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HOMOCYSTEINE AND CARDIOVASCULAR DISEASE

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ABSTRACT

An elevated level of total homocysteine (tHcy) in blood, denoted hyperhomocysteinemia, is emerging as a prevalent and strong risk factor for atherosclerotic vascular disease in the coronary, cerebral, and peripheral vessels, and for arterial and venous thromboembolism. The basis for these conclusions is data from about 80 clinical and epidemiological studies including more than 10,000 patients. Elevated tHcy confers a graded risk with no threshold, is independent of but may enhance the effect of the conventional risk factors, and seems to be a particularly strong predictor of cardiovascular mortality. Hyperhomocysteinemia is attributed to commonly occurring genetic and acquired factors including deficiencies of folate and vitamin B12. Supplementation with B-vitamins, in particular with folic acid, is an efficient, safe, and inexpensive means to reduce an elevated tHcy level. Studies are now in progress to establish whether such therapy will reduce cardiovascular risk.

INTRODUCTION

The total homocysteine (tHcy) level in plasma or serum is a sensitive indicator of vitamin B12 and folate deficiencies (1). It is related to pregnancy complications (2), neural tube defects (3), mental disorders (4, 5), and cognitive impairment in the elderly (6, 7). Furthermore, data from about 80 clinical and epidemiological studies provide ample evidence that an elevated tHcy level is a common cardiovascular risk factor in the general population (8–10).

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In this article, we review the biochemical, experimental and clinical literature on tHcy in relation to cardiovascular disease, with emphasis on the genetics of hyperhomocysteinemia, the interactions between tHcy and conventional risk factors, and the possible role of tHcy as a thrombogenic factor. Finally, we summarize the evidence that vitamins, in particular folic acid, are effective means to normalize an elevated tHcy level, which may aid in the prevention of cardiovascular disease.

BIOCHEMISTRY AND MOLECULAR GENETICS

Homocysteine (Hcy) is formed from methionine as a product of numerous S-adenosylmethionine-dependent transmethylation reactions (11). Three enzymes utilize Hcy (Figure 1), and its distribution among them depends on metabolic status (11, 12). When methionine is in excess, Hcy is directed to the transsulfuration pathway that irreversibly converts Hcy to cysteine. The first reaction in this pathway is catalysed by the vitamin B6-dependent enzyme cystathionine β-synthase (CBS) (EC 4.2.1.22) (11). Under conditions of negative methionine balance, Hcy is primarily disposed via two methionine conserving pathways (11, 12). In the liver, a substantial proportion of Hcy is remethylated by betaine-homocysteine methyltransferase (BHMT) (EC 2.1.1.5) (11), which uses betaine as a methyl donor. In most tissues, however, the remethylation of Hcy is catalyzed by the ubiquitous methionine synthase (MS) (EC 2.1.1.13), which uses vitamin B12 as a cofactor and methyltetrahydrofolate as a substrate. The formation of methyltetrahydrofolate is catalyzed by methylenetetrahydrofolate reductase (MTHFR) (EC 1.7.99.5), a vitamin B2 (FAD)-dependent enzyme (13) that has an indirect but strong influence on Hcy remethylation (14).

If one or more of the Hcy metabolizing pathways are inhibited due to enzymatic defects or vitamin deficiencies, Hcy accumulates, thereby causing an increased tHcy level in plasma (9). This is the metabolic basis for using elevated tHcy as a functional marker of vitamin B12 and folate status (1), and explains the high tHcy levels in the various inborn errors of Hcy metabolism collectively termed homocystinuria (15).

During the last few years, CBS (16), MTHFR (17), MS (18–20), and BHMT (21) have been cloned. Mutations causing homocystinuria have been identified in both CBS (22–24) and MS (19). Recently, a prevalent 68-bp insertion was described in the CBS gene (23, 25), but its functional and clinical importance is uncertain (26). In 1988, Kang et al described a thermolabile variant of the MTHFR that caused elevated tHcy levels (27, 28), and this variant was later ascribed to a C677T mutation in MTHFR gene (29). Subjects with homozygosity for this mutation (TT genotype) usually have a higher tHcy

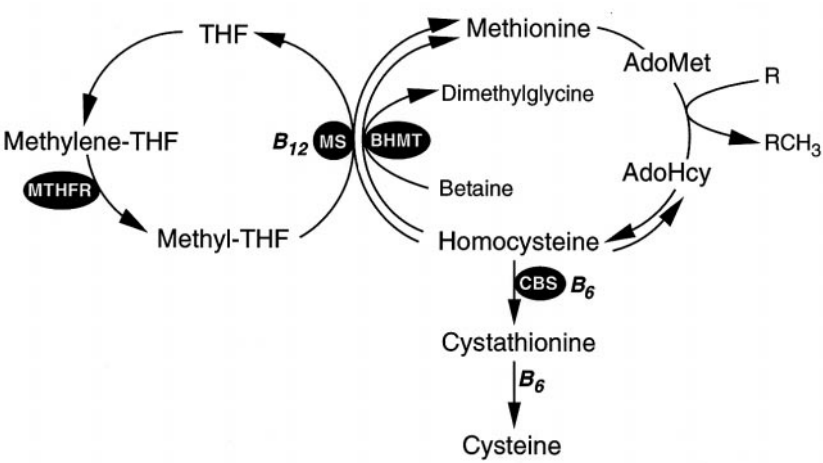

Figure 1 Homocysteine metabolism. AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; B6, vitamin B6 (pyridoxal phosphate); B12, vitamin B12 (methylcobalamin); CBS, cystathionine β-synthase; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; THF, tetrahydrofolate.

level than those who are heterozygous (CT genotype), or have a normal (CC genotype) variant of the enzyme (14, 30).

ASSESSMENT OF HOMOCYSTEINE STATUS

Methodology

Homocysteine is a sulfur amino acid with a sulfhydryl group that makes it susceptible to oxidation at physiologic pH, thereby forming disulfides with other thiols. In this article, the abbreviation Hcy refers to both homocysteine itself (reduced Hcy) and its oxidized species. In plasma, trace amounts (~1%) exist in reduced form, about 70% is bound to albumin, and the remaining 30% forms low molecular weight disulfides, predominantly with cysteine. The sum of all these Hcy species is termed total Hcy, abbreviated tHcy (31).

Methods for tHcy determination were introduced during the 1980s (32), and the problems related to the determination of multiple and unstable Hcy species were thereby avoided. In these assays, plasma or serum is treated with a reductant. This procedure converts all Hcy species into reduced Hcy, which is either directly quantitated or derivatized (32). The principles for derivatization, separation, and detection have been described in a previous review (32). Most assays are based on chromatographic techniques (high performance liquid chromatography or gas chromatography with mass spectrometry). An im-

munoassay (33) may soon be commercially available, and this may allow for widespread use of tHcy determination in laboratory diagnostics.

Optimal procedures for blood sample collection and handling are critical when tHcy is used for cardiovascular risk assessment; inadequate procedures may result in artificial elevation of tHcy, which may be interpreted as increased risk. Sampling in the fasting state is recommended. A small breakfast will probably not affect the plasma tHcy level, whereas a protein-rich meal may cause an increase of 15–20% (34, 35). The posture of the subject during blood collection should be standardized since this affects albumin concentration, which is a determinant of protein-bound Hcy (36, 37).

In the presence of blood cells, there is a time and temperature-dependent increase in the plasma level; at room temperature, tHcy increases 5–15% per hour (32). Immediate centrifugation of the blood is preferable, but the increase can be prevented by keeping the sample on ice (38) or by adding a stabilizer like fluoride (34, 39). After removal of the blood cells, tHcy in serum or plasma is stable for days at room temperature, for weeks at 0–2°C, and for years when kept frozen at −20°C (32).

For investigation of the relation between oxidized and reduced Hcy species, techniques involving trapping of thiols have been developed (31). These methods are not practical for clinical laboratories.

Methionine and Homocysteine Loading

Methionine loading involves intake of a high dose of methionine (0.1 g/kg or 3.8 g/m^2), and tHcy is measured immediately before and usually 2, 4, or 6 h after ingestion (9, 40). The tHcy response induced by protein-rich food (35) may represent the physiologic corollary of the methionine load. The test was originally introduced to detect heterozygosity for CBS deficiency (41, 42), and subjects with a mild disturbance of the transsulfuration pathway often have a normal fasting tHcy level but an elevated postmethionine load (PML) tHcy level (43–45). Recent data suggest that variable tHcy responses in heterozygous subjects may be due to different phenotypic expression of various CBS mutations (46).

The fasting and PML tHcy levels are strongly correlated. They discriminate between vascular patients and controls equally well, but the results do not completely overlap. Thus, determination of only fasting tHcy will fail to identify the substantial proportion of subjects who have normal fasting but elevated PML tHcy levels (47–49).

By performing peroral Hcy loading (65 μmol/kg), elimination of Hcy from plasma can be investigated (50). Subjects with severe cobalamin/folate deficiency have normal tHcy clearance (51), whereas a markedly reduced clearance is observed in renal failure (52).

Determinants of the tHcy Level

Determinants of tHcy include genetic and acquired factors as summarized in Table 1.

AGE, SEX, AND RENAL FUNCTION Women have lower tHcy than men, and tHcy increases with age (37, 53, 54). This may partly be due to differences in vitamin status (55), but also to the influence of sex hormones. Plasma tHcy levels increase after menopause (53, 56), which may explain the steeper age-related increase in women compared with men (54). The sex difference may also be related to stoichiometric formation of Hcy in connection with the creatine/creatinine synthesis that is proportional to muscle mass, and therefore higher in men than in women (57).

Renal function is a strong determinant of the tHcy level (58, 59). This is probably related to Hcy clearance via renal metabolism (60) rather than urinary excretion, which is minor (52, 61). The physiologic decline in renal function may partly explain the age effect (62, 63).

LIFESTYLE Dietary intake of vitamin B6, B12, and folate is inversely correlated to plasma tHcy (55). Smoking and caffeinated coffee consumption cause a shift of the distribution towards higher tHcy values, whereas physical activity is associated with low tHcy levels (54, 64). Notably, the effect of these lifestyle factors on the tHcy level seems to be more pronounced in women than in men (37, 54, 64). Chronic, high ethanol consumption is associated with elevated tHcy levels (65), possibly through its effect on vitamin status. In contrast, a moderate consumption seems to be associated with a lower tHcy level (66).

GENETIC DETERMINANTS Homocystinuria usually refers to the inborn errors of Hcy metabolism associated with severe hyperhomocysteinemia. Homozygosity for CBS deficiency is the most common cause, with a birth prevalence of 1/300,000, but with marked geographic differences (15). Rare forms of homocystinuria include severe defects of MTHFR (67) and impaired Hcy remethylation due to inborn errors of cobalamin metabolism (68).

Heterozygosity for CBS deficiency is present in <1% of the general population (15). These subjects usually have a normal fasting tHcy level, but the PML tHcy level may be elevated (43, 44). Recent genetic studies have shown that heterozygosity for CBS deficiency occurs only sporadically in vascular patients (14, 69, 70) and suggest that this mild genetic defect is not a frequent cause of hyperhomocysteinemia in these patients.

The common C677T mutation in the MTHFR gene shows ethnic differences with a high allele frequency of about 40% (10% TT genotype) in Caucasians (71), whereas it is almost absent in African-Americans (72). This polymorphism causes reduced enzyme activity and thermolability and predisposes

Table 1 Determinants of the plasma total homocysteine level

	Effect	Reference(s)
Genetic factors		
Homozygosity for CBS defects	↑↑↑	15, 80
Homozygosity for MTHFR defects	↑↑↑	81, 82
Cobalamin mutations (C, D, E, F, G)	↑↑↑	68, 83
Down's syndrome	↓	84
Thermolabile MTHFR	↑	14, 73, 74
Heterozygosity for CBS defects[1]	↑	85
Heterozygosity for MTHFR defects	↑	81, 82
Physiologic determinants		
Increasing age	(↑)	53, 54
Male sex	(↑)	53, 54
Renal function, reduced GFR	(↑)	86
Increasing muscle mass	(↑)	57
Lifestyle determinants		
Vitamin intake	↓	55
Smoking	(↑)	54
Coffee consumption	(↑)	64
Ethanol consumption	↑↓	65, 66
Physical activity	↓	54
Clinical conditions		
Folate deficiency	↑↑	1, 75
Vitamin B12 deficiency	↑↑↑	1
Vitamin B6 deficiency[1]	↑	87
Renal failure	↑↑	59, 76
Hyperproliferative disorders	↑	88, 89
Hypothyroidism	↑	90
Drugs		
Folate antagonists (methotrexate)	↑	77
Vitamin B12 antagonists (nitrous oxide)	↑↑	91, 92
Vitamin B6 antagonists[1]	↑	45, 93
AdoHcy hydrolase inhibition	↓	94
Antiepileptic drugs	↑	8
Contraceptives, hormone therapy	↓	95–97
Aminothiols (acetylcysteine, penicillamine)	↓	78, 79
Others (L-dopa, cholestyramine, niacine)	↑	98–100

↓ = Reduction of the total homocysteine level; (↑) = increase within normal reference range; ↑, ↑↑, ↑↑↑ = moderate hyperhomocysteinemia (15–30 μM), intermediate hyperhomocysteinemia (30–100 μM), and severe hyperhomocysteinemia (>100 μM), respectively. AdoHcy = S-adenosylhomocysteine; CBS = cystathionine β-synthase; MTHFR = methylenetetrahydrofolate reductase.

[1]In subjects with vitamin B6 deficiency or mild defects in CBS, the fasting total homocysteine level is usually normal but the postmethionine load level is increased.

to moderate (15–30 μM) and intermediate (30–100 μM) hyperhomocysteinemia under conditions of impaired folate status (29, 73, 74). A low daily dose (0.2 mg) of folic acid will probably enable maintenance of a normal tHcy level in most of these subjects (73).

CLINICAL CONDITIONS AND DRUGS Folate or cobalamin deficiency are common causes of hyperhomocysteinemia in the general population (1, 75). Elevated tHcy levels are also observed in renal failure (59, 76), and in various other clinical conditions (Table 1) (9). Hyperhomocysteinemia is induced by certain drugs, especially those affecting the vitamins related to Hcy metabolism (77). Aminothiols, like penicillamine (78) and acetylcysteine (79), reduce the plasma tHcy level.

Reference Ranges

FASTING tHcy The normal range in adults is usually 5–15 μM, with a mean level of about 10 μM (Table 2) (32). Hyperhomocysteinemia is defined as a plasma tHcy >15 μM and is denoted as moderate (15–30 μM), intermediate (30–100 μM) and severe (>100 μM) hyperhomocysteinemia (101).

Reported reference ranges differ markedly (32). These variations are probably related to a skewed tHcy distribution toward higher values, varying definitions of the upper threshold, differences in sample handling and methodology, and variations in determinants of the tHcy levels among different populations.

It has been suggested that reference ranges should be determined in a population with adequate vitamin status (102–104). Such individuals have markedly lower tHcy levels (with an upper limit of about 12 μM) than the general population. The 2.5–97.5 percentiles for 40–42-year-old subjects in the Hordaland population (n = 18,043) were 5.6–17.4 μM for women and 6.9–20.9 μM for men. The corresponding values for non-smokers with high folate intake and low coffee consumption were 4.7–11.4 μM and 6.3–13.1 μM, respectively (105).

Only sparse data exist on tHcy levels in children (88, 106–108). Recently, Tonstad et al found that tHcy levels in 8–12-year-old boys and girls are about half of that observed in adults. There was no sex difference, and the tHcy frequency distribution was nearly gaussian, with a mean tHcy level of 5.25 μM and a reference range (mean ±2SD) of 2.9–7.6 μM (107). At puberty, tHcy increases markedly, and the distribution becomes skewed as in adult populations (108).

PML tHcy Relatively few investigations have addressed the reference range for PML tHcy levels (48, 53, 109, 110), and various definitions for an abnormal response have been used (9). Relative to the fasting level, the PML tHcy and the increase in tHcy measured after 4 to 6 h are usually about 3 and 2 times

Table 2 Fasting, postmethionine load (PML) and increase after loading in plasma total homocysteine level in the control group of the European Concerted Action Project on homocysteine and vascular disease (49)

	Percentile								
	2.5	5	10	20	50	80	90	95	97.5
	(μmol/L)								
All (n=800)									
Fasting	5.9	6.4	7.0	7.7	9.5	12.1	13.4	15.7	18.5
PML	17.4	19.2	21.5	24.2	29.9	38.0	43.7	51.3	58.1
Increase	10.4	11.6	13.1	15.4	19.8	27.3	31.6	37.2	42.8
Men <45 year (n=232)									
Fasting	6.3	6.8	7.2	7.8	9.6	11.9	12.9	15.8	18.8
PML	17.3	19.0	20.9	23.8	29.6	39.8	47.5	56.4	60.9
Increase	10.4	11.4	12.3	15.2	19.3	28.2	34.1	39.1	44.7
Women <45 year (n=135)									
Fasting	4.8	5.3	6.0	6.6	8.1	10.4	12.6	13.6	16.9
PML	16.0	16.6	17.9	20.0	26.0	34.1	40.2	44.7	48.2
Increase	8.9	9.9	11.3	13.1	17.8	23.9	30.1	32.1	34.9
Men ≥45 year (n=338)									
Fasting	6.8	7.2	7.8	8.5	10.3	12.6	13.7	15.9	18.6
PML	21.0	22.0	23.6	25.7	31.0	38.0	42.9	50.3	57.9
Increase	12.8	13.8	14.9	16.2	20.6	26.6	30.1	35.5	41.7
Women ≥45 year (n=95)									
Fasting	5.8	6.3	6.7	7.1	9.1	11.4	12.9	13.7	15.1
PML	16.8	19.6	21.5	24.0	31.2	36.9	42.4	50.1	54.2
Increase	10.1	12.0	13.7	15.3	21.4	28.2	33.3	40.3	43.2

higher, respectively (Table 2). PML tHcy is higher in men than in women and increases with age, especially in women (109, 110).

HOMOCYSTEINE AND CARDIOVASCULAR DISEASE

History and Summary of Highlights up to 1995

Homocystinuria was described in 1962 in mentally retarded children (111, 112). About two years later, the defect in CBS was identified (113), and it was reported that such patients frequently had thromboembolic events (114, 115). Severe MTHFR deficiency (67, 116) and certain defects in intracellular cobalamin metabolism (68, 117) were later reported to cause a similar clinical

picture. In 1969, McCully described the vascular pathology of homocystinuria (117). He noted that thromboembolic disease was a characteristic feature of homocystinuria independent of the site of the metabolic defect, pointing to Hcy as the causal agent. This is the basis for his Hcy theory of atherosclerosis, which implies that moderately elevated tHcy may be a cardiovascular risk factor in the general population (118).

In 1976, Wilcken & Wilcken published the first report that coronary artery patients frequently have abnormal Hcy metabolism (119). For the following 15 years, there were only scattered reports (8, 9) on the relation between Hcy levels and coronary artery disease (120–122), cerebrovascular disease (123–125), peripheral artery disease (124, 126, 127), and venous thrombosis (128, 129). In the same period, the most important determinants of tHcy were identified, including age and sex (121, 130), renal function (76, 131), and folate and vitamin B12 status (75, 132). In 1988, Kang et al reported on thermolabile MTHFR and its relation to cardiovascular risk and hyperhomocysteinemia (27, 28).

Since 1990, there has been an exponential increase in the publication rate on tHcy and cardiovascular disease (Figure 2). This is related to the recognition of elevated tHcy as an independent cardiovascular risk factor (169). But equally important was the introduction of various assays for tHcy determination (32), which are more practical in the clinical setting and allow the use of stored blood samples. The first positive prospective studies on tHcy and coronary heart disease were reported in 1992 (170) and 1993 (171). One negative prospective study on myocardial infarction and stroke from Finland (172) caused some concern about the validity of the Hcy theory, but the hypothesis was later substantiated by several reports which indicated that tHcy is a risk factor for cardiovascular disease. In 1995, Selhub et al reported on a strong relation between tHcy and extracranial carotid-artery stenosis in the elderly (173), and a prospective study demonstrated that tHcy conveyed a graded risk for stroke in middle-aged British men (162). The same year, the C677T mutation in the MTHFR gene was identified and proposed as a candidate risk factor for vascular disease (29).

In 1995, Boushey et al reviewed most studies on Hcy and cardiovascular disease. Their meta-analysis, based on 27 studies including about 4000 patients, showed that Hcy was an independent, graded risk factor for atherosclerotic disease in the coronary, cerebral and peripheral vessels (10). Since then, there have been about 40 additional studies on tHcy as a risk factor for cardiovascular disease and its complications (Table 3); the majority of them support the conclusions formulated by Boushey et al. The strongest evidence derives from ten prospective studies: Eight demonstrate an increase in risk of stroke (162, 174), coronary heart disease (163, 170), venous thrombosis (164), car-

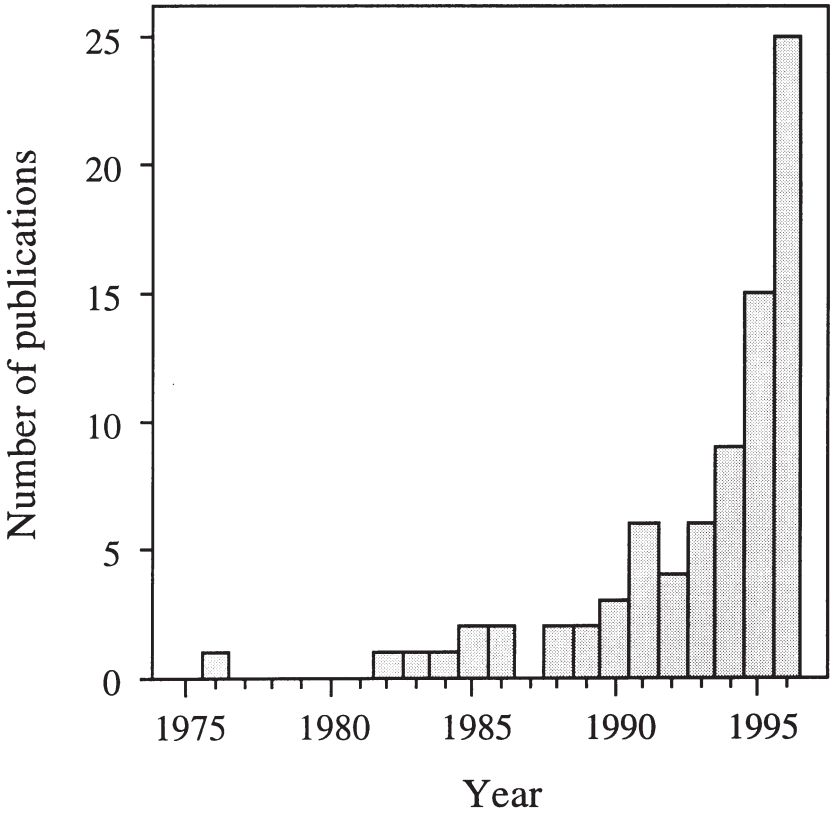

Figure 2 Number of clinical and epidemiological studies on homocysteine and cardiovascular disease in the years 1976 to 1996. Nine publications on methylenetetrahydrofolate and cardiovascular disease are included.

diovascular complications (166, 167) or mortality (168), whereas only two are negative (165, 172). Recent data have been reviewed and subjected to frequent editorial comments (175–179).

Hcy and Conventional Risk Factors

Hcy is one of more than 200 identified risk factors for cardiovascular disease (180). Thus, a frequently asked question is whether the reported tHcy association is due to confounding with established factors.

A correlation between tHcy level and total, HDL or LDL cholesterol has been shown in some studies (9, 54, 62, 135, 163, 181). Two recent studies have

demonstrated that tHcy is an independent predictor of atherosclerotic events (135) and of carotid intimal-medial thickness (138) in hyperlipidemic subjects.

Plasma tHcy level is positively associated with blood pressure in a healthy population (54, 125, 182), in diabetic patients (183), and possibly in vascular patients (181). The relation between the tHcy level and carotid wall thickness is stronger in hypertensive than normotensive individuals (184).

The use of tobacco is associated with reduced intake of nutrients (185) and lower blood levels of folate (186), vitamin B12 (187) and pyridoxal 5'-phosphate (188). Despite these findings, a relationship between smoking and tHcy levels has infrequently been reported. In the Hordaland population, tHcy levels increased almost proportionally to the number of cigarettes smoked per day, and smoking was one of the strongest determinants of tHcy levels (54). Plasma tHcy is elevated in vascular patients who smoke (47, 156, 189), but it is a consistent finding that the relationship between tHcy and vascular disease remains strong after adjustment for smoking.

In patients with chronic renal failure, where atherosclerotic complications are a leading cause of death, an elevated tHcy level occurs more frequently than any of the conventional risk factors (59). A recent prospective study showed that elevated tHcy levels may contribute to the high incidence of nonfatal and fatal cardiovascular events in end-stage renal disease (167). In diabetic patients with intact renal function, the plasma tHcy level is normal (183, 190) or even low (191), possibly due to the glomerular hyperfiltration frequently observed in these subjects (192). In contrast, in diabetic patients with proteinuria and macrovascular disease, tHcy levels are increased (141, 183, 190), which may contribute to accelerated atherogenesis in these patients.

The European Concerted Action Project on homocysteine and vascular disease (49) is a 19-center case-control study of 750 vascular disease patients (coronary artery, cerebrovascular, and peripheral vascular) and 800 controls. In this study, the interaction between tHcy and the three most important cardiovascular risk factors (180) (cholesterol, smoking, and high blood pressure) were systematically investigated. The risk conferred by tHcy was similar to and independent of the conventional risk factors. An elevated tHcy level interacted strongly with hypertension and smoking; the combined effect was more than multiplicative in both sexes, but was most pronounced in women (49).

In conclusion, there is a positive relationship between tHcy and several of the conventional risk factors. However, the association between tHcy and cardiovascular endpoints remains strong after adjustment, and in various subgroups. Moreover, there are pronounced interactive effects with conventional risk factors, especially with smoking and hypertension, suggesting that tHcy may further enhance the cardiovascular risk in these patients.

Table 3 Clinical and epidemiological studies on total homocysteine and cardiovascular disease published after the review by Boushey et al in 1995 (10)

Study type and outcome	Sample size	Cases/ events	Controls	Age (year)	Sex	tHcy	Result	Ref.
ECOLOGICAL								
CVD mortality in 11 countries	260			40–49	M	B	Pos	133
CROSS-SECTIONAL								
Severity of atherosclerosis in PAD pt.	185			<55	M/F	B,PML	Pos,Pos	134
Atherosclerosis in hyperlipidemic pt.	482			mean 60	M/F	B	Pos	135
Occlusive arterial dis. in HD pt.	50	24		26–84	M/F	B	Pos	136
Intervention failure in PAD pt.	66			mean 52	M/F	B	Pos	137
Carotid wall thickness in FH/healthy subjects	115			10–19	M/F	B	Pos	138
Atherosclerotic compl. in HD pt.	176	85		mean 56	M/F	B	Pos	139
Extent of CAD in pt. with suspected CAD	367			mean 73	M/F	B	Neg	140
Cardiovasc. death in male relatives of children	756	42		8–12	M/F	B	Pos	107
Cardiovasc. dis. in male relatives of FH children	165	39		7–17	M/F	B	Pos	108
Macrovascular dis. in diabetic pt.	28	17		<60	M/F	B,PML	Neg, Pos	141
Confirmed VT(E) in pt. with suspected VT(E)	208	60		19–91	M/F	B	Pos	142
CHD, PAD, CVD in the general population	630			≥55	M/F	B	Pos[1]	143
CASE-CONTROL								
Recurrent VT(E) - General practice ctr.		185	220	23–88	M/F	B,PML	Pos, Pos	144
Acute MI - Population ctr.		68	80	28–81	M/F	B	Neg	145
Acute stroke - Population ctr.		162	60	51–98	M/F	B	Neg	146
CAD - Healthy workers		150	584	<60	M/F	B	Pos	147
CHD - Mixed ctr. source		162	155	38–68	M/F	B	Pos	148
Venous/arterial oclusions - Mixed ctr. source		157	60	mean 33	M/F	B,PML	Pos, Pos	149
VT(E) - Blood donors		35	39	20–56	M/F	B,PML	Neg, Neg	150
CAD - Healthy executives		304	231	mean 62	M/F	B	Pos	151
PAD - Population ctr.		65	65	36–62	M/F	B,PML	Pos, Pos	47
PAD - Mixed ctr. source		50	45	mean 46	M	B	Neg	152

VT(E) - Neighbor ctr.		269	269	<70	M/F	B	Pos	153
First MI - Population ctr.		130	118	<76	M/F	B	Pos	154
CAD - Population ctr.		70	45	28–79	M/F	B	Pos	155
CAD - Mixed ctr. source		45	23	mean 48	M/F	B,PML	Pos, Pos[2]	156
CHD - Mixed ctr. source		111	105	<55	M/F	B,PML	Pos, Neg	157
Thrombangitis obliterans - Healthy subjects		12	30	mean 33	M/F	B	Pos	158
MI in Northern Ireland - General practice ctr.		191	171	25–64	M	B	Neg[3]	159
MI in France - Population ctr.		229	315	25–64	M	B	Pos	159
CHD, PAD, CVD - General practice ctr.		58	111	13–68	M/F	B,PML	Pos, Pos	70
CAD, PAD, CVD - Mixed ctr. source		750	800	<60	M/F	B	Pos	49
CAD - Healthy workers		152	121	<60	M/F	B	Pos	160
CAD - Mixed ctr. source		131	189	25–65	M/F	B	Pos	161
NESTED CASE-CONTROL								
Stroke (fw up 12.8 year)		107	118	40–59	M/F	B	Pos	162
CHD (fw up 4 year)		122	478	12–61	M/F	B	Pos	163
VT(E) (fw up 10 year)		145	646	25–68	M	B	Pos	164
CAD without prior MI (fw up 9 year)		149	149	25–68	M	B	Neg	165
COHORT								
Thrombotic events in SLE pt. (fw up 4.8 year)	337	94		mean 35	M/F	B	Pos[4]	166
Cardiovasc. events in HD pt. (fw up 1.4 year)	37	16		mean 56	M/F	B	Pos	167
Mortality in CAD pt. (fw up 4.6 year)	587	64		32–80	M/F	B	Pos	168

B; basal or fasting tHcy level; CAD, angiographically verified coronary artery disease; CHD, coronary heart disease; CVD, cerebrovascular disease; FH, familial hypercholesterolemia; HD, hemodialysis; MI, myocardial infarction; PAD, peripheral artery disease; PML, postmethionine load tHcy level; SLE, systemic lupus erythematosus; VT(E), venous thrombotic(embolic) events

[1]Negative in subjects ≥75 year

[2]Reported as negative by the authors, but mean tHcy is significantly higher in patients than controls

[3]Positive in univariate analysis

[4]tHcy is a risk factor for atherothrombotic events but not venous thrombosis

Clinical Genetics and Familial Vascular Disease

FAMILY STUDIES The role of familial factors in the determination of plasma tHcy levels has been demonstrated by studies of twins (193), by investigation of family members (135, 194), and by the frequent finding of hyperhomocysteinemia in patients with familial vascular disease (62, 135, 148).

Recently, Tonstad et al performed a series of studies on tHcy levels in healthy children and in children with familial hypercholesterolemia. In one study, lipid-related factors and tHcy levels were determined in 756 school children aged 8–12 years. In this study, the lipid levels in the children were associated with a family history of hyperlipidemia, but not with vascular disease. In contrast, a modest tHcy elevation in the children was significantly related to premature cardiovascular death in their male relatives. In this group, there was a complete shift of the tHcy distribution curve towards higher values, with the serum folate level as the strongest determinant (107). In a similar study of 155 children with hypercholesterolemia, we found a higher tHcy level in children whose parents had vascular disease, compared with those who did not. Moreover, homozygosity for the C677T mutation in the MTHFR gene was associated with a higher tHcy level and tended to be more frequent in children with parental history of cardiovascular disease (108).

The available data, therefore, suggest that an elevated tHcy level is a familial trait that may contribute to increased risk in persons with a positive family history of cardiovascular disease.

CBS AND MTHFR The early studies in vascular patients often concluded that an abnormal response to a methionine load was due to heterozygosity for CBS deficiency (9, 124, 169). However, obligate heterozygotes for CBS deficiency apparently have no excess risk of vascular disease (195, 196), and genetic analyses indicate that the hitherto identified CBS mutations occur only sporadically in patients with vascular disease (69, 70, 197). The prevalence of the 68 bp insertion in the CBS gene is somewhat higher in coronary artery disease patients, but this genetic variant is not associated with hyperhomocysteinemia, and its clinical significance is unknown (26).

In 1995, Frosst et al suggested that the C677T polymorphism in the MTHFR gene is a candidate risk factor for vascular disease (29). This has been supported by some (70, 157, 198) but not most studies (189, 199–203). A recent meta-analysis on eight different case-control studies suggests that the TT genotype is a modest but significant risk factor for coronary artery disease (204).

It is an apparent paradox that the TT genotype in the MTHFR gene, a strong predictor of hyperhomocysteinemia in the general population (29, 73, 74), is not unequivocally associated with increased cardiovascular risk. There are

some possible explanations. Presumably, the TT genotype affects folate distribution and thereby causes elevated tHcy, which in turn may be responsible for the vascular lesion. If so, the predictive value of the tHcy level is expected to be stronger than that of a more remote genetic defect. In addition, the plasma tHcy level only becomes elevated in folate-deficient subjects with the TT genotype (74, 189, 205, 206). Finally, the slope of regression lines relating tHcy to folate increases in the order of CC, CT, and TT genotypes (73, 202), which suggests that subjects with the TT genotype and high serum folate have a low tHcy level. This points to a protective effect for the combination of TT genotype and positive folate homeostasis, as shown for colorectal cancer (207). Thus, the low frequency of the TT genotype combined with its possible dual effect requires large studies to investigate the interaction between the C677T polymorphism and folate status in cardiovascular patients.

Atherosclerosis versus Thrombosis

HOMOCYSTINURIA In subjects with homocystinuria, independent of the site of the metabolic defect, the main cause of death is arterial and venous thromboembolic events (15). In untreated CBS-deficient subjects, the annual risk of such an event is 4% (15). On autopsy, the macroscopic findings include arterial and venous thromboses, arteriosclerotic lesions in large and medium-sized arteries, and multiple infarctions in different organs. Microscopic examinations show intimal thickening and fibrosis, proliferation of the connective tissue, lesions of the internal elastic membrane, and narrowing of the arterial lumen. Fatty atheromatous plaques in the arteries are, however, not a common finding (9, 15). Ultrasound imaging has revealed that intimal-medial thickness and blood flow velocity is normal in patients with homocystinuria, which contrasts to the diffuse and focal thickening of carotid arteries in patients with familial hypercholesterolemia (208). Thus, in inborn errors of metabolism with severe hyperhomocysteinemia, the thromboembolic events dominate the clinical picture, and the histological findings differ from the typical atheromatous changes related to hyperlipidemia.

ARTERIAL DISEASE Most clinical and epidemiologic studies on tHcy and vascular disease have suggested a role of Hcy in atherogenesis (10). Cross-sectional studies have shown that the tHcy level is related to the extent of atherosclerotic disease in the carotid (173, 184), coronary (161, 209, 210), and peripheral (134) arteries. In teenagers, tHcy is associated with intimal-medial thicknesses in the carotid artery, suggesting that the tHcy level may be a marker of early carotid atherosclerosis (138).

There is now increasing evidence suggesting that elevated tHcy may provoke arterial thromboembolic events. In patients with systemic lupus erythe-

matosus, tHcy is positively related to increased risk of arterial thrombosis (166). Moreover, tHcy is associated with an increased risk of placental infarction (211), which usually is explained by multiple thrombi (212). One recent study in coronary patients points to a strong relation between tHcy and acute cardiovascular events (168).

VENOUS THROMBOSIS The strongest evidence in favor of a thrombotic effect of Hcy is provided by the clinical studies on patients with venous thrombosis (128, 129, 142, 144, 150, 153, 164, 213–217); most of these studies conclude that elevated tHcy is a risk factor. In a meta-analysis including 8 studies (830 patients and 819 controls), the pooled odds ratio for venous thrombosis was 2.8 in subjects with hyperhomocysteinemia (109). In some of these studies, the PML tHcy level was more strongly related to disease than the fasting tHcy level (144, 214, 215), but the meta-analysis indicates that they distinguish equally well between cases and controls (109).

The most frequent cause of familial venous thrombosis is resistance to activated protein C due to the factor V Leiden mutation (218). In a study of Israeli-Arab families with homocystinuria caused by CBS deficiency, thrombosis occurred only in patients who also had the factor V Leiden mutation (219). Notably, a prospective study of apparently healthy men demonstrated that tHcy above the 95th percentile was associated with increased risk of idiopathic venous thromboembolism (RR = 3.4) (164). In subjects who also had the Leiden mutation, the risk was markedly increased (RR = 21.8). Thus, it is plausible that hyperhomocysteinemia causes venous thrombosis only in the presence of additional thrombotic risk factors. However, controversy exists since others have demonstrated that CBS deficiency (220) as well as hyperhomocysteinemia (153, 221) are risk factors for thromboembolism in the absence of the factor V Leiden mutation.

MORTALITY There is a strong relation between the tHcy level in various countries and cardiovascular mortality rates (133). We recently investigated the relation between the tHcy level and mortality in 587 patients with confirmed coronary artery disease (168). Plasma tHcy measured at the time of angiography was strongly associated with previous myocardial infarction, but only weakly related to the extent of coronary artery disease. Notably, after a follow up time of 4.6 years, only 3.6% of those with a tHcy level <9 μM had died, whereas the mortality was 24.7% in patients with a tHcy level >15μM. After adjustment for possible confounders, there was still a graded increase in mortality with increasing tHcy levels. Using tHcy below 9 μM as reference, the mortality rate increased 1.9-, 2.8-, and 4.5-fold among those with tHcy levels of 9–15, 15–20, and ≥20 μM, respectively (168). Subgroup analyses showed that the tHcy level predicts mortality independent of age, gender,

smoking habits, blood pressure (Figure 3), and serum creatinine. Our data suggest that an increased tHcy level may contribute to acute thromboembolic events leading to death.

Mechanisms

Observations in homocystinuria patients, animal experiments, and in vitro studies have identified several potential sites where hyperhomocysteinemia may induce vascular lesions. These targets include connective tissue and smooth-muscle cells, platelets, endothelial cells, the vessel wall, blood lipids, coagulation factors, and nitric oxide.

In vitro (222) and in vivo (223) experiments suggest that Hcy promotes aggregation of platelets, but this has been contested (224, 225). Endothelial damage mediated through H_2O_2 production has been proposed (226–229), but cysteine induces similar effects, and the validity of these observations for in vivo atherogenesis has been questioned (230). Hcy increases DNA synthesis,

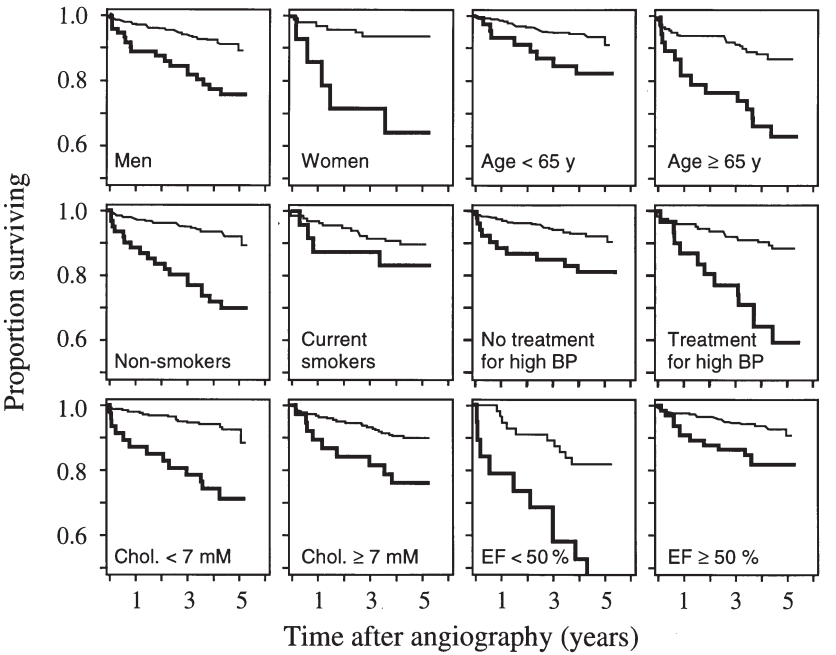

Figure 3 The relation between plasma total homocysteine (tHcy) and mortality. Kaplan-Meier survival plots comparing patients with tHcy below (thin line) and above (thick line) 15 μM in various subgroups. The tHcy level was measured at the time of the angiography (168). BP, blood pressure; Chol., serum total cholesterol; EF, left ventricular ejection fraction.

growth, and cyclin A gene expression (231) in cultured vascular smooth muscle cells, and cyclin-dependent kinase expression in the aorta of rats (232). Oxidative modification of LDL by Hcy has been demonstrated in vitro (233, 234) but has not been observed in hyperhomocysteinemic patients (235, 236). Physiologic levels of Hcy may enhance the binding of lipoprotein(a) to fibrin (237), but cysteine and other thiols have a similar effect. High concentrations of Hcy in vitro activate factor V (238), reduce protein C activation (239, 240), inactivate the cofactor activity of thrombomodulin (241), suppress thrombomodulin (240) and anticoagulant heparan sulfate expression (242), and block tissue plasminogen activator binding to human endothelial cells (243). Hcy rapidly reacts with endothelium-derived relaxing factor/nitric oxide (NO) to form S-nitroso-Hcy, which acts as a potent antiplatelet agent and vasodilator. The formation of this adduct may attenuate H_2O_2 production from Hcy and, thereby, protect against the atherogenic properties of Hcy. According to this model, vascular injury is caused by an imbalance between NO production from dysfunctional endothelial cells and the levels of Hcy (225). Notably, impaired endothelium-dependent vasodilatation associated with elevated tHcy has been demonstrated in vivo (244, 245). The possible etiologic mechanisms of Hcy in vascular disease have been summarized in recent review articles (225, 246).

The data cited above show that there is no unifying hypothesis explaining the atherogenic and thrombogenic effects of circulating Hcy. This may reflect its diversity of effects, but may also be due to flaws in study design or interpretation of data. In general, it is difficult to mimic the slow atherosclerotic effect by short-term in vitro studies. Another problem is related to the high millimolar concentrations of Hcy frequently used (229, 239, 243, 247, 248) that are 100- to 1000-fold higher than observed in moderate hyperhomocysteinemia (249). Moreover, the complex redox reactions involving the various Hcy forms and their relation to other aminothiols in vivo (31) contrast to the in vitro testing of a single Hcy species (249). Finally, the specificity of some Hcy effects should be questioned because they can be obtained by other thiols (230, 233, 250), in particular cysteine, which is the most abundant aminothiol in plasma, with a total concentration about 25-fold higher than tHcy (31).

HOMOCYSTEINE-LOWERING THERAPY

Increased intake of folic acid, vitamin B12, and B6 will probably reduce the tHcy level in nearly all individuals independent of their pretreatment tHcy level (251). Vitamin therapy partly prevents the vascular complications of homocystinuria (15), and use of vitamins is associated with lower risk of vascular disease in the general population (49). A high intake of fruits and vegetables, which are good sources of dietary folate, protects against cardiovascular dis-

ease (252), and an observational study suggests that vitamin B6 may delay the progress of coronary heart disease (253). However, it remains to be shown in randomized placebo-controlled clinical trials that a reduction of the tHcy level has an overall beneficial effect.

Vitamin and Drug Therapy

FOLIC ACID AND FOLATE INTAKE In 1988, Brattström et al showed that healthy subjects responded to a high dose of folic acid (5 mg/d) with a marked reduction in their tHcy levels (254). Since then, several studies have demonstrated that 0.65–10 mg/d of folic acid alone or together with vitamin B12 and/or B6 reduce the fasting and PML tHcy level by 25–50%, both in healthy and in hyperhomocysteinemic subjects and in vascular patients (251, 255–257). Data on dose response are sparse, but no difference was found for 2.5 versus 10 mg/d in patients with myocardial infarction (145) or for 0.5 versus 5 mg/d in healthy subjects (109).

Generally, a total folate intake from food and supplements below 200–250 μg/d is occasionally associated with hyperhomocysteinemia, whereas an intake of 300–400 μg/d usually ensures a normal to low tHcy level in the majority of the population (55, 258–260). A higher dose may be required to obtain a maximal tHcy reduction in subgroups such as patients with reduced renal function (261) or subjects with low folate status (73).

Folic acid is considered nontoxic and well tolerated for chronic use in high doses (262), but may mask the symptoms of vitamin B12 deficiency (see below).

VITAMIN B12 The effect of vitamin B12 on the tHcy level is modest with maximum 10–15% reduction (109, 254, 256), except in vitamin B12-deficient subjects (1). However, a low serum B12 level may prevent an optimal response to folic acid (73, 251). Moreover, there is high prevalence of vitamin B12 deficiency in elderly subjects (263). The concern that folic acid supplementation may alleviate hematological signs of B12 deficiency or even precipitate neuropathy has been thoroughly debated (10, 264–266). A daily vitamin B12 supplement of 200 μg/d can probably prevent the clinical symptoms of pernicious anemia (267).

VITAMIN B6 Oral treatment with pyridoxine up to 300 mg/d does not lower the fasting tHcy level in healthy subjects or vascular patients (251, 254, 257, 268). However, pyridoxine (10–250 mg/d) lowers an abnormal PML tHcy level in most patients, and, when combined with folic acid, nearly all obtain a normal PML tHcy level (45, 127, 255, 257, 269). Chronic use of vitamin B6 may precipitate peripheral neuropathy, but a daily dose of 100 mg or less is probably safe (270).

BETAINE AND DRUGS Except in homocystinuria, the effect of betaine has only sporadically been investigated. It may be equally (255), or in some instances, more efficient (269) than folic acid and pyridoxine in reducing an abnormal PML Hcy level. In renal failure patients, betaine had no effect on the fasting tHcy level (271).

Tamoxifen (96), estrogen replacement therapy (97), and aminothiol drugs (78, 79) may also reduce the plasma tHcy level. Compared with efficient, safe and inexpensive vitamins, these are not the first choice for patients with cardiovascular disease.

Intervention Strategies

A consensus on intervention strategies to reduce tHcy does not exist. Folic acid will presumably be used in all trials on vascular patients, and vitamin B12 will frequently be added as a safety measure against pernicious anemia. The inclusion of vitamin B6 is controversial. This vitamin participates in more than 100 reactions (272), and its relation to vascular disease is independent of the tHcy level (151), suggesting that the cardioprotective effect may be mediated via other mechanisms than improved Hcy metabolism. The design of randomized placebo-controlled trials, which now are under way, differ markedly in target population, endpoint(s), and the doses and combinations of vitamins. Thus, within a few years, we hope we will have gained experience with several therapeutic regimes, regarding both efficiency and side effect profile.

Since 10% of all coronary artery disease events may be explained by tHcy (10), a primary prevention strategy needs to be considered. In the United States, the folic acid fortification of flour and cereal products starting in 1998 will probably reduce the proportion of the population with hyperhomocysteinemia (266). This large-scale population intervention may provide data on whether food fortification has an overall beneficial effect and, therefore, can be recommended in other countries as well. An alternative strategy is life-style intervention. High dietary folate intake, low coffee consumption, smoking cessation, and increased physical activity may all contribute to lower tHcy levels in the general population (54, 64). Thus, while waiting for the outcome of the clinical trials, we can safely recommend previously accepted guidelines for a cardioprotective lifestyle.

CONCLUSION

Epidemiologic studies have unequivocally established that an elevated plasma tHcy level both predicts and precedes the occurrence of cardiovascular disease. This hypothesis also meets the criteria of causality (273), such as consistency, strength, temporality, and biologic plausibility. The relation between

the tHcy level and cardiovascular disease is graded without an apparent threshold and remains strong after adjustment for potential confounders. The joint effect of an elevated tHcy level with conventional factors such as hypertension or smoking may confer a particularly high risk.

The high prevalence of moderate hyperhomocysteinemia, combined with identified acquired and genetic determinants, makes it an ideal target for intervention in vascular patients, as well as in the general population. Vitamins are an efficient and safe means to reduce an elevated tHcy level, but randomized placebo-controlled clinical trials are yet to be undertaken, despite repeated appeals (175, 176). The commercial incentives to test inexpensive and nonpatentable vitamins are low, and health authorities carry a special responsibility to promote such trials.

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