisms have not been elucidated. In women, tHcy levels are lowered by tamoxifen [145, 146], possibly by hormone replacement therapy [147–149], and some oral contraceptives [150–152], and increased by androgen administration [153]. In men, oestrogen plus antiandrogen administration has a substantial tHcy lowering effect [153].

Aminothiols such as penicillamin [154, 155], acetylcysteine [156] and ifosfamide/mesna [157] reduce the plasma tHcy level, probably by increasing renal clearance or by displacing Hcy from the protein binding sites [154, 155]. Such drugs have been suggested for the treatment of homocystinuria [154, 155]. In patients with moderately elevated tHcy levels, aminothiols are not, however, the drugs of choice since long-term therapy with these agents may have side-effects related to disulphide exchange

and redox reactions. Vitamin therapy seems to be a more effective, safer and less expensive alternative.

Various diseases

Several clinical conditions are associated with elevated tHcy levels. This can usually be explained by low vitamin status, impaired enzyme function or renal failure.

Hyperproliferative disorders, such as acute lymphoblastic leukaemia [158] and severe psoriasis [24, 159–161] are associated with elevated tHcy levels. This is probably explained by an increased Hcy export from the proliferating cells.

Recently, an increased prevalence of hyperhomocysteinemia has been reported amongst patients with hypothyroidism [162]. Acute hyperinsulinemia is associated with a decrease in tHcy concentration in normal subjects [163]. In diabetes mellitus, elevated tHcy is observed concomitant with the onset of nephropathy [164], which occurs more frequently amongst those with early onset of the disease and with a poor metabolic control [165]. Nephropathy is predicted by microalbuminuria [166], which may be a reliable index of vascular damage both in diabetic and other patient groups [167]. Recent studies show that microalbuminuria is strongly related to plasma tHcy in diabetic patients [168–170], although the relation may be independent of the diabetes per se [170].

Vitamin therapy

Hyperhomocysteinemia, as a risk factor for CVD, represents a strong incentive for investigating folic acid, vitamin B₁₂ and B₆, and possibly also betaine, as tHcy lowering therapy. Based on the role of these vitamins in Hcy metabolism (Fig. 1), a differential effect on tHcy levels in relation to fasting and methionine intake is expected. Supplementation with folic acid lowers high fasting as well as postmethionine load tHcy in most subjects, whereas vitamin B₁₂ only has an effect in subjects with vitamin B₁₂ deficiency [77, 171–173]. Vitamin B₆, even in very high doses, does not affect fasting tHcy levels, but usually reduces abnormal elevation in tHcy after a methionine load. The effect of betaine is less known, but may be effective in some patients with elevated postload tHcy levels [174, 175].

The marked tHcy lowering effect of high doses of

folic acid (5 mg day⁻¹) in the absence of folate deficiency was first reported by Brattström *et al.* in 1988 [176]. This and later studies [172, 174, 177–179] have demonstrated that 0.5–10 mg folic acid, alone or in combinations, reduces fasting as well as postload tHcy levels, usually by about 25–35%. In contrast to vitamin B₆, vitamin B₁₂ has a small additional effect, observed in vascular patients as well as in healthy subjects, and it is most pronounced in subjects with hyperhomocysteinemia [179].

In subjects with markedly elevated tHcy ($\geq 40 \mu\text{mol L}^{-1}$) and without overt vitamin B₁₂ deficiency, a daily supplement of 200 μg folic acid led to a normal tHcy level in 70% of the subjects whereas 5 mg day⁻¹ was effective amongst the remainder in this study. Most hyperhomocysteinemic individuals were homozygous for the C677T mutation of the MTHFR gene [82]. The question of the folic acid responsiveness of subjects with the C677T genotype was elaborated further in a recent study demonstrating that these subjects were more responsive than the CC subjects to the tHcy-lowering effects of 1–2 mg folic acid [180].

The effect of low doses of folic acid has been investigated in healthy subjects with normal tHcy levels. Doses of 0.3–0.4 mg day⁻¹ seem sufficient to maintain low or normal tHcy in most subjects [181, 182]. The minimal dose required probably depends on the individual's uptake, utilization and stores of folate. Thus, amongst the subjects in the lowest tertile for tHcy, a daily folic acid dose up to 400 μg induced a nonsignificant reduction in the tHcy level (from 7.1 to 6.4 $\mu\text{mol L}^{-1}$). Individuals with a tHcy level in the two upper tertiles responded to a 100- μg folic acid per day with some reduction in the tHcy level, and a further reduction was observed when the dose was increased to 200 μg and 400 μg [183].

The possible tHcy lowering effects of other vitamins have been tested in subjects with mildly elevated tHcy, but treatment with the antioxidant vitamins ascorbic acid, α -tocopherol or β -carotene did not lower the tHcy levels [184].

Cardiovascular disease

Homocystinuria

Biological plausibility of Hcy as an atherothrombotic

agent is derived from the clinical presentation of patients with homocystinuria characterized by severely elevated tHcy. Untreated, 50% of these patients suffer a thromboembolic event (half of which are venous thromboembolic events), and 20% die before the age of 30 years [6, 9]. On autopsy, findings include arteriosclerotic lesions in large and medium-sized arteries, arterial and venous thrombosis and multiple infarctions in different organs. The microscopic findings differ from the atheromatous changes in patients with hyperlipidemia, and include loosening and fragmentation of the internal elastic lamina, intimal hyperplasia and narrowing of the arterial lumen [3, 6, 57]. This difference from hyperlipidemia is also demonstrated *in vivo*; ultrasound assessed carotid intima-media thickness and blood flow velocity of middle cerebral arteries are normal in young patients with homocystinuria but clearly pathologic in familial hypercholesterolemia [185].

Arterial disease

Since the first study by Wilcken and Wilcken in 1976 [12], the results from about 100 clinical and epidemiological studies have shown that even a mild or moderate elevation of tHcy is associated with an increased risk of CVD [13].

Several pooled analyses or meta-analyses based primarily on retrospective studies have been performed. Ueland *et al.* summarized in 1992 the results of 17 studies including approximately 1500 CVD patients and 1400 controls [57]. The findings were consistent across the various forms of CVD, and showed that the fasting tHcy concentration was 32% higher amongst patients. A methionine load test had been performed in some of the studies, and an abnormal postload tHcy was detected in 24% of the patients compared to only 2% of the controls.

In a report by Boers 2 years later, the analysis was restricted to studies with postload Hcy determinations [186]. Amongst the 750 CVD patients, mild hyperhomocysteinemia was detected in 21% with CAD, 24% with cerebrovascular disease and 32% with peripheral vascular disease, compared to 2% amongst 200 controls.

The meta-analysis by Boushey *et al.* in 1995 [187] was based on 24 retrospective and 3 prospective studies and included approximately

4100 patients and a similar number of controls. In this analysis, it was estimated that the relative risk associated with a 5- $\mu\text{mol L}^{-1}$ increment in the tHcy level, was 1.7 (95% CI, 1.5–1.9) for coronary heart disease, 1.9 (95% CI, 1.6–2.3) for cerebrovascular disease and even higher for peripheral artery disease. It was further calculated that at least 10% of the population's risk of CAD might be attributable to tHcy.

Recently, Wald *et al.* summarized the results of eight retrospective studies on tHcy and myocardial infarction [188]. The combined odds for a 5- $\mu\text{mol L}^{-1}$ increase in the tHcy level was 1.84 (95% CI, 1.52–2.23), which was reduced to 1.65 if one study with an atypically high estimate was excluded.

The relationship between CVD and tHcy was studied in a large European case-control study of 800 cases and 750 controls [55]. Fasting as well as postload tHcy concentrations were about 20% higher in cases compared to controls, and similar increases in risk was observed with elevation in any of the two types of tHcy determinations. A particularly high risk was found in subjects with increased levels of both fasting and postload tHcy concentrations. After adjustment for conventional risk factors, the relative risk per 5- $\mu\text{mol L}^{-1}$ increment in fasting tHcy level was 1.35 (95% CI, 1.2–1.6) for men and 1.42 (95% CI, 0.99–2.05) for women. Notably, users of supplements containing folic acid, vitamin B₆ or vitamin B₁₂ had a relative risk of 0.38 (95% CI, 0.2–0.7) compared with nonusers.

Cross-sectional studies using angiography or ultrasound imaging have shown that tHcy is related to the extent of atherosclerosis in the carotid [189–192], peripheral [193] and coronary [32, 194–200] arteries, and tHcy is related to graft vasculopathy in transplant recipients [201].

A recent prospective study also shows that tHcy is related to the progression of coronary atherosclerosis [202]. This may indicate that tHcy promotes the atherosclerotic process. In addition, some prospective studies have shown that tHcy is related to the acute thromboembolic event such as myocardial infarction [203–206], stroke [207] or arterial thrombosis [208]. However, plasma tHcy is low in the acute phase of myocardial infarction [113, 209] and stroke [210]. Because the major part of tHcy is probably bound to albumin [6], this may be related to lower albumin levels in bedfast patients [37,

211] or to other haemodynamic changes due to the acute stress.

Mortality

Whereas most previous prospective investigations have studied presumably healthy individuals, we evaluated mortality in a cohort of 587 patients with angiographically verified CAD in 1991–92 [32]. This distinction may explain the particularly strong effect of tHcy amongst the patients. Five years follow up revealed that, together with left ventricular ejection fraction and creatinine levels, tHcy was the strongest determinant of overall and cardiovascular mortality. The difference in tHcy between those who died and those who survived was 35% in women and 20% in men (Table 1). Notably, plasma tHcy was the strongest modifiable determinant of mortality, and the mortality rate amongst individuals with tHcy <9.0 $\mu\text{mol L}^{-1}$ was less than 1% per year compared with 6% per year in those with tHcy $\geq 15 \mu\text{mol L}^{-1}$. In addition, the relation of tHcy with mortality was present in subgroups (Fig. 5).

We also found a strong relationship between tHcy and previous myocardial infarction, which probably explains the strong relationship between tHcy and ejection fraction. Plasma tHcy was only weakly related to the number of stenosed coronary arteries. These observations suggest that elevated tHcy is more strongly related to the acute event than to atherosclerosis.

Two recent studies confirm our finding that tHcy is an important risk factor for mortality in vascular patients [212, 213]. In one of these studies of patients with acute coronary syndromes, tHcy was not related to short-term mortality within 1 month but was related to mortality after a median follow up time of 3.5 years [213]. Three recent community

Table 1 Plasma total homocysteine (tHcy) levels according to mortality amongst 587 patients with angiographically verified coronary artery disease diagnosed at Haukeland University Hospital 1991–92. Mean follow up was 4.6 years

Gender	Number	Mortality (%)	Survivors tHcy ($\mu\text{mol L}^{-1}$)	Deceased tHcy ($\mu\text{mol L}^{-1}$)
Women	109	10.1	10.9	14.7
Men	478	12.1	11.7	14.0

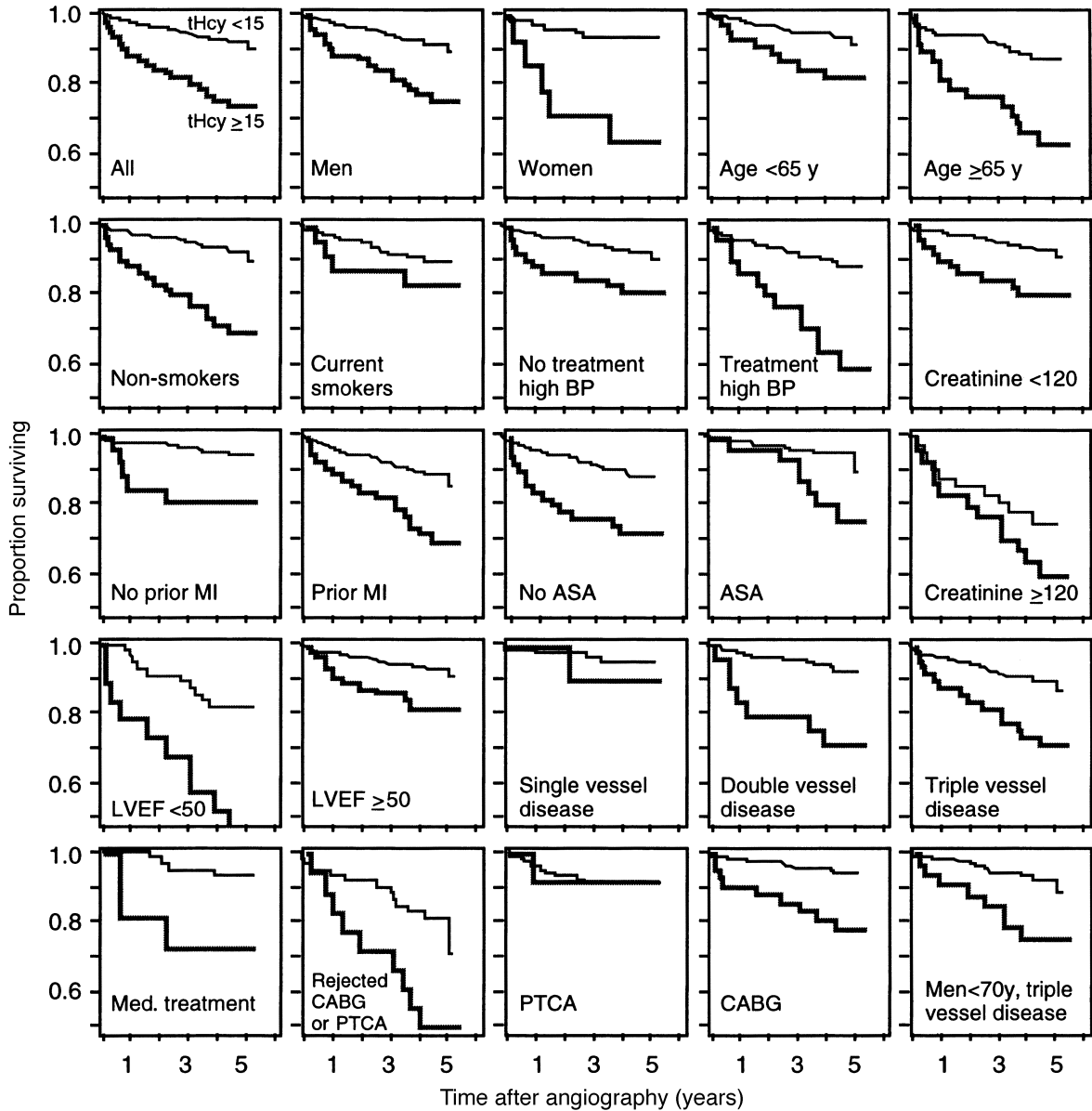


Fig. 5 Kaplan–Meier survival plots comparing patients with plasma total homocysteine (tHcy) above (bold line) and below (thin line) $15 \mu\text{mol L}^{-1}$ in various subgroups of patients with coronary artery disease [32]. BP denotes blood pressure, MI myocardial infarction, ASA acetylsalicylic acid, LVEF left ventricular ejection fraction, CABG coronary artery bypass grafting, and PTCA percutaneous transluminal coronary angioplasty.

studies now show that plasma tHcy is a risk factor for mortality, also in the general population [188, 214, 215].

Venous thromboembolic disease

Besides its relation with arterial occlusive disease, there is recent evidence that hyperhomocysteinemia is related to venous thrombosis as well [15, 216–

227]. This is perhaps the strongest evidence of a thrombotic effect of elevated tHcy.

Cardiovascular risk factors

Like the overall risk for CVD, plasma tHcy is higher in men than in women and increases with age. In women, menopause may confer an increase in tHcy beyond the effect of age [228]. There is an

association between tHcy and several established risk factors, including serum cholesterol [31, 229, 230], blood pressure [31, 92, 230], cigarette smoking [31, 204, 231], diabetes mellitus [164] and renal function [6, 25, 99, 105, 108, 232]. Notably, amongst patients with renal failure, hyperhomocysteinemia is more prevalent than other cardiovascular risk factors [104] and is associated with increased risk of CVD [233]. Elevated tHcy may confer a particularly high risk of CVD in diabetic patients [170]. tHcy is also elevated in patients with hypothyroidism [162], who have a high incidence of CVD [234]. Furthermore, tHcy is related to fibrinogen [195], von Willebrand factor (vWF) [235–237] and the intercellular adhesion molecule ICAM-1 [238]. The effect of high concentrations of tHcy on a number of recognized risk factors related to haemostasis, endothelial function and vascular smooth muscle cells have been investigated in mechanistic studies, and will be discussed below.

Recent studies indicate that elevated tHcy may interact with some hereditary atherothrombotic disorders [239, 240]. The factor V Leiden mutation, which is the most frequent cause of familial venous thrombosis, produces an increased risk of combined venous and arterial thrombosis amongst patients with homocystinuria [241]. Moderate hyperhomocysteinemia, in some but not all studies, seems to interact with the factor V Leiden mutation to increase the risk of idiopathic venous thrombosis beyond that already conferred by the Leiden mutation [220, 222, 240, 242, 243].

Familial hyperhomocysteinemia

The plasma level of tHcy is frequently elevated in subjects with a family history of CVD. This has been documented through various studies on twins, siblings, parents, children, grandchildren and relatives of patients with CVD [93, 100, 244–248]. Even in young children aged 8–12 years, a higher tHcy level is observed in those who reported premature vascular death in a male relative [100]. Hence, the available data strongly suggest that the plasma tHcy is a genetic trait, which may contribute to a family history of CVD.

Experimental evidence and mechanisms

Elevated tHcy is related to numerous processes

involved in atherosclerosis or thrombosis. This evidence relates both to *in vitro* and *in vivo* studies, and elevated tHcy is associated with the development of atherosclerosis in animals. There is no unifying hypothesis on the molecular and cellular mechanisms whereby Hcy might influence the pathogenesis of CVD. An overview of the possible mechanisms is presented.

Endothelium. Increasing evidence points to a relationship between elevated Hcy and endothelial dysfunction, which reflects an imbalance between factors involved in vasomotor function, cellular growth, coagulation or thrombolysis [249, 250]. A dysfunctional endothelium is an early marker of atherosclerosis and thrombotic risk [251, 252], and the vasomotor function improves during regression of diet-induced atherosclerosis in monkeys [252, 253]. Umbilical endothelial cells from patients with homocystinuria have normal endothelial function markers [254]. However, a large series of investigations link elevated Hcy levels with injury and dysfunction of the endothelium, and a direct pathophysiological response to Hcy is therefore implied [254].

Endothelium and coagulation, in vitro studies. The vascular endothelium has a vital function in regulating the balance between pro- and anticoagulant factors [255, 256], and several of these factors have been used as markers of endothelium function. Studies with cultured endothelial cells show that elevated Hcy may activate coagulation factor V [257], inhibit the binding of tissue plasminogen activator to endothelial cells [258, 259] by hampering the binding to annexin II [259], reducing the thrombomodulin-dependent activation of protein C [260–262], suppressing the expression of anticoagulant heparan sulphate [263], stimulating the expression of procoagulant tissue factor [264], and decreasing the production of the two important vasorelaxant and antiaggregatory substances, nitric oxide [265] and prostacyclin [266–268]. These results indicate that elevated tHcy may modulate the endothelium in a way that promotes thrombosis. One study indicates an opposite effect by demonstrating *in vitro* inhibition of vWF production [269],

but this finding has been contested in later *in vivo* studies [235, 236].

Endothelium and coagulation, in vivo studies. Diet-induced hyperhomocysteinemia is associated with altered levels of endothelial markers in animals. Thus, elevated levels of vWF and a concomitant decrease in angiotensin converting enzyme levels have been observed in hyperhomocysteinemic rats [236], whereas a decreased thrombomodulin-dependent protein C activation was detected in the thoracic aorta of monkeys [270].

In humans, elevated levels of tHcy, vWF and thrombomodulin have been observed in patients with peripheral artery disease, and the concentrations decrease after 12 weeks daily treatment with vitamin B₆ plus folic acid [235]. However, a recent study from this group suggests that elevated protein markers are a consequence rather than a cause of endothelial injury [237].

Endothelial-dependent vasodilatation. Endothelial dysfunction also causes an imbalance between relaxing and contracting forces [271], which is ascertained by impaired vasomotor responses to various stimuli [252]. A marked impairment of endothelium-dependent vasodilatation in response to acetylcholine and adenosine diphosphate (ADP) has been demonstrated in hyperhomocysteinemic monkeys [270], but normalization of plasma tHcy by B-vitamins is insufficient to correct the vascular dysfunction when the animals persist on a hypercholesterolemic diet [272].

Impairment of flow-mediated endothelium-dependent vasodilatation was demonstrated in children with homocystinuria, by using high-resolution ultrasound [273]. Similar observations have recently been made in adults with moderately elevated tHcy [274–277], and the endothelial dysfunction is improved by folic acid supplementation [278].

Cytotoxic effect on endothelial cells. Elevated Hcy has been associated with endothelial damage in several *in vivo* [79–282] and *in vitro* studies [283–288]. Although negative studies have been published [289], the data may suggest a cytotoxic effect of Hcy on the endothelial cells. This is supported by a recent finding of a marked increase in circulating endothelial cells (endothelium) in vascular patients subjected to methionine loading [290]. Other studies

indicate that Hcy directly inhibits the growth of such cells [291, 292] as well as other cell types (HeLa cell line) [293].

Inflammation. As an initial step in atherosclerosis, endothelial injury involves inflammation and targeting of monocytes [294]. In the study by de Jong *et al.* [237], tHcy was not associated with signs of inflammation as evaluated by CRP. In contrast, amongst the participants of the Physicians' Health Study, plasma tHcy was significantly related to the concentration of the intercellular adhesion molecule ICAM-1 [238].

Oxidative stress. The underlying mechanisms for the Hcy-associated endothelial injury and dysfunction are not established. *In vivo* studies indicate that hyperhomocysteinemia induces oxidative stress [295, 296]. This is further elaborated by *in vitro* studies showing that oxygen and catalytic activity of copper [284, 285, 287, 288], caeruloplasmin [285] or hypoxanthine/xanthine [287] are required to provoke the Hcy-induced cellular injury. The response may be inhibited by the metal chelator, desferal [288]. Because the concentration of the copper containing protein caeruloplasmin is increased in patients with homocystinuria [297], copper-catalysed oxidation of Hcy with subsequent hydrogen peroxide formation has been proposed to be involved in the pathogenesis of CVD [284, 285].

The Hcy effects are also related to elevated levels of the oxidation products thiobarbituric acid reactive substances [288]. In addition, catalase, which breaks down hydrogen peroxide, inhibits the response [283, 285, 287, 288]. This indicates a central role for hydrogen peroxide in mediating the oxidative stress on endothelial cells [283, 285, 287, 288].

The observation that endothelium-independent vasodilatation is not affected whereas endothelium-dependent response is impaired in hyperhomocysteinemic humans, indicates that high tHcy reduces endothelial nitric oxide activity [273–277]. Hcy may decrease the bioavailability of nitric oxide by forming S-nitroso-Hcy [265]. Another potential mechanism is impaired function of the intracellular antioxidant enzyme glutathione peroxidase, which catalyses the reduction of hydrogen peroxide and lipid peroxides [298, 299]. This effect of Hcy may be specific since it is not provoked by cysteine [299].

Prolonged Hcy administration may also reduce intracellular levels of glutathione [293].

The initial response to elevated Hcy appears, however, to be stimulation of intracellular production of glutathione [299], nitric oxide or S-nitroso-Hcy [300]. The scavenging potential of glutathione and nitric oxide may be saturated after prolonged Hcy elevation. This may lead to toxic effects of Hcy by increased formation of hydrogen peroxide [293, 300–302] or other reactive oxygen species, such as the superoxide radical or hydroxyl radical [265, 301, 303].

Peroxidation of lipids. The oxidation hypothesis of atherosclerosis implies a causal role of oxidized lipoproteins [304, 305]. Signs of increased low-density lipoprotein (LDL) peroxidation by Hcy have been demonstrated in cell-free systems [306, 307] and *in vivo* in animals [308, 309]. However, other *in vivo* and *in vitro* studies [310–313] refute these findings, and one *in vitro* study indicates that elevated Hcy may actually serve as an antioxidant and protect LDL against oxidative modification [314]. In contrast, a recent human study suggests an increased lipid peroxidation by elevated tHcy after methionine load [315].

Smooth muscle cells and collagen. Several investigations have shown that hyperhomocysteinemia promotes the growth of vascular smooth muscle [279, 291, 316–320] and other cells in the aortic wall [236]. Although diverging results have been obtained [292], Hcy may provoke this effect by induction of cyclin A gene expression [316], enhancement of platelet derived growth factor [318], or stimulation of protein kinase C activation [321]. In addition, Hcy promotes the production of collagen [317, 320], which may account for the intimal hyperplasia in patients with homocystinuria [3]. The fragmentation of the internal elastic lamina observed in these patients [3, 322] may be related to serine elastase induction by Hcy [322].

Platelet aggregation. Some *in vitro* [266] and *in vivo* [279, 280, 282, 308, 323] experiments indicate that Hcy enhances platelet aggregation. This is also supported by the finding of an increased thromboxane biosynthesis amongst patients with homocystinuria [324, 325] and in hyperhomocysteinemic rats [308], which is believed to reflect *in vivo* platelet

activation. However, results from several other studies argue against enhanced platelet aggregation in hyperhomocysteinemia [265, 325–327].

Miscellaneous mechanisms. Other mechanisms linking elevated tHcy with CVD or thrombosis include activation of coagulation factor XII [328], enhanced binding of lipoprotein(a) to fibrin [329], stimulation of the macrophage tissue factor activity [308, 323], and impaired anticoagulant pathway as indicated by elevated levels of prothrombin fragments [330].

Validity and clinical relevance of the suggested mechanisms. The validity and clinical relevance of the different observations have been questioned. Most experiments were carried out with one particular chemical form of Hcy and do not reflect the complex redox reactions or interconversion of the different Hcy species in blood [24]. In addition, millimolar concentrations were often applied, which is more than 100-fold higher than observed in moderate hyperhomocysteinemia [331]. This may also explain why many *in vitro* observations have not been reproduced *in vivo* [6, 286]. Finally, often the effects provoked by high Hcy are not specific since they can be obtained with other thiols [286, 306, 332].

Is homocysteine causally related to CVD?

A critical question is whether elevated tHcy is a cause of CVD or just an epiphenomenon. The evidence supporting a causal relationship as well as that disputing it, will therefore be critically reviewed.

Observational clinical and epidemiological evidence

The belief that Hcy may be an atherothrombotic agent was originally derived from the clinical presentation of patients with homocystinuria [3, 6, 8]. The assumption is supported by an extensive series of cross-sectional or case-control studies almost consistently demonstrating a higher risk of CVD amongst subjects with moderate tHcy elevations [13, 55, 57, 186, 187, 189–191, 193–200]. In addition, elevated tHcy potentiates the risk conferred by other risk factors such as hypertension [55, 333] and smoking [55].

Prospective studies. The most reliable epidemiological evidence originates from prospective studies because samples for tHcy determination are collected before disease occurrence. Presently, when investigations with mortality as endpoint are included, results from at least 24 prospective studies have been reported (Table 2). Sixteen of the studies are based primarily on healthy subjects, including five from the Physicians' Health Study and two from the British Regional Heart Study. All the community or occupational cohort studies have used a nested case-control design, which rely upon the quality of tHcy determinations in frozen samples. This approach is probably appropriate [23, 40–42]. The tHcy level was significantly related to subsequent CVD or mortality in nine of the studies [188, 203, 204, 206, 207, 214, 215, 222, 334], non-significantly related in three studies [42, 335, 336], whereas no association was observed in the remaining four [194, 337–339].

In eight of the prospective studies, patients with systemic lupus erythematosus, renal disease or various types of CVD were followed, and tHcy significantly predicted the prognosis [32, 208, 212–214, 340, 341] or progression of atherosclerosis [202] in all these investigations. The stronger predictive power of tHcy in clinical cohorts, compared to the community-based studies may be due to a shorter follow up. In the Physicians' Health

Study, the tHcy relation with myocardial infarction was attenuated when follow up was extended from 5 [203] to 9 years [336]. The five studies from this cohort of US physicians included men who presumably are better nourished and with better vitamin status than the general population [336]. This may impede the detection of any tHcy-associated risk. Estimates of the reliability constant of tHcy suggest that the magnitude of the risk associations may be underestimated by more than 10% when they are based on a single individual tHcy determination [44, 45, 342].

Dose-response. A strong dose-response relationship between tHcy and mortality was observed in our patient cohort [32] (Fig. 6, left panel). The tHcy-associated risk was slightly strengthened with cardiovascular mortality as endpoint in this study. We noticed a particularly strong mortality relation at tHcy levels above $15 \mu\text{mol L}^{-1}$, but the relationship at lower tHcy was similar to that observed in the meta-analyses by Boushey *et al.* [187] and Wald *et al.* [188].

The association between tHcy and CVD or mortality cannot be used to determine the benefits that might be derived from tHcy intervention trials. However, the data can be used in the design of such studies, and we have calculated the hypothetical mortality reduction associated with a decrease in

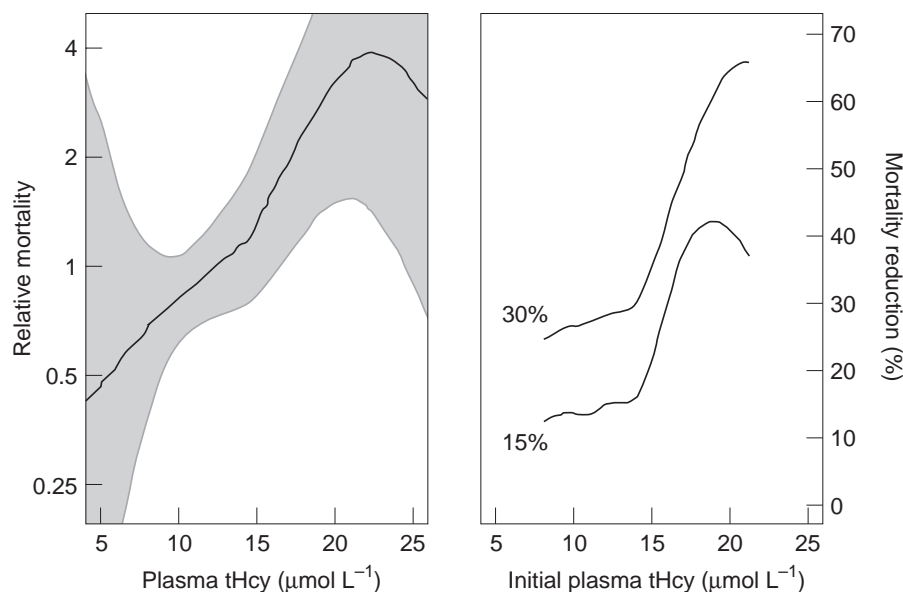


Fig. 6 Dose-response relation between plasma total homocysteine (tHcy) and mortality. The left panel shows the dose-response relationship between plasma tHcy and mortality adjusted for age, sex, left ventricular ejection fraction, creatinine, total cholesterol, and presence of single, double or triple vessel disease, using generalized additive logistic regression. Relative mortality was approximated by the odds ratio. The solid line indicates the estimated dose-response curve and the shaded area the 95% confidence interval. Based on the dose-response relationship of the left panel, the right panel shows the estimated potential reduction in mortality associated with a reduction in tHcy of 15 and 30%. The Initial plasma tHcy refers to the tHcy level before the hypothetical reduction.

Table 2 Prospective studies of the relationship between tHcy and CVD or mortality

Cohort type or population [reference], year	Outcome	Sample size	Cases/events	Controls	Age (years)	Sex	Follow up (years)			Result	Significant	Difference in tHcy ($\mu\text{mol L}^{-1}$) ^a
							mean	max	min			
Community/occupational cohorts^b												
Physicians' Health Study [203], 1992	AMI + CADM	271	271	271	40-84	M	5	≈7	Pos	Yes	0.6	
Malmö Study [42], 1993	Stroke/AMI	13/15	21/27	21/27	NA	M	11	16	Pos	No	2.8/1.1	
North Karelia Project [337], 1994	AMI + stroke	265	269	269	40-64	MF	9	14	Neg	No	0.2	
Physicians' Health Study [335], 1994	Ischemic stroke	109	427	427	40-84	M	5	≈7	Pos	No	0.5	
British Regional Heart Study [207], 1995	Stroke	107	118	118	40-59	M	12.8	≈14	Pos	Yes	1.8	
Tromsø Study [204], 1995	CHD	122	478	478	NA-61	MF	4	≈5	Pos	Yes	1.4	
Physicians' Health Study [336], 1996	AMI	333	333	333	40-84	M	7.5	13	Pos	No	NA	
Physicians' Health Study [222], 1997	VTE	145	646	646	40-84	M	10	12	Pos	Yes ^c	0.6	
Physicians' Health Study [343], 1997	CAD ^d	149	149	149	40-84	M	9	11	Neg	No	0.5	
MRFIT [338], 1997	AMI + CADM	240	472	472	35-57	MF	7/15 ^e	≈17	Neg	No	-0.5	
Framingham [214], 1998 (abstract)	Mortality	653	1280	1280	60-91	MF	10	NA	Pos	Yes	NA	
ARIC [339], 1998	CHD	232	537	537	NA	MF	3.3	NA	Neg	No	NA	
BUPA [188], 1998	Mortality	229	1126	1126	35-64	M	8.7	12	Pos	Yes	1.3	
The Hoorn Study [215], 1998 abstract	Mortality	188	631	631	50-75	MF	NA	7	Pos	Yes	1.2	
British Regional Heart Study [206], 1998 abstract ^f	AMI	386	454	454	40-59	M	NA	NA	Pos	Yes	0.7	
Scottish Heart & MONICA [334], 1998 abstract	CHD	335	335	335	35-64	MF	7.6	NA	Pos	Yes	1.5/2.7 ^g	
Patient cohorts												
SLE pts [208], 1996	AVTE	337	94	94	mean 35	MF	4.8	8	Pos ^h	Yes	2.9	
Hemodialysis pts [233], 1997	CVE	73	16	16	mean 56	MF	1.4	2.1	Pos	Yes	NA	
CAD pts [32], 1998	Mortality	587	64	64	32-80	MF	4.6	5.2	Pos	Yes	2.5	
End stage renal disease pts [340], 1998	CVD + mortality	167	86	86	mean 56	MF	1.5	NA	Pos	Yes	1.6	
Peripheral artery disease [341], 1998 abstract	New CVD event	162	48	48	NA	MF	1.7	5.3	Pos	Yes	NA	
CAD pts [202], 1998 (abstract)	CAD progression	218	218	218	NA	M	2	2	Pos	Yes	NA	
Vascular disease pts [212], 1998 abstract	CVDM	641	27	27	<60	MF	4.5	NA	Pos	Yes	NA	
Acute coronary syndromes [213], 1998 abstract	CVDM + AMI	420	NA	NA	NA	MF	3.5	NA	Pos ⁱ	Yes	NA	

AMI; acute myocardial infarction, CAD; coronary artery disease, CADM; mortality due to CAD, NA; not available, CHD; coronary heart disease, VTE; venous thrombotic or embolic event, AVTE; arterial and VTE, SLE; systemic lupus erythematosus, CVE; cardiovascular event (nonfatal and fatal), CVDM; CVD mortality.

^aBetween cases and noncases. ^bThe Physicians' Health Study is a cohort study of US physicians. All community cohort studies are nested case-control. ^ctHcy is a risk factor for idiopathic venous thrombosis but not for any venous thrombosis. ^dPatients referred for coronary surgery; none had had a prior AMI. ^eAMI; follow up 7 years, CADM; follow up 15 years. ^fResults on essentially the same patients are reported in [205]. ^gDifference for men/women. ^htHcy is a risk factor for atherothrombotic events but not venous thrombosis. ⁱtHcy is not a risk factor for short-term mortality (28 days).

tHcy if all the associated effects can be reversed by tHcy lowering (Fig. 6, right panel). Although highly speculative, and likely to exaggerate any effect of tHcy lowering, the calculation shows that a moderate reduction in tHcy may be associated with a substantial mortality reduction even amongst patients with tHcy $<15 \mu\text{mol L}^{-1}$, further suggesting that the entire CAD population may benefit from tHcy lowering therapy.

Vitamin intake and tHcy lowering therapy

Observational studies have shown that high intakes of fruit and vegetables, the major sources of folates [344], are associated with lower rates of stroke and coronary heart disease [345]. A protective effect on CVD from folate or vitamin B₆ is also supported by recent observational prospective studies [339, 346, 347].

A strong evidence of a beneficial effect related to increased vitamin intake is provided by clinical observations amongst patients with homocystinuria due to cystathionine β -synthase deficiency. In these patients, the cardiovascular morbidity is markedly reduced in those responding to Hcy lowering therapy with vitamin B₆ [6]. This effect is achieved even though tHcy is not normalized. Betaine seems to have a similar protective effect in vitamin B₆-nonresponsive patients [58, 348].

Amongst patients with mild to moderate hyperhomocysteinemia, the data on the effect of tHcy lowering therapy are sparse. One study on non-randomized patients recently showed that a combination regimen of folic acid, vitamins B₆ and B₁₂ reduced the progression of carotid atherosclerosis [349]. In another study of patients with premature peripheral arterial occlusive disease, the patients with initial hyperhomocysteinemia were treated with vitamins B₆ and folic acid and tended to have a lower incidence of new cardiovascular events than patients with normal tHcy levels [341].

CVD and genetic causes of mildly elevated tHcy

Recent studies [338, 339] have added to the ongoing debate of whether Hcy itself is a cause of occlusive vascular disease or just an epiphenomenon [89, 350]. In this paragraph we discuss how genetic causes of hyperhomocysteinemia may complement the discussion of whether tHcy is a causal

factor for CVD, just a marker of risk, or a combination of the two.

If mild hyperhomocysteinemia itself is responsible for vascular injury, genetic causes of mildly elevated (fasting or postload) tHcy are candidate CVD risk factors. There is no increased risk of CVD in obligatory heterozygote homocystinuria due to CBS deficiency [351], and the prevalence of common mutations in the CBS gene in CVD patients with an abnormal response to methionine is not higher than expected [352–355]. However, heterozygosity for the CBS mutations is rare, and a majority of these subjects have a normal fasting tHcy level, and often normal postload tHcy [6].

The CVD risk associated with the common C677T MTHFR polymorphism has, since 1995, been extensively studied. In this review, we have extended a published metaanalysis [356] on the C677T MTHFR polymorphism, plasma tHcy concentration and CVD risk, by including the most recent articles.

Based on 17 studies of European and North American populations, we found that subjects with the TT genotype ($n = 614$) have on average $3.5 \mu\text{mol L}^{-1}$ (32%) higher mean plasma tHcy concentration than those with the CC genotype ($n = 2491$; Table 3). This difference in tHcy concentration between the TT and CC genotypes is larger than the difference usually found between CVD patients and control subjects (see Table 2 for comparison). In 28 studies, comprising 6944 cardiovascular patient and 7764 control subjects, the TT genotype is found in 12.5% of the patients and 11.9% of the control subjects (Table 4), giving an odds ratio for CVD of 1.15 (95% CI 0.97–1.38), which is not significant. Interestingly, subgroup analysis of three Japanese studies [372, 379, 386], including 826 CVD patients and 1289 control subjects, shows a significantly increased risk for CVD in those with the TT genotype (OR 2.04, 95% CI 1.55–2.68).

The available data on MTHFR and CVD risk, summarized above, do not suggest a link between mild fasting hyperhomocysteinemia, mediated through the TT genotype, and CVD, at least not in European, North American, and Australian populations. However, there are several reasons why high tHcy may still confer CVD risk whilst MTHFR is not a major risk factor. First, the TT genotype may protect against CVD by mechanisms independent of Hcy, as

Table 3 Plasma total homocysteine (tHcy) concentration in 17 different studies in relation to the C677T/MTHFR genotypes (TT genotype = mutant homozygotes, CT genotype = mutant heterozygotes, CC genotype = normal homozygotes)

Author [reference], year	Mean plasma tHcy concentration in $\mu\text{mol L}^{-1}$		
	TT genotype (n)	CT genotype (n)	CC genotype (n)
Frosst <i>et al.</i> [357], 1995	22.4 (12)	13.8 (9)	12.6 (19)
van der Put <i>et al.</i> [358], 1995	17.1 (34)	13.2 (164)	13.4 (194)
Jacques <i>et al.</i> [80], 1996	9.9 (45)	8.4 (170)	8.7 (150)
Harmon <i>et al.</i> [359], 1996	9.5 (72)	7.1 (273)	6.8 (280)
Kluijtmans <i>et al.</i> [352], 1996	16.3 (15)	13.4 (61)	12.3 (93)
Schmitz <i>et al.</i> [360], 1996	9.1 (14)	10.6 (46)	9.9 (67)
Ma <i>et al.</i> [361], 1996	12.6 (72)	10.9 (240)	10.6 (271)
Deloughery <i>et al.</i> [362], 1996	17.2 (22)	13.6 (111)	13.0 (114)
Verhoef <i>et al.</i> [363], 1997	15.5 (30)	12.3 (150)	11.4 (138)
Kluijtmans <i>et al.</i> [364], 1997	15.4 (51)	13.4 (233)	12.6 (231)
Christensen <i>et al.</i> [365], 1997	12.8 (22)	11.0 (98)	10.3 (89)
Schwartz <i>et al.</i> [366], 1997	13.5 (43)	10.8 (141)	10.9 (154)
Kluijtmans <i>et al.</i> [243], 1998	17.7 (51)	13.3 (229)	12.6 (258)
Legnani <i>et al.</i> [367], 1997	13.0 (12)	7.8 (31)	7.4 (20)
Arai <i>et al.</i> [368], 1997	19.8 (22)	15.5 (22)	15.4 (22)
Gudnason <i>et al.</i> [85], 1998	16.5 (88)	10.4 (338)	9.9 (359)
Lalouschek <i>et al.</i> [369], 1998	10.4 (9)	9.8 (35)	9.4 (32)
Approximate means of all studies	14.3 (614)	11.1 (2351)	10.8 (2491)
Elevation in tHcy compared with the CC genotype	3.5 (32%)	0.3 (3%)	

suggested by lower blood pressure in TT subjects [388]. Secondly, high tHcy may cause vascular lesion only in subjects with coexisting risk factors, and such interactive effects have actually been demonstrated in the COMAC study [55]. Interactions of the MTHFR C677T polymorphism with nutrition [389] and genetic traits seem likely and may explain the association found in the Japanese.

The interactions between the MTHFR genotype and conventional risk factors have been addressed in a recent study from the European Concerted Action Project based on genotyping and tHcy measurement in 711 cases and 747 controls. The TT genotype was associated with $3.5 \mu\text{mol L}^{-1}$ higher tHcy and a 26% higher CVD risk than the CC genotype. The unexpected finding was made in the hyperhomocysteinemic subgroup (tHcy $>15 \mu\text{mol L}^{-1}$); TT subjects had a lower prevalence of CVD risk factors than CC subjects. After adjustment for these risk factors, the TT genotype was significantly associated with a 50% increased risk for overall CVD and an even higher risk for CAD, compared with the CC genotype. The increase in risk was markedly attenuated after adjustment for tHcy (Meleady *et al.*

1998, submitted). This suggests that the TT associated risk is mediated through elevated tHcy. Furthermore, the unadjusted relative risk associated with the TT genotype agrees with what has been calculated (20–40%) for a tHcy increment of $3.5 \mu\text{mol L}^{-1}$ [187], and the adjusted relative risk observed in the COMAC study actually exceeds this expected relative risk. Statistical sample size calculations show that at least 2500 patients and an equal number of controls are required to detect an unadjusted 25% risk increase associated with TT genotype, if the prevalence of the genotype is about 10%.

Conclusions

Observational studies show that plasma tHcy is a strong risk factor for CVD and mortality, and genetic causes of mildly elevated tHcy may be associated with increased risk of CVD when other risk factors are accounted for. However, mechanisms are uncertain and results from adequately sized trials with tHcy lowering therapy and clinical endpoints

Table 4 Numbers and frequencies of the three different C677T/MTHFR genotypes (TT genotype = mutant homozygotes, CT genotype = mutant heterozygotes, and CC genotype = normal homozygotes) in 28 studies of patients with cardiovascular disease and controls

Author [reference], year	Type ^a	Cases [Genotype (numbers (%))]				Controls [Genotype (numbers (%))]			
		TT	CT	CC	All	TT	CT	CC	All
Kluijtmans <i>et al.</i> [352], 1996	Mixed	9 (15.0)	21 (35.0)	30 (50.0)	60	6 (5.4)	42 (37.8)	63 (56.8)	111
Schmitz <i>et al.</i> [360], 1996	CHD	29 (15.3)	66 (34.7)	95 (50.0)	190	27 (14.4)	90 (47.8)	71 (37.8)	188
Ma <i>et al.</i> [361], 1996	CHD	33 (11.3)	124 (42.3)	136 (46.4)	293	39 (13.4)	116 (40.0)	135 (46.6)	290
Deloughery <i>et al.</i> [362], 1996 ^b	Mixed	42 (9.9)	199 (46.8)	184 (43.3)	425	94 (15.8)	262 (44.0)	240 (40.2)	596
Verhoef <i>et al.</i> [363], 1997	CHD	13 (10.0)	59 (45.0)	59 (45.0)	131	7 (7.0)	48 (48.0)	45 (45.0)	100
Kluijtmans <i>et al.</i> [364], 1997	CHD	70 (9.5)	328 (44.6)	337 (45.9)	735	106 (8.5)	527 (42.2)	617 (49.3)	1250
Christensen <i>et al.</i> [365], 1997	CHD	22 (14.5)	68 (44.7)	62 (40.8)	152	13 (10.7)	61 (50.4)	47 (38.8)	121
Schwartz <i>et al.</i> [366], 1997	CHD	7 (10.1)	34 (49.3)	28 (40.6)	69	43 (12.7)	141 (41.7)	154 (45.6)	338
Kluijtmans <i>et al.</i> [243], 1998	VTE	47 (10.0)	213 (45.2)	211 (44.8)	471	47 (9.9)	203 (42.8)	224 (47.3)	474
Gallagher <i>et al.</i> [370], 1996	CHD	19 (17.1)	48 (43.2)	44 (39.7)	111	7 (6.7)	45 (42.9)	53 (50.4)	105
Adams <i>et al.</i> [371], 1996	CHD	32 (10.3)	145 (46.8)	133 (42.9)	310	29 (13.1)	97 (43.7)	96 (43.2)	222
Izumi <i>et al.</i> [372], 1996	CHD	50 (20.0)	110 (44.0)	90 (36.0)	250	25 (12.4)	102 (50.8)	74 (36.8)	201
Wilcken <i>et al.</i> [373], 1996	CHD	53 (11.6)	217 (47.6)	186 (40.8)	456	24 (10.7)	113 (50.2)	88 (39.1)	225
de Franchis <i>et al.</i> [374], 1996	Mixed	19 (29.7)	32 (50.0)	13 (20.3)	64	39 (15.1)	129 (50.0)	90 (34.9)	258
Narang <i>et al.</i> [375], 1996	CHD	6 (6.5)	31 (34.0)	55 (59.5)	92	5 (10.0)	19 (38.0)	26 (52.0)	50
Brulhart <i>et al.</i> [376], 1997 ^c	CHD	23 (11.9)	84 (43.5)	86 (44.6)	193	73 (16.0)	195 (42.7)	188 (41.3)	456
van Bockxmeer <i>et al.</i> [377], 1997	CHD	56 (10.1)	234 (42.2)	265 (47.7)	555	15 (10.5)	58 (40.5)	70 (49.0)	143
Brugada <i>et al.</i> [378], 1997	CHD	10 (6.5)	69 (44.5)	76 (49.0)	155	12 (7.7)	73 (47.1)	70 (45.2)	155
Morita <i>et al.</i> [379], 1997	CHD	57 (15.8)	188 (51.9)	117 (32.3)	362	79 (10.2)	361 (46.4)	338 (43.4)	778
Verhoef <i>et al.</i> [380], 1997	CHD	61 (12.2)	209 (41.8)	230 (46.0)	500	72 (14.4)	200 (40.0)	228 (45.6)	500
Tosetto <i>et al.</i> [381], 1997	VTE	8 (12.3)	36 (55.4)	21 (32.3)	65	17 (13.1)	71 (54.6)	42 (32.3)	130
Malinow <i>et al.</i> [180], 1997	CHD	17 (12.1)	83 (59.3)	40 (28.6)	140	8 (7.8)	45 (44.1)	49 (48.0)	102
Markus <i>et al.</i> [382], 1997	CBV	37 (10.7)	146 (42.3)	162 (47.0)	345	22 (13.7)	63 (39.1)	76 (47.2)	161
Salden <i>et al.</i> [383], 1997	VTE	26 (12.0)	102 (47.2)	88 (40.8)	216	18 (11.0)	75 (45.7)	71 (43.3)	164
Reinhardt <i>et al.</i> [384], 1998	CHD	23 (13.0)	66 (36.5)	91 (50.5)	180	9 (8.7)	46 (44.2)	49 (47.1)	104
Abbate <i>et al.</i> [385], 1998	CHD	24 (28.6)	41 (48.8)	19 (22.6)	84	32 (30.2)	48 (45.3)	26 (24.5)	106
Ou <i>et al.</i> [386], 1998	CHD	61 (28.5)	84 (39.3)	69 (32.2)	214	42 (13.5)	158 (51.0)	110 (35.5)	310
Kostulas <i>et al.</i> [387], 1998	CBV	13 (10.3)	50 (39.7)	63 (50.0)	126	13 (10.3)	50 (39.7)	63 (50.0)	126
Total numbers of genotypes		867	3087	2990	6944	923	3438	3403	7764
Genotype frequencies (%)		12.5	44.5	43.0	11.9	44.3	43.8		
Allele frequency (%)			34.7			34.0			

^aCHD = coronary heart disease, CBV = cerebrovascular diseases, VTE = venous thromboembolism. Mixed: different types of atherothrombotic cardiovascular disease. ^bPatients with venous thrombosis are included in cases. ^cDiabetic cases and controls.

do not exist. Because elevated tHcy may be just a marker of individuals at high risk, a preventive action with B-vitamin supplementation in the general population or in the general CVD population is not justifiable until the results of intervention trials with tHcy lowering therapy are available.

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