Review Article

Genetics of hyperhomocysteinaemia in cardiovascular disease

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Abstract

Homocysteine, a sulphur amino acid, is a branch-point intermediate of methionine metabolism. It can be degraded in the transsulphuration pathway to cystathionine, or remethylated to methionine via the remethylation pathway. In both pathways, major genetic defects that cause enzyme deficiencies are associated with very high plasma homocysteine concentrations and excretion of homocystine into the urine. Mildly elevated plasma homocysteine concentrations are thought to be an independent and graded risk factor for both arterial occlusive disease and venous thrombosis. Genetic defects in genes encoding enzymes involved in homocysteine metabolism, or depletion of important cofactors or (co)substrates for those enzymes, including folate, vitamin B_{12} and vitamin B_6, may result in elevated plasma homocysteine concentrations. Plasma homocysteine concentrations are also influenced by dietary and lifestyle factors. In the last decade, several studies have been conducted to elucidate the genetic determinants of hyperhomocysteinaemia in patients with cardiovascular disease. We report on both environmental and genetic determinants of hyperhomocysteinaemia and give a detailed overview of all the genetic determinants that have been reported to date.

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Introduction

About 30 years ago, McCully postulated that mildly elevated homocysteine concentrations could increase the risk of cardiovascular disease[1] after observing artery wall lesions in two different metabolic disorders of methionine metabolism which resulted in elevated plasma homocysteine concentrations.[2] Since then many studies have been conducted to investigate whether elevated plasma homocysteine concentrations are associated with an increased risk of cardiovascular disease. A modest elevation of plasma homocysteine concentration, commonly referred to as hyperhomocysteinaemia, is generally,[3,4] although not universally,[5,6] accepted as an independent and graded risk factor for both arterial occlusive diseases and venous thrombosis.[7,8] In this review, we report on both environmental and genetic determinants of hyperhomocysteinaemia and give a detailed overview of all the genetic determinants that have been reported to date.

Homocysteine metabolism

Homocysteine is a sulphur amino acid that is formed by the demethylation of the essential amino acid methionine via *S*-adenosylmethionine (AdoMet) and *S*-adenosylhomocysteine (AdoHcy) (*see* Fig. 1). Methionine adenosyltransferase catalyses the formation of AdoMet, which is the methyl donor in numerous reactions (e.g. methylation of DNA, RNA, hormones and lipids). The transmethylation reactions form AdoHcy, which is an inhibitor of many methyltransferases. Adenosine and homocysteine are formed from the hydrolysis of AdoHcy by *S*-adenosylhomocysteine hydrolase. Homocysteine can be further metabolized in two pathways: it may be irreversibly degraded to cystathionine and cysteine in the transsulphuration pathway, or remethylated to methionine in the so-called remethylation pathway.

Transsulphuration

The first step in the transsulphuration pathway is the condensation of homocysteine and serine to give cystathionine. Cystathionine β-synthase (CBS) catalyses this initial step and requires pyridoxal 5′-phosphate (PLP, the active form of vitamin B_6) for its activity. Cystathionine is subsequently hydrolysed to cysteine by another PLP-requiring enzyme, γ-cystathionase. These enzymatic steps are limited to the liver and kidney.

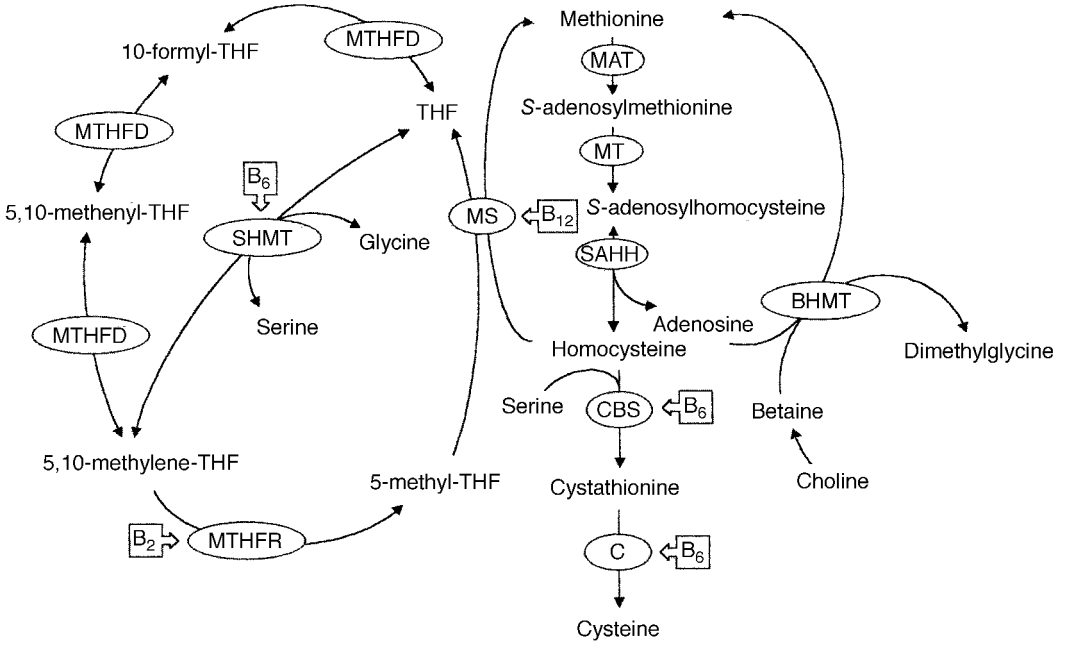

Figure 1. *Homocysteine metabolism. MAT = methionine adenosyltransferase; MT = methyltransferases; SAHH = S-adenosylhomocysteine hydrolase; BHMT = betaine–homocysteine methyltransferase; MTHFD = methylenetetrahydrofolate dehydrogenase; SHMT = serine hydroxymethyltransferase; CBS = cystathionine β-synthase; C = γ-cystathionase; MS = methionine synthase; MTHFR = methylenetetrahydrofolate reductase; THF = tetrahydrofolate; B_2, B_6, B_{12} = vitamins B_2, B_6, B_{12}, respectively.*

Remethylation

Homocysteine can be remethylated to methionine by two different enzymatic reactions: methionine synthase (MS), with 5-methyltetrahydrofolate (5-methyl-THF) as methyl donor; or betaine–homocysteine methyltransferase (BHMT), with betaine (trimethylglycine) as methyl donor. MS is dependent on methylcobalamin (a biologically active form of vitamin B_{12}) as a cofactor for its enzymatic activity. 5-Methyl-THF is formed upon the reduction of 5,10-methylenetetrahydrofolate, a reaction that is catalysed by the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR). MTHFR uses flavin adenine dinucleotide (FAD; the active form of vitamin B_2) as cofactor.[9] The reversible conversion of serine and tetrahydrofolate (THF) to glycine and 5,10-methylene-THF is catalysed by serine hydroxymethyltransferase (SHMT), another PLP-dependent enzyme in homocysteine metabolism. Methylenetetrahydrofolate dehydrogenase (MTHFD) has several enzymatic properties and catalyses three sequential reactions in the interconversion of one-carbon derivatives of THF. The remethylation by BHMT occurs primarily in the liver and kidney, whereas folate- and vitamin B_{12}-dependent remethylation of homocysteine take place in every cell of the human body apart from red blood cells (RBCs).

B vitamins as cofactors

Several B vitamins serve as cofactors in homocysteine metabolism: PLP (vitamin B_6) as a cofactor for CBS, γ-cystathionase and SHMT; methylcobalamin (vitamin B_{12}) for MS; and FAD (vitamin B_2) for MTHFR. In addition, 5-methyl-THF, the active form of folate, is required by MS as a co-substrate; it is formed upon reduction of 5,10-methylene-THF by MTHFR.

Vitamin B_6

Vitamin B_6 consists of different related forms: pyridoxine, pyridoxal and pyridoxamine, and their phosphate esters.[10] Vitamin B_6 is absorbed in the upper small intestine by diffusion and transported to the liver where phosphorylation takes place.[11] PLP is the main circulating form which, bound by albumin, is exported from the liver[12] and is the only active form that can be used by enzymes. Uptake into tissue is by extracellular dephosphorylation followed by metabolic trapping intracellularly as PLP.[13]

Vitamin B_{12}

Dietary vitamin B_{12} is first released from food by pepsin. In the stomach it is bound to haptocorrin. Vitamin B_{12} is released from haptocorrin by pancreatic proteases and is subsequently bound to intrinsic factor (IF).[14] In the ileum, the IF–vitamin B_{12} complex binds

to its receptor cubulin[15] and is absorbed by receptor-mediated endocytosis. Vitamin B_{12} is transported in the circulation by the protein transcobalamin (TC), which also delivers vitamin B_{12} to the cells.[16] The vitamin B_{12}–TC complex is taken up by receptor-mediated endocytosis via the receptor TC-R,[16] which is expressed on the cell surface of most tissues. Intracellularly, vitamin B_{12} is released and metabolized in different steps before it can function as a methyl carrier in homocysteine metabolism. Various inborn errors of metabolism involving each of these necessary steps have been described. These have been subdivided into different groups on the basis of complementation experiments: cobalamin (cbl) F, cblC/D, cblE and cblG.

Folate

Folate occurs naturally as a mixture of polyglutamate derivatives.[17] However, dietary folate polyglutamates must be hydrolysed to monoglutamates in the intestine prior to absorption. Folylpoly-γ-glutamate carboxypeptidase, which is anchored to the intestinal brush border membrane, hydrolyses these polyglutamylated folates into monoglutamylfolates, which can be actively transported across the membrane by the reduced folate carrier. Once inside the cell the monoglutamylfolates have to be converted back to polyglutamates by folylpolyglutamate synthetase, since the enzymes that utilize folate as cofactor have higher affinities for polyglutamated folate species.[18] Folylpolyglutamates cannot cross or are only poorly transported across cell membranes and are important for intracellular retention of folates, whereas monoglutamates, in particular methyl-THF, are the transport form of the vitamin.[18]

Determinants of homocysteine concentrations

Disturbances in intracellular homocysteine metabolism may lead to elevated plasma homocysteine concentrations. These defects can have a genetic background, such as an inherited enzyme deficiency, a variation in the genes encoding these enzymes or an environmental aetiology (*see* Box 1), such as diet and lifestyle factors, which could lead to depletion of important cofactors or substrates involved in homocysteine metabolism or even inhibited enzyme activity.

Environmental factors

Renal function

A strong determinant of homocysteine concentration is kidney function.[19] Patients with renal failure have markedly elevated plasma homocysteine concentrations, but the underlying pathophysiological mechanism is not completely understood. However, the extent to which homocysteine is excreted in the urine is very low in these patients. It is possible that homocysteine metabolism is influenced or even regulated by kidney function, or that the kidney itself converts a major amount of homocysteine present in blood. Serum creatinine concentrations, as an indicator of altered renal function, have been shown to be strongly positively associated with homocysteine concentrations.[20]

Box 1. *Environmental factors associated with hyperhomocysteinaemia*

- Renal function
- Increasing age
- Male gender
- Dietary
  - Low folate status
  - Low vitamin B_{12} status
  - Low vitamin B_6 status
- Lifestyle
  - Coffee consumption
  - Smoking
  - Alcohol
  - Lack of exercise
  - High body mass index
- Other
  - Drugs

Age and sex

Increasing age[21,22] and male gender[22,23] have been found to be associated with increased homocysteine concentrations, although the influence of gender was found to be most pronounced in the lower concentration range.[23] The sex difference may be explained by vitamin status,[24] muscle mass and hormonal factors.[25]

Dietary factors

Several dietary factors have been investigated as possible determinants of homocysteine concentrations in the Hordaland Homocysteine Study,[26] the Framingham Offspring Cohort[20] and the MORGEN Study.[27] Obviously, folate status and that of other B vitamins are important determinants of homocysteine concentrations in the general population since they play an important role in homocysteine metabolism as substrate or cofactors. Both folic acid supplementation and a high dietary folate intake[22,24,28–30] have been shown to decrease plasma homocysteine concentrations and this relationship was found to be dose-responsive. However, folic acid in supplements appears to be more effective than natural food folate in improving folate status,[22] presumably because the synthetic form of the vitamin is more stable and more bioavailable than the natural food

folate.[31] Regular users of multivitamin preparations also have lower homocysteine concentrations,[23] although the intake of supplements that contain only vitamin B_6 and cobalamin, but not folic acid, was not related to homocysteine concentrations.[22] In the Framingham Offspring Cohort, folate and vitamin B_{12} concentrations were associated with low homocysteine concentrations, whereas dietary vitamin B_{12} intake was not.[20] In a Dutch population, folate intake was the only B vitamin that was independently and inversely associated with plasma homocysteine concentrations.[27]

Lifestyle factors

Coffee consumption and smoking have also been associated with increased homocysteine concentrations[32–34] and, in combination with low folate intake, greatly exceeded the effect of each factor alone.[22] The association between alcohol consumption and homocysteine appears J-shaped:[35] alcoholics have high homocysteine concentrations,[36] whereas moderate alcohol users have lower homocysteine concentrations compared with non-drinkers.[37] The effect of alcohol consumption was also shown to be dependent on the type of alcoholic beverage.[38]

Miscellaneous

Several drugs, such as antiepileptic drugs, methotrexate or lipid-lowering drugs, have been shown to increase plasma homocysteine to mildly elevated concentrations.[39] Also, lack of exercise,[40] body mass index[20] and cardiovascular risk factors such as cholesterol[40,41] and blood pressure[42] have been mentioned as determinants of homocysteine concentrations.

Genetics of homocysteine

Homocystinuria

Severe hyperhomocysteinaemia or homocystinuria, which is characterized by the accumulation of homocysteine in the blood and homocysteine excretion in the urine, was first described by Carson and Neill.[43] CBS deficiency is the most common cause of homocystinuria.[44] The most common clinical features of CBS deficiency include dislocation of the optic lens, osteoporosis, marfanoid features, mental retardation and an early onset of vascular disease. Most CBS-deficient patients have clearly reduced CBS enzyme activity in extracts of cultured fibroblasts. In addition to homocysteine, methionine accumulates in the body and is excreted via the kidney. More than 100 mutations in the CBS gene have been described in CBS-deficient patients, and most of them have been found to decrease CBS enzyme activity significantly.[45] The two most frequent mutations are 833T→C (I278T), which accounts for about one-quarter of all homocystinuric alleles and is the most common cause of homocystinuria in the Netherlands,[46] and 919G→A (G307S), which is the leading cause of homocystinuria in Ireland.[47]

Reduced MS activity due to inborn errors of methylcobalamin transport or synthesis have been described as causing severe hyperhomocysteinaemia, but such cases are very rare.[48,49]

In MTHFR deficiency, the clinical severity is correlated with the degree of enzyme deficiency, the most common clinical manifestation of MTHFR deficiency being developmental delay.[48] In contrast to CBS deficiency, the major biochemical findings are moderate homocystinuria and homocystinaemia, with low or relatively normal concentrations of plasma methionine; the excretion of homocysteine is much less than in homocystinuria due to CBS deficiency. Deficiency of CBS and MTHFR as well as of MS are associated with an increased risk for vascular disease.

Hyperhomocysteinaemia

In a study among family members of 21 post-load hyperhomocysteinaemic vascular patients, it was shown that, after exclusion of individuals with vitamin deficiencies, liver and renal diseases, the number of family members with hyperhomocysteinaemia was much higher than might be expected in the normal population.[50] Furthermore, post-load mild hyperhomocysteinaemia was established in at least one other family member in 71% of the families, indicating a strong genetic basis for hyperhomocysteinaemia. The conclusion that hyperhomocysteinaemia is, at least partially, genetically based is in line with previous reports.[51–55] Thus, genetic variation in genes coding for enzymes involved in the regulation of homocysteine may affect plasma homocysteine concentrations.

Obviously, the genes coding for the enzymes MTHFR, CBS and MS are possibilities because of their direct catalytic involvement in homocysteine and folate metabolism, but other enzymes involved in homocysteine metabolism, such as BHMT, SHMT and MTHFD, are also important candidates. In addition, enzymes involved in the metabolism of cofactors such as vitamin B_6 or vitamin B_{12}, or substrates such as folate, could be of great importance in maintaining homocysteine homeostasis.

Numerous variations in genes involving homocysteine metabolism have already been investigated (*see* Table 1), of which 677C→T (A226V) in the MTHFR gene is most extensively described. The requirements of high-throughput and ease of a polymorphism genotyping method will become increasingly important. A large number of good genotyping methods are

Table 1. *Possible genetic determinants of plasma homocysteine concentrations*

Gene	Polymorphism	Amino acid substitution	Frequency (%) (allele)	Effect on total homocysteine*
MTHFR	677C → T	A226V	30–40 (T)	+ (25% ↑)
	1298A → C	E433A	30–40 (C)	±
CBS	844ins68	–	5–12 (ins)	0
	14037 31-bp VNTR	–	NA**	+
	−5707GT STR	–	NA**	0
	699C → T	Y233Y	30–40 (T)	0
	1080C → T	A360A	30–45 (T)	0
MS	2756A → G	D919G	15–20 (G)	±
MTRR	66A → G	I22M	50 (G)	±
BHMT	595G → A	G199S	1 (A)	0
	716G → A	Q239R	32 (A)	0
	1218G → T	Q406H	< 1 (T)	0
cSHMT	1420C → T	L474F	32 (T)	±
MTHFD	1958G → A	R653Q	44 (A)	0
TC	67A → G	I23V	13 (G)	±
	280G → A	G94S	< 1 (A)	0
	776C → G	P259R	50 (G)	±
	1043C → T	S348F	11 (T)	0
	1196G → A	R399Q	2 (A)	0
GCPII	1561C → T	H475Y	5–10 (T)	±
RFC	80G → A	R27H	48 (A)	0

*+ = clear association; ± = possible association; 0 = no association.
**NA = not applicable.
MTHFR = methylenetetrahydrofolate reductase; CBS = cystathionine β-synthase; bp = base pair; VNTR = variable number of tandem repeats; STR = short tandem repeat; MS = methionine synthase; MTRR = methionine synthase reductase; BHMT = betaine–homocysteine methyltransferase; cSHMT = cytosolic serine hydroxymethyltransferase; MTHFD = methylenetetrahydrofolate dehydrogenase; TC = transcobalamin; GCPII = glutamate carboxypeptidase gene II; RFC = reduced-folate carrier.

available; these have been extensively and systematically explained in two recent review articles.[56,57]

Cystathionine β-synthase

In 1985, Boers *et al.*[58] reported that heterozygosity for CBS deficiency accounted for the hyperhomocysteinaemia in patients with vascular disease. Subsequently, Clarke *et al.*[59] reported a similar observation. However, results from Mudd *et al.*[60] showed no statistically significant increase in the incidence of heart attacks or strokes in parents and grandparents of homocystinuric children. Furthermore, the estimated frequency of heterozygosity for CBS deficiency is much lower than the frequency of mild hyperhomocysteinaemia, which is approximately 10–20% in the normal population and is too low to account for the number of vascular patients with mild hyperhomocysteinaemia.[61] In addition, the finding of decreased CBS activity in cultured fibroblasts from hyperhomocysteinaemic patients with vascular disease in the range of obligate heterozygotes could not be reproduced[62,63] and still requires clarification. The isolation and characterization of CBS cDNA by Kraus *et al.*[64] permitted the molecular genetic analysis of CBS cDNA in patients with vascular disease. In 60 Dutch patients with arterial occlusive disease, we were unable to detect the 833C→T (I278T) mutation in the CBS gene, a mutation that is present in approximately 50% of alleles of Dutch homozygotes for CBS deficiency.[63] In a comparable study, Gallagher *et al.*[65] did not detect the 'Celtic' 919G→A (G307S) mutation in 100 patients with vascular disease, whereas this mutation is the predominant cause of homocystinuria in the Irish population.[47] These observations were supported by the results of Kozich *et al.*,[66] who analysed the CBS cDNA of four hyperhomocysteinaemic patients with peripheral arterial occlusive disease and decreased CBS activity in extracts of cultured fibroblasts. Neither pathogenic mutations nor a defective stimulation by Adomet were noticed as a possible cause of decreased enzymatic activity. In summary, all genetic studies that have been performed to date have failed to detect any involvement of heterozygosity for CBS deficiency in hyperhomocysteinaemia and premature vascular disease.

844ins68

Several polymorphisms have been reported in the CBS gene. A 68-base pair (bp) insertion (844ins68) was first reported in a patient with homocystinuria,[67] but this subsequently appeared to be a common variant.[68] This insertion always segregates in *cis* with the 833T→C mutation, but both are skipped by alternative splicing of the CBS mRNA.[68,69] The 844ins68 polymorphism does not seem to affect CBS enzyme activity[69] or homocysteine concentration,[69,70] although, in 1999, Tsai *et al.* reported that carriers of the 844ins68 insertion had even lower post-methionine-load increases when vitamin B_6 concentrations were below the sample median of 38·0 nmol/L.[71]

31-base pair variable number of tandem repeats polymorphism

In 1998, Kraus *et al.* described the complete nucleotide sequence of the human CBS gene and reported a variable number of tandem repeats (VNTR) polymorphism of 31 bp in intron 13.[72] However, after closer examination we found that this 31-bp VNTR spans the exon 13–intron 13 boundary and could therefore contain multiple splice donor sites, with possible repercussions on CBS protein and, consequently, function. We found that this 31-bp VNTR is associated with a significant increase in post-methionine-load homocysteine concentrations and showed evidence of alternative splicing in individuals with different VNTR genotypes.[73] In addition, we observed a negative correlation between CBS enzyme activity and an increasing number of repeat units, which corroborates the positive association between the VNTR and post-load homocysteine concentrations. We later confirmed the positive association between post-methionine-load homocysteine concentrations and number of repeat units in more than 1400 subjects of the Framingham Offspring Study (Lievers *et al.*, unpublished results). Yang *et al.*[74] reported lower post-methionine-load homocysteine concentrations in the 17-18 as well as the 18-19 genotype compared with the most common 18-18 genotype.

699C→T (Y233Y) and 1080C→T (A360A)

These two silent polymorphisms in the CBS gene have been studied as possible determinants of homocysteine concentrations and risk of coronary artery disease (CAD) or as markers for possible functional variants in the CBS gene.[75–77] De Stefano *et al.*[75] found no association between these CBS variants and plasma homocysteine concentrations, while Aras *et al.*[77] reported decreased post-methionine-load homocysteine concentrations in individuals who were heterozygous or homozygous for the 699T allele compared with 699CC subjects. This association became more significant when individuals carrying 844ins68 and the 1080T allele were excluded. With regard to the 1080C→T polymorphism, the 1080T allele was associated with lower post-methionine-load homocysteine concentrations only when individuals carrying 844ins68 and the 699T allele were excluded from the analysis. Aras *et al.* speculated that the 699C→T and 1080C→T CBS variants may be in linkage disequilibrium with regulatory elements that upregulate CBS gene transcription.[77] In 2000, Kruger *et al.*[76] failed to find an association between the two silent CBS variants and homocysteine concentrations, while in their study population of 142 CAD patients and 105 controls, the homozygous 699TT individuals as well as the 1080CC subjects were significantly underrepresented in the patients. We, however, did not observe any association between homocysteine concentrations or CAD risk and the different genotypes of these two CBS variants.[129]

Methionine synthase

In the MS gene, a single nucleotide polymorphism was identified at position 2756 (an A to G transition, D919G).[78] Harmon *et al.*[79] reported a relative risk of 1·58 [95% confidence interval (95% CI): 1·14–2·19] of the 2756AA genotype compared with the AG and GG genotypes, for having a homocysteine concentration in the top half of the distribution. Several years later, Hyndman *et al.*[80] calculated that the 2756AG heterozygotes were less likely to have a recurrent vascular event than the AA individuals [odds ratio (OR): 3·4; 95% CI: 1·09–10·9]. Furthermore, this 2756A→G polymorphism did not affect homocysteine concentrations, but the RBC folate concentrations were significantly elevated in the 2756AG heterozygotes compared with the AA subjects. Chen *et al.*[81] also investigated the association between this MS variant and plasma homocysteine concentrations, folate levels and risk of myocardial infarction (MI). They found a non-significant reduction of MI risk in the GG individuals compared with the AA individuals and a trend towards decreased homocysteine concentrations. In our study population of patients with cardiovascular disease and healthy controls, the 2756GG genotype was significantly associated with increased risk of coronary heart disease (CHD), whereas the AG genotype was inversely, although not significantly, associated with CHD risk. The 2756A→G polymorphism was, in our study population, not associated with fasting or post-methionine-load homocysteine concentrations (Klerk *et al.*, unpublished results).

Methionine synthase reductase

The enzyme methionine synthase reductase (MTRR) is needed to maintain MS in its active state. Polymorphisms of MTRR might therefore influence homocysteine concentrations. Mutation analysis of the MTRR gene in homocystinuric patients belonging to the cblE complementation group led to the discovery of a polymorphism, an A to G substitution at bp 66 (I22M), which is common in the general population.[82] In a subsequent study, the 66GG genotype appeared to increase the risk of a neural tube defect (NTD) pregnancy outcome in women with low vitamin B_{12} concentrations, while no association between the 66A→G genotype and homocysteine concentrations was reported.[82] This polymorphism was also reported to be a risk factor for the development of premature CAD, with a relative risk of 1·49 (95% CI: 1·10–2·03) in 66GG subjects compared with 66AA individuals. Again, there was no difference in plasma homocysteine, plasma folate and vitamin B_{12} concentrations between the three 66A→G genotypes.[83] In a study of 601 Northern Irish men, Gaughan *et al.*[84] established a relative risk of 1·59 (95% CI: 1·10–2·25) for having a plasma homocysteine concentration in the top half of the distribution. This homocysteine-elevating effect was found to be independent of serum folate, vitamin B_{12} and B_6 concentrations, and the estimated effect of the 66AA genotype on cardiovascular disease (CVD) risk was an approximately 4% increase compared with the 66GG genotype.

Betaine–homocysteine methyl transferase

Betaine–homocysteine methyl transferase is the enzyme which, along with MS, remethylates homocysteine to methionine, but is primarily expressed in liver and kidney. There is only one study that reported polymorphisms in the BHMT gene and the influence of three BHMT variants on homocysteine concentrations and CVD risk.[85] We found one variant, the 1218G→T (Q406H) substitution, in only one patient and one control in the heterozygous state. The other two variants, the 595G→A (G199S) and the 716G→A (Q239R) transitions, were found to be more frequent but showed no associations with homocysteine concentrations and were not associated with an increased CVD risk.

5,10-methylenetetrahydrofolate

677C→T (A226V)

In 1988, Kang *et al.*[86] detected a variant of the MTHFR enzyme which was associated with decreased enzyme activity, reduced stability after heating at 46°C and increased homocysteine concentrations. A few years later these authors demonstrated that this thermolabile form of the MTHFR enzyme was more common among CVD patients (17%) than among controls (5%).[87] In our study this thermolabile MTHFR enzyme was identified in patients with different forms of premature vascular disease and was associated with fasting as well as post-methionine-load homocysteine concentrations.[62] In 1995, Frosst *et al.*[88] identified the single base pair substitution of C to T at nucleotide 677 to be responsible for this thermolabile MTHFR enzyme. Since then, numerous studies have been reported which investigated this MTHFR variant and its association with homocysteine concentrations and CVD risk.[89] Although an association between the 677C→T variant and elevated homocysteine concentrations was universally found,[63,65,88,90–96] an increased risk for CVD was found in only some studies.[63,65] The association between the 677C→T variant and elevated homocysteine concentrations was reported to exist only in individuals with low folate status.[91,92] In 1998, the hypothesis that this variant is associated with altered distribution of RBC folates was tested by a chromatographic method *in vitro*.[97] This method involves the analysis of RBC folates by affinity/high-performance liquid chromatography with electrochemical (coulometric) detection.[98] Probably due to the reduced MTHFR enzyme activity, formylated tetrahydrofolate polyglutamates were present at the expense of methyl-THF in most 677TT individuals.

Thermolabile MTHFR accounts for 25% of the mild hyperhomocysteinaemia observed in patients with vascular disease,[62] indicating that additional mutations in the MTHFR gene or other genes may also affect homocysteine concentrations. Moreover, it appears that the 677TT genotype is associated with increased homocysteine concentrations only in individuals with low folate status.[91,92] Thus, possible gene–environment interactions also play an important role in modulating plasma homocysteine concentrations.

1298A→C (E433A)

In 1998, a second common polymorphism in the MTHFR gene was described, the 1298A→C transition, which mandates an amino acid substitution of glutamate by alanine.[99] This variant was observed only in *trans* with the 677C→T variant and was associated with decreased MTHFR enzyme activity. We have described the associations of this 1298A→C variant with MTHFR enzyme activity, plasma homocysteine concentrations and risk of CVD.[100] We again detected a decrease in enzyme activity in individuals with the 1298AC and 1298CC genotypes, but noted no effect on the thermostability of the enzyme or on plasma homocysteine concentrations.[100] Although all studies confirm that the 1298A→C variant is associated with

decreased MTHFR activity,[99–102] supported by expression analysis in *Escherichia coli*,[103] an association with homocysteine concentrations has not been detected.[101,102,104–109] Probably, other factors that affect homocysteine concentrations, such as nutritional status, play a role, or the decreased MTHFR enzyme activity must reach a certain threshold below which increased plasma homocysteine concentrations result.

Serine hydroxymethyl transferase

In humans, SHMT is present as two isoenzymes, one located in the mitochondria and the other in the cytoplasm.[110] In 1999, the genes for both isoforms were sequenced by our group and several variations were found in both genes.[111] One polymorphism in the cytosolic SHMT, i.e. 1420C→T (L474F), was associated with increased homocysteine concentrations and decreased plasma and RBC folate levels in 1420CC homozygotes. This polymorphism may cause a shift in distribution of the various folate derivatives, and its consequences for homocysteine/folate metabolism deserve further attention (e.g. by *in vitro* expression analysis).

Methylenetetrahydrofolate dehydrogenase

In the MTHFD gene, a 1958G→A (R653Q) substitution was detected by us in NTD patients and controls, but no association between this variant and homocysteine concentrations was found.[112]

Transcobalamin

Since TC is the transporter of vitamin B_{12} to the cells and vitamin B_{12} is the cofactor for MS, variation in the TC gene could affect homocysteine concentrations. Several variants have been reported in the TC gene,[113–115] of which the 779C→G (P259R) polymorphism is the most studied. In 1998, Namour *et al.*[116] reported a lower vitamin B_{12} concentration as well as lower apo-TC (the proportion of TC that does not contain vitamin B_{12}) concentrations in subjects carrying the 779G allele. Several years later, they reported the same results with respect to apo-TC levels, but observed no differences in vitamin B_{12} concentrations between the 779C→G genotypes and concluded that the 779C allele might enhance intracellular vitamin B_{12} availability.[117] McCaddon *et al.*[118] studied this TC 779C→G variant in 73 healthy elderly volunteers and in 71 dementia patients and observed no association with either vitamin B_{12} levels or with homocysteine concentrations. Lower holo-TC (the proportion that contains vitamin B_{12}) concentrations for the 779G allele were reported by Afman *et al.*[119] in a population of 46 mothers with an NTD child and 73 controls, but no association between the 779C→G genotypes and homocysteine concentrations was observed. Later, in the same study population, the 779G allele was found to be associated with a lower total-TC, lower apo-TC and a lower holo-TC/total-TC ratio, suggesting that the TC 779C→G variant may result in a disturbance of vitamin B_{12} binding to TC.[120]

Our results[121] showed an association between 259PP individuals and decreased homocysteine concentrations only when their vitamin B_{12} status was greater than 299 pmol/L. These data indicate that 259PP individuals, in particular, benefit from high vitamin B_{12} concentrations with respect to lowering of total homocysteine, in contrast to their 259PR and 259RR peers, and suggest a gene–environment interaction between this TC variant and vitamin B_{12} concentrations. We therefore also investigated whether the P259R variant influenced the slope of the curve relating plasma total homocysteine concentration and we observed a clear negative correlation between vitamin B_{12} concentrations and plasma total homocysteine concentrations in 259PP individuals as well as in 259PR heterozygotes, whereas in subjects with the 259RR genotype no such association was found. Possibly, the 259P allele affects TC transcription or binding and transport of vitamin B_{12} by TC with positive repercussions on intracellular availability of vitamin B_{12}.[128]

Other variants in the TC gene are the M198T, I219L, S376L,[113] the Q234R,[114] the I23V[115] and the G94S, S348F and R399Q.[120] The latter four were also examined by us for their possible role in homocysteine metabolism,[121] but only the I23V polymorphism seemed to have an association with homocysteine concentrations, although this was only observed in the three homozygous 23VV individuals. These TC variants have also been studied, but none of them was found to be associated with homocysteine concentrations.[120]

Glutamate carboxypeptidase gene II

Folate plays an important role in homocysteine metabolism as the substrate for MTHFR. Dietary folates are a mixture of polyglutamylated folates and, since polyglutamates are poorly transported across membranes; they have to be hydrolysed in the digestive system to monoglutamylfolates by the action of folylpoly-γ-glutamate carboxypeptidase (FGCP). This enzyme is expressed by the glutamate carboxypeptidase gene II (*GCPII*). Variation in this gene could therefore affect the intestinal absorption of dietary folates, resulting in altered blood folate levels and, consequently, homocysteine concentrations. In 2000, Devlin *et al.*[122] reported a C to T substitution at bp 1561 (H475Y) and determined the functional significance of this variant

in vitro. Compared with FGCP activity in wild-type transfectants, the 1561C→T variant resulted in a significant reduction in FGCP activity by 53%. In addition, among 75 healthy subjects six subjects heterozygous for the 1561C→T variant had lower plasma folate levels and higher homocysteine concentrations than the 1561CC individuals. They postulated that the presence of the 1561C→T variant impaired the intestinal absorption of dietary folates, resulting in relatively low blood folate concentrations and hyperhomocysteinaemia. These results, however, were contradicted by our results.[123] We investigated the relationship between the 1561C→T variant in *GCPII* with fasting, post-methionine-load plasma homocysteine, folate and vitamin B_{12} concentrations and the risk of CVD in 190 patients with vascular disease and 601 apparently healthy controls. Although no clear association was seen between the variant and plasma homocysteine concentrations and vitamin B_{12} levels, the 1561C→T polymorphism significantly increased both red blood cell folate and plasma folate concentrations (ANOVA $P = 0{\cdot}013$, test for linear trend $P = 0{\cdot}03$, respectively).

Reduced-folate carrier

A polymorphism, 80G→A (R27H), in the reduced-folate carrier gene, the candidate gene for 5-methyl-THF internalization within cells, was reported by Chango *et al.*[124] However, this variant was not associated with a change in homocysteine, plasma and RBC folate concentrations.

Other

In NTD patients, a polymorphism in the folate receptor-α gene has been investigated, but its association with homocysteine concentrations was not investigated.[125] We also explored this folate receptor-α gene as well as the folate receptor-β gene for variants in spina bifida patients, mothers of a spina bifida child and controls, but found no polymorphisms.[126]

Gene–gene interactions

A combination of multiple variants was studied by Tsai *et al.*[96] They studied the combined effect of the CBS 844ins68 variant, the 677C→T MTHFR variant and the 2756A→G polymorphism in the MS gene, on either fasting or post-methionine-load homocysteine concentrations, and found that the effects of these three variants were additive and therefore demonstrated a polygenic regulation of plasma homocysteine concentrations.[96] Another genetic interaction analysis was carried out by Dekou *et al.*[127] to determine whether the effects of three variants, the MTHFR 677C→T, the MS 2756A→G and the CBS 844ins68, on homocysteine concentrations were independent and additive. They observed that the homocysteine-raising effect of the 677TT genotype was absent in 23 men who also carried the CBS 844ins68 insertion. In the group of men homozygous for the MS 2756A→G variant, those carrying the CBS 844ins68 allele had lower homocysteine concentrations than those lacking the insertion. A similar analysis for the effects of MTHFR and MS genotypes suggested that the homocysteine-increasing effect of both variants was additive. For all of these gene–gene effects, the possible involvement of nutritional status was explored but these effects were not substantially altered by these adjustments. Recently, Feix *et al.*[128] reported that the 2756A→G transition in combination with the MTHFR 677TT/1298AA and 677CT/1298AC is associated with high homocysteine concentrations. However, when they confined their analysis to only the MS variant, they did not observe any association with homocysteine concentrations whatsoever.[128]

Thus, although the majority of studies on genetic regulation of plasma homocysteine concentrations have been confined to a single polymorphism, the few studies on gene–gene interaction document a polygenic influence on plasma homocysteine concentrations. Therefore, studies that do not confine their analyses to a single polymorphism will be of great interest for studying the genetic basis of hyperhomocysteinaemia.

Gene–environment interactions

Many studies have demonstrated the importance of nutritional regulation of plasma homocysteine concentrations.[24] An interaction between micronutrient deficiencies and variants in genes regulating homocysteine metabolism has in particular been demonstrated for the 677C→T polymorphism in the MTHFR gene.[91] Since many nutritional factors such as folate or B vitamins play an important role in homocysteine metabolism as cofactors or substrate, further research on an interactive role between these factors and genetic variation is warranted.

In conclusion, many variants in the genes involved in homocysteine metabolism have now been studied. Besides the 677C→T polymorphism in the MTHFR gene, which has a pronounced effect on plasma homocysteine concentrations, several other variants have been reported to influence homocysteine metabolism by affecting enzyme activity (e.g. the MTHFR 1298A→C and CBS 31-bp VNTR), or by showing an association with plasma homocysteine concentrations (e.g. the MS 2756A→G, cSHMT 1420C→T, the MTRR 66A→G and the GCPII 1561C→T variants). Despite the large number of publications on the association between elevated plasma homocysteine concentrations and various polymorphisms, our understanding of the aetiology of moderately elevated plasma homocysteine

concentrations is still incomplete. Although there is a wide agreement that both genetic and nutritional factors are implicated, all the genetic variations described so far do not seem to explain the genetic component of hyperhomocysteinaemia in the general population. In a recent study, Kluijtmans *et al.*[130] found that these known polymorphisms explained only 9% of the overall variance in the plasma homocysteine concentrations, which was almost entirely explained by the MTHFR 677C→T polymorphism. It is possible that more genetic variation in genes involved in homocysteine metabolism is present. Genes that could be eligible as candidate genes are involved in transport or absorption of folate, such as the folate-binding protein, or cofactors such as vitamin B_{12} and B_6. Also (other) variants, in the genes mentioned here, can be further examined for their role and effect on homocysteine concentrations and/or CVD risk. In addition to the genes of the key enzymes in homocysteine metabolism, other genes coding for enzymes that might be relevant for homocysteine homeostasis could be important. Therefore, a genome-wide approach using linkage and association studies in large family cohorts is a powerful tool in the discovery of unknown polymorphisms that influence homocysteine concentrations in humans.

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