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Cystathionine β -synthase polymorphisms and hyperhomocysteinaemia: an association study

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Hyperhomocysteinaemia is generally accepted as an independent and graded risk factor for both arterial occlusive disease and venous thrombosis. The only way of homocysteine degradation is conversion to cysteine in the transsulfuration pathway in which the regulating step is catalysed by cystathionine β -synthase (CBS). Mild impairment of CBS function could therefore affect homocysteine concentration, in particular after methionine loading, and consequently cardiovascular disease (CVD) risk. We analysed two silent polymorphisms and one short tandem repeat in the CBS gene (ie 699C→T, 1080C→T and –5697 (GT) STR) as genetic markers potentially in linkage disequilibrium with a functional polymorphism. We assessed their association with fasting and post-methionine load homocysteine in 190 patients with arterial occlusive disease, and in 381 controls. No differences in CBS genotype frequencies between cases and controls were found, nor was a particular CBS genotype associated with an elevated risk of arterial occlusive disease. Although we did find a high rate of linkage disequilibrium between the two single nucleotide polymorphisms and the GT STR, none of the genotypes defined by the three CBS variants studied showed an association with elevated fasting, post-load or increase upon methionine loading homocysteine concentrations. In conclusion, we did not find any indication that genetic variation in the CBS gene is associated with increased homocysteine concentrations.

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Introduction

Established risk factors for cardiovascular disease (CVD) include elevated blood cholesterol, hypertension, diabetes mellitus and tobacco smoking. A modest elevation of plasma homocysteine concentration, commonly referred to as hyperhomocysteinemia, is generally,^{1,2} though not universally^{3,4} accepted as an independent and graded risk factor for both arterial occlusive diseases and venous thrombosis.^{5,6} Homocysteine, an intermediate sulfur amino acid in

methionine metabolism, may either be irreversibly degraded to cysteine via cystathionine in the transsulfuration pathway or remethylated to methionine. The remethylation by methionine synthase requires 5-methyltetrahydrofolate for methyl donation and vitamin B12 for methyl transport, while the remethylation by betaine-homocysteine-methyltransferase requires betaine as methyl donor. The regulating step in the transsulfuration pathway is catalysed by cystathionine β -synthase (CBS), a pyridoxal 5'-phosphate-dependent enzyme. The inherited metabolic disorder CBS deficiency is acknowledged to be the most frequent cause of homocystinuria in humans.⁷

In 1985, Boers *et al*⁸ postulated heterozygosity for CBS deficiency as a major cause of hyperhomocysteinaemia in

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CVD patients. This was based on the decreased CBS activities they found in extracts of cultured fibroblasts of hyperhomocysteinaemic vascular disease patients and their increased post-methionine loading homocysteine concentrations, both resembling those of obligate heterozygotes for CBS deficiency. Similar results were described by Clarke *et al.*⁹ Both studies however, do not reconcile with an earlier observation by Mudd *et al.*,¹⁰ who described a similar incidence of heart attacks or strokes in parents and grandparents of homocystinuric children compared with relatives of children with either new-mutation achondroplastic dwarfism or with impaired phenylalanine metabolism. Furthermore, the finding of decreased CBS activities in cultured fibroblasts from hyperhomocysteinaemic vascular disease patients in the range of obligate heterozygotes could not be reproduced by us,^{11,12} and still needs clarification. In addition to fasting homocysteine concentrations, also post-methionine load homocysteine concentrations were found to be correlated among family members of patients with hyperhomocysteinaemia and vascular disease.^{13–15} So, it can be concluded that hyperhomocysteinaemia is, at least partially, genetically based. Still, elevated post-methionine load homocysteine concentrations are associated with increased CVD risk,^{16–18} but its genetic determinants remain obscure.

Over 100 mutations have been described in the CBS gene in classical homocystinuria patients.¹⁹ A 68 bp insertion in exon 8, first reported as a causal mutation in an Italian patient, was found to be polymorphic and not to be associated with plasma homocysteine concentrations.^{20,21} No other potentially functional polymorphisms associated with elevated homocysteine levels were detected in the CBS gene in the analyses of the genetic basis of homocystinuria. However, most studies performed thus far only examined the coding region of the CBS gene, leaving the non-coding regions unexplored.

Very recently, we described an inverse correlation between the number of tandem repeats defined by a 31 bp VNTR (variable number of tandem repeats),²² and CBS enzyme activities in fibroblasts.²³ Furthermore, a positive association was observed between the number of repeat units and post-methionine load plasma homocysteine concentrations.²³

Genetic association studies are widely used for the identification of candidate genes of complex, ie non-mendelian traits.²⁴ In such studies, the association is investigated between a phenotype and genomic markers close to or, preferably, within the gene of interest. Such a marker in itself is not necessarily functionally related to the phenotype but may be in linkage disequilibrium with a functional variant.

In the present study, we investigated the potential involvement of genetic variants in non-coding regions or of silent mutations in the CBS gene in hyperhomocysteinaemia and vascular disease, as possible markers which may

be in linkage disequilibrium with a functional polymorphism. We analysed two silent single nucleotide polymorphisms (699C→T, 1080C→T) and a short tandem repeat (STR) –5697(GT),²² in the CBS gene in 190 vascular disease patients and in 381 population-based controls. We report the allele frequencies and genotype distributions of these polymorphic variants in both study groups, and describe their association with fasting, post-methionine load and delta (ie increase upon methionine loading) plasma homocysteine concentrations. Also, the extent of linkage disequilibrium between the several CBS variants are calculated.

Materials and methods

Study population

We studied 190 patients with coronary, peripheral or cerebral vascular disease; a total of 130 cases were recruited from a cohort of patients who underwent coronary angiography in the Zuiderziekenhuis Hospital in Rotterdam, The Netherlands.¹⁸ Reasons for angiography were myocardial infarction or angina pectoris and cases were defined as those having $\geq 90\%$ occlusion in one and $\geq 40\%$ occlusion in one additional coronary artery. The other 60 patients had documented premature cardiovascular disease of which 10 patients had suffered from myocardial infarction, 32 from cerebral arterial occlusive disease and 18 from peripheral arterial occlusive disease.¹²

The control group consisted of 381 controls, of whom 101 were included from the general population in Rotterdam and comprised subjects with no history of cardiovascular disease¹⁸ and 280 were recruited from a general practice in The Hague, The Netherlands to take part in a health survey.²⁵

Biochemical parameters

After an overnight fast, blood was collected in EDTA tubes for measurement of fasting total homocysteine. The EDTA-blood samples were placed on ice immediately, and centrifuged for 10 min at 3000 g with minimal delay. All individuals were subjected to a standardised methionine loading test.²⁶ Briefly, L-methionine (0.1 g/kg body weight) was dissolved in 200 mL orange juice and administered orally. All study subjects received a standardised low protein breakfast and luncheon, and were asked not to consume any milk-containing beverages. After 6 h, another blood sample was drawn for assessment of the post-methionine load homocysteine concentration. Total homocysteine was measured in plasma using a high-performance liquid chromatography (HPLC) procedure, with reverse phase separation and fluorescence detection, as described by Te Poele-Pothoff *et al.*²⁷ All homocysteine measurements were conducted at the University Medical Center Nijmegen, The Netherlands, and fasting and post-load plasma homocysteine levels were obtained from 555 and 525 individuals, respectively.

Detection of CBS polymorphisms

Genomic DNA was isolated from buffy coats of EDTA blood by a standard method,²⁸ and stored at 4°C until analysis. Genomic DNA was available from 561 individuals.

699C→T transition

This polymorphism does not create a restriction enzyme recognition site, and was therefore analysed by Primer Introduced Restriction Analysis. A mutagenic sense oligonucleotide (5'-CAGCAACCCCCTGGCTCAGT-3') introduces a *RsaI* site in the 699C allele and together with an antisense oligonucleotide (5'-TTATCGTTTGTGCCCGTACCG-3') a genomic DNA fragment of 287 bp was amplified using approximately 100 ng genomic DNA and 100 ng of both oligonucleotides in a standard PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl) containing 2 mM MgCl₂ and 200 μM dNTPs. PCR parameters were as follows: 3 min initial denaturation at 92°C, followed by 35 cycles of 92°C/60 s, 64°C/60 s, 72°C/30 s, and a final extension of 7 min at 72°C. After digestion with *RsaI* and resolution of the fragments on a 20% polyacrylamide gel, the 699 CC genotype results in fragments of 171, 92, 20 and 4 bp, whereas the 699 TT genotype shows fragments of 171, 112 and 4 bp.

1080C→T transition

A genomic DNA fragment of 88 bp was amplified by PCR using both 100 ng forward (5'-CTGGCAGCACGGTGGC-GG-3') and 100 ng reverse oligonucleotides (5'-CGCACTG-AGTCGGGCAGAATG-3'), and approximately 100 ng genomic DNA in a standard PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl) containing 2 mM MgCl₂ and 200 μM dNTPs. All samples were cycled 35 times: 1 min /92°C denaturation, 1 min/55°C annealing, 30 s/72°C extension, preceded by an initial denaturation of 3 min/92°C and followed by a final extension of 7 min/72°C. The PCR fragment was analysed by *Bst*UI restriction enzyme analysis followed by separation on a 2% agarose gel. The 1080 TT genotype results in an uncut fragment of 88 bp, the 1080 CC genotype in two fragments of 55 and 33 bp, whereas the heterozygous CT genotype is displayed by three fragments (88, 55, and 33 bp).

–5697 (GT) STR

A PCR was performed with a 5'FAM labelled forward primer (5'-GAGCTGAAATCACACCACT-3') and a reverse primer (5'-GTTTTACTACATTTGCTCCC-3') in a standard PCR buffer containing 2 mM MgCl₂ and using an annealing temperature of 58°C. The 193–211 bp fluorescently labelled PCR products were separated on a 4% denaturing polyacrylamide gel using the ABI Prism 377, according to the manufacturer's recommendations. Data-analysis was performed using the Genescan and Genotyper Software (Perkin Elmer).

Statistical analysis

Plasma homocysteine concentrations showed positive skewness, therefore log-transformed homocysteine levels were

used in all analyses. Differences in genotype distributions, allele frequencies and gender were assessed by Pearson's χ^2 analysis. Differences in continuous variables between cases and controls were assessed by Student's *t*-test, adjusted for age and gender by means of linear regression analysis. One-way analysis of variance (ANOVA) was used to assess the differences of continuous variables between different genotypes, followed by Bonferroni-corrected *t*-tests. Odds ratios (OR) and 95% confidence intervals (95% CI) as estimates of the relative risk for arterial occlusive disease for the different CBS genotypes, were calculated with logistic regression analysis. We also investigated the association between these three genetic variants and the 31 bp VNTR, of which alleles 19 and 21 were shown to be associated with increased post-methionine load homocysteine concentrations.²³ The haplotype frequencies and linkage disequilibria were estimated by the EH program.²⁹ *D'* reflects the extent of the linkage disequilibrium, which is reported as the ratio between the actual value of *D* (ie the departure from linkage disequilibrium) and the maximum value it can have for the given allele frequencies. The sign in front of the coefficients indicates whether the rare alleles are associated (+) or the rare allele is associated with the frequent allele (–). All *P*-values reported are two-tailed, and statistical significance was accepted at *P* < 0.05.

Results

Table 1 depicts age, gender, and total plasma homocysteine concentrations (ie fasting, post methionine load, and net increase upon methionine loading) of cases and controls. Age, expressed as the geometric mean, and the percentage males did not differ between the two groups. A higher fasting homocysteine (+7.5%; *P*=0.011) and post-methionine loading homocysteine concentration (+5.6%; *P*=0.045) was seen in cases compared with controls. Cases and controls showed a comparable net increase upon methionine loading (ie post-load minus fasting homocysteine concentration).

Both 699C→T, 1080C→T as well as the GT STR genotype distributions were in Hardy-Weinberg equilibrium (data not shown). Genotype distributions for each of the three CBS polymorphisms were similar in cases and controls (Table 2, left side). The allele frequency of the 699T allele was 0.37 and 0.35 in patients and controls, respectively ($\chi^2=0.30$, *P*=0.58); the 1080T allele had an allele frequency of 0.32 in patients and of 0.37 in controls ($\chi^2=2.89$, *P*=0.09). In our study population, we identified 10 different alleles defined by the highly polymorphic GT STR (which were named after the number of repeat units) that led to the observation of 22 different genotype combinations (Table 2).

CBS genotypes were investigated as risk factors for arterial occlusive disease. The differences in CBS genotype frequencies between the case and control group were not statistically significant (Table 2). Odds ratios for the 699TT

Table 1 Characteristics and plasma homocysteine concentration (tHcy) in cases and controls

	Cases (n=190)	Controls (n=381)	<i>P</i> ^a
Age (years)	49.0 ± 10.2	50.8 ± 11.9	0.11
Gender (% male)	75.3	74.3	0.80 ^b
Fasting tHcy (μmol/L)	14.4 (13.7–15.1)	13.4 (13.0–13.9)	0.011 ^c
Postload tHcy (μmol/L)	41.3 (39.4–43.3)	39.1 (38.0–40.3)	0.045 ^c
Postload increase ^d (μmol/L)	26.1 (24.6–27.7)	24.7 (23.6–25.9)	0.13 ^c

Age is expressed as mean ± SD and tHcy as geometric means (95% CI). ^aStudent's *t*-test. ^bPearson χ^2 test. ^cAdjusted for age and gender. ^dIncrease upon methionine loading (ie postload minus fasting tHcy).

Table 2 CBS genotype distribution in cases and controls and the relationship between plasma homocysteine concentration (tHcy) and CBS genotypes (patients and controls combined)

CBS genotype	Patients N (%)	Number of Controls N (%)	Fasting tHcy μmol/L	Postload tHcy μmol/L	Δ tHcy ^a
699CC	76 (40.0)	156 (42.9)	13.9	40.0	25.1
699CT	87 (45.8)	158 (43.4)	13.5	39.6	25.5
699TT	27 (14.2)	50 (13.7)	14.2	40.0	25.1
	$\chi^2=0.41$; <i>P</i> =0.81		<i>P</i> =0.60 ^b	<i>P</i> =0.92 ^b	<i>P</i> =0.91 ^b
1080CC	93 (49.2)	150 (40.4)	14.0	40.6	25.9
1080CT	70 (37.0)	164 (44.2)	13.6	39.1	24.9
1080TT	26 (13.8)	57 (15.4)	13.5	39.7	24.4
	$\chi^2=3.98$; <i>P</i> =0.14		<i>P</i> =0.58 ^b	<i>P</i> =0.38 ^b	<i>P</i> =0.44 ^b
GT STR					
14-14	1 (0.5)	1 (0.3)	20.1	44.3	24.2
11-16	0	1 (0.3)	11.4	31.8	20.5
12-16	1 (0.5)	0	11.6	-	-
13-16	0	1 (0.3)	9.6	24.3	14.8
14-16	9 (4.8)	33 (9.6)	14.3	40.6	26.1
15-16	10 (5.3)	11 (3.2)	15.0	41.7	25.9
16-16	75 (40.1)	146 (42.7)	13.5	39.4	25.2
14-17	2 (1.1)	3 (0.9)	15.4	41.6	25.8
16-17	47 (25.1)	79 (23.1)	13.6	40.3	26.1
17-17	3 (1.6)	4 (1.2)	18.3	46.7	28.1
14-18	1 (0.5)	4 (1.2)	13.1	37.5	23.6
15-18	1 (0.5)	1 (0.3)	13.4	47.6	33.5
16-18	14 (7.5)	25 (7.3)	13.7	38.7	24.7
17-18	2 (1.1)	8 (2.3)	15.3	38.1	22.5
18-18	2 (1.1)	1 (0.3)	11.6	34.3	22.6
14-19	1 (0.5)	2 (0.6)	13.6	45.3	30.5
16-19	11 (5.9)	16 (4.7)	15.1	39.4	20.6
17-19	5 (2.7)	3 (0.9)	13.2	44.8	30.5
18-19	0	1 (0.3)	12.8	38.5	25.8
19-19	0	1 (0.3)	7.8	20.7	12.8
16-20	1 (0.5)	0	18.9	46.1	27.0
17-20	1 (0.5)	1 (0.3)	13.0	38.2	25.0
	$\chi^2=17.74$; <i>P</i> =0.67		<i>P</i> =0.42 ^b	<i>P</i> =0.78 ^b	<i>P</i> =0.73 ^b

tHcy expressed as geometric means (95% CI). ^aNet increase upon methionine loading (ie postload minus fasting tHcy). ^bANOVA on log transformed data.

and 699CT genotype, with the 699CC genotype as reference category, were 1.07 (95% CI: 0.81–1.40) and 1.12 (95% CI: 0.77–1.64) respectively. Odds ratios for the 1080TT and 1080CT genotype, with 1080CC as reference category, were 1.07 (95% CI: 0.89–1.52) and 0.94 (95% CI: 0.54–1.62) respectively.

In addition, the association between CBS genotypes and fasting and post-load plasma homocysteine concentrations

was studied (Table 2, right side). We combined cases and controls to increase statistical power, since associations between CBS genotypes and plasma homocysteine did not differ between cases and controls (all *P*-values >0.14). For the 699C→T polymorphism no differences in fasting, post-load or delta homocysteine concentrations were observed between the three genotypes (Table 2). Furthermore, none of the genotypes defined by the 1080C→T

polymorphism showed an association with elevated fasting, post-load or delta homocysteine concentration (Table 2). The different GT STR genotypes were also not associated with a change in plasma homocysteine concentrations (Table 2). We also investigated the association between these three genetic variants and the 31 bp VNTR, of which alleles 19 and 21 were shown to be associated with increased post-methionine load homocysteine concentrations.²³ The positive association between alleles 19 and 21 and post-load homocysteine concentrations was however not reflected in one of the genotypes defined by the three variants studied here. We examined whether alleles 19 and 21 were located on particular haplotypes, but found out that this was not the case; six different haplotypes were observed containing allele 19, and three containing allele 21 (Table 3).

Linkage disequilibria were calculated between the 699C→T, 1080C→T and the GT STR and all were found to be highly significant ($P < 0.001$; data not shown). All pairwise linkage disequilibria between the different CBS alleles are depicted in Table 4.

Discussion

In the present study, we investigated the potential involvement of variants in non-coding regions or silent mutations

in the CBS gene by analysing the 699C→T and 1080C→T variants and a GT STR, which is located in the promotor region, as markers of possible genetic CBS defects contributing to hyperhomocysteinaemia and to the risk of arterial occlusive disease. No differences in genotype distributions or allele frequencies were found between cases and controls, indicating that no particular CBS genotype is over-represented in arterial occlusive disease patients compared with controls. We investigated the associations between CBS genotypes and plasma homocysteine concentrations, especially after methionine loading. The two silent mutations as well as the GT STR did not show an association with homocysteine concentrations, neither fasting, nor post-load, nor on the increase upon methionine loading.

Besides fasting plasma homocysteine concentrations,^{1,18} also post-methionine load homocysteine concentrations have been associated with an increased CVD risk.^{16,18,30} Verhoef *et al*¹⁸ calculated that the risk of coronary artery disease increased with 20% for each 12 $\mu\text{mol/L}$ increase in post-load homocysteine levels. Graham *et al*³¹ reported a relative risk for vascular disease of 1.5 for a subject with a post-load homocysteine level above the 80th percentile of controls. Since methionine loading particularly promotes the transsulfuration of homocysteine into cysteine, in which CBS is the regulating enzyme, it has been postulated that hyperhomocysteinaemia after methionine loading reflects the involvement of suboptimal CBS function.³² Several studies excluded the involvement of heterozygosity for CBS deficiency in hyperhomocysteinaemia and premature vascular disease,^{12,33,34} but the results of these studies do not exclude an involvement of a mildly impaired CBS function. No potentially functional polymorphisms associated with hyperhomocysteinaemia after methionine loading were detected in the CBS gene in the analyses of the genetic basis of homocystinuria. However, most studies performed thus far only examined the coding region of the CBS gene, leaving the non-coding regions unexplored. The effect of a potential functional polymorphism may be reflected in other, non-functional, polymorphisms, in case they are in linkage disequilibrium.

Recently, Kruger *et al*³⁵ calculated odds ratios of the 699TT and 1080CC genotypes versus the other two genotypes defined by each variant on the risk of coronary artery disease and found decreased risks, ie protective effects. However, individuals with the 699TT or 1080CC genotype had similar fasting homocysteine concentrations as the other genotypes and, unfortunately, no post-load homocysteine concentrations were available. They postulated that the 699T and 1080T alleles, may be linked to other yet unidentified functional polymorphisms in the CBS gene. These results were similar to those of De Stefano *et al*³⁶ who also did not find an effect of both CBS 699C→T and the 1080C→T CBS variants on fasting homocysteine concentrations. They did not examine the association between the CBS variants and post-methionine load homo-

Table 3 Haplotypes of the CBS 31 bp VNTR alleles 19 and 21 and their relative frequencies

Haplotype	31 bp VNTR	Polymorphisms			Relative frequency
		GT STR	699C→T	1080C→T	
A	19	15	T	C	0.036
B	19	16	C	C	0.074
C	19	16	C	T	0.036
D	19	16	T	C	0.448
E	19	17	C	T	0.036
F	19	17	T	C	0.085
G	21	16	C	C	0.190
H	21	16	T	C	0.038
I	21	17	C	C	0.058

Table 4 Linkage disequilibrium coefficients (D') between different alleles of CBS polymorphisms. The sign in front of the coefficients indicates whether the rare alleles are associated (+) or whether the rare allele is associated with the frequent allele (-)

Polymorphism	Allele	699C→T	1080C→T
1080C→T		-0.75	-
-5697 (GT) STR	14	0.71	-0.80
	15	0.25	-0.35
	16	0.25	-0.08
	17	-0.35	0.52
	18	-0.47	0.67
	19	-0.32	-0.36

cysteine concentrations either. Aras *et al*³⁷ reported decreased post-methionine load homocysteine concentrations in individuals who were heterozygous or homozygous for the 699T allele compared with the 699CC subjects. This association became more significant when individuals carrying the 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 levels only when individuals carrying the 844ins68 and the 699T allele were excluded from the analysis. They also speculated that the 699C→T and 1080C→T CBS variants may be in linkage disequilibrium with regulatory elements that upregulate CBS gene transcription.³⁷ In the current study however, the two silent polymorphisms as well as the GT STR did not show any association with homocysteine concentrations, neither fasting, nor post-load, nor on the increase upon methionine loading. Both etiologic and genetic heterogeneity may play an important role in genetic association studies. Since the populations used in the different studies may have a different background, it is possible that the actual range of etiologies vary across samples. Allele frequencies of the 699C→T and 1080C→T polymorphisms were comparable between the three studies published earlier^{35–37} and the present one, ranging from 31.8 to 39.4% for the 699T allele and from 32.4 to 35.5% for the 1080T allele.

We also investigated the association between these three genetic variants and the 31 bp VNTR, of which the alleles containing 19 and 21 repeat units were shown to be associated with increased post-methionine load homocysteine concentrations.²³ However, this positive association between alleles 19 and 21 and post-load homocysteine concentrations was not reflected in one of the genotypes defined by the three variants studied here. One explanation is that alleles 19 and 21 are rare (allele frequencies of 10.5 and 2.7%, respectively) and are therefore not picked up by a genetic association analysis described here. Furthermore, we observed that alleles 19 and 21 were both not located on one particular haplotype, but that different haplotypes were observed containing allele 19, which was also the case for allele 21 (Table 3).

In the present study, we detected strong linkage disequilibrium between the CBS variants studied. For each pair of alleles, defined by the three CBS variants, linkage disequilibrium coefficients (D') were calculated (Table 4). D' between the 699C→T and the 1080C→T polymorphisms of -0.75 in our study is similar to that calculated by De Stephano *et al*³⁶ of -0.83 in almost 800 subjects recruited from 11 European countries.

In conclusion, we did not find any genotype, defined by the three CBS variants studied here, associated with elevated homocysteine concentrations. Although we observed strong linkage between these three polymorphisms, no evidence for a common CBS polymorphism

leading to elevated homocysteine concentrations and increased CVD risk was found.

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