

Betaine-Homocysteine Methyltransferase (BHMT): Genomic Sequencing and Relevance to Hyperhomocysteinemia and Vascular Disease in Humans

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Received July 7, 2000, and in revised form August 7, 2000

Elevated homocysteine levels have been associated with arteriosclerosis and thrombosis. Hyperhomocysteinemia is caused by altered functioning of enzymes of its metabolism due to either inherited or acquired factors. Betaine-homocysteine methyltransferase (BHMT) serves, next to methionine synthase, as a facilitator of methyl group donation for remethylation of homocysteine into methionine, and reduced functioning of BHMT could theoretically result in elevated homocysteine levels. Recently, the genomic sequence of the BHMT gene was published. Mutation analysis may reveal mutations of the BHMT gene that could lead to hyperhomocysteinemia. In the present study we performed genomic sequencing of the BHMT gene of 16 vascular patients with hyperhomocysteinemia and detected three mutations in the coding region of this gene. The first was an amino acid substitution of glycine to serine (G199S), which was found only in the heterozygous state. The second mutation was a substitution of glutamine to arginine (Q239R), and the last mutation was an amino acid substitution of glutamine to histidine (Q406H). The latter was also found only in the heterozygous state. The relevance of these mutations was tested in a study group, which consists of 190 cases with vascular disease and 601 controls. The influence of these three mu-

tations on homocysteine levels was investigated. None of the three mutations led to significantly changed homocysteine levels. In addition, no differences in genotype distribution between cases and controls were found. So far, our results provide no evidence for a role of defective BHMT functioning in hyperhomocysteinemia or subsequently in vascular disease. © 2000 Academic Press

Key Words: betaine; betaine-homocysteine methyltransferase (BHMT); choline; homocysteine; hyperhomocysteinemia; mutation analysis; vascular disease.

Hyperhomocysteinemia (HHcy) is caused by a combination of genetic and nutritional disturbances. Homocysteine (Hcy) may accumulate due to altered functioning of the remethylation pathways, but also from a defect in the transsulfuration pathway due to defective functioning of cystathionine β -synthase (CBS) (Fig. 1). HHcy can be classified into two forms, severe and mild HHcy. Patients with severe HHcy (Hcy > 50 μ mol/L) due to deficiency of methylenetetrahydrofolate reductase (MTHFR), methionine synthase (MS), or CBS (Fig. 1) often suffer from vascular disease. Mild HHcy has also been associated with vascular disease (1,2). Recently, a common mutation in the MTHFR gene (677 C > T) has been found, leading to mildly elevated Hcy levels. This mutation, however, can only explain the observed increased Hcy levels in some cases (3,4). Therefore, deficiencies of other enzymes of methionine/homocysteine

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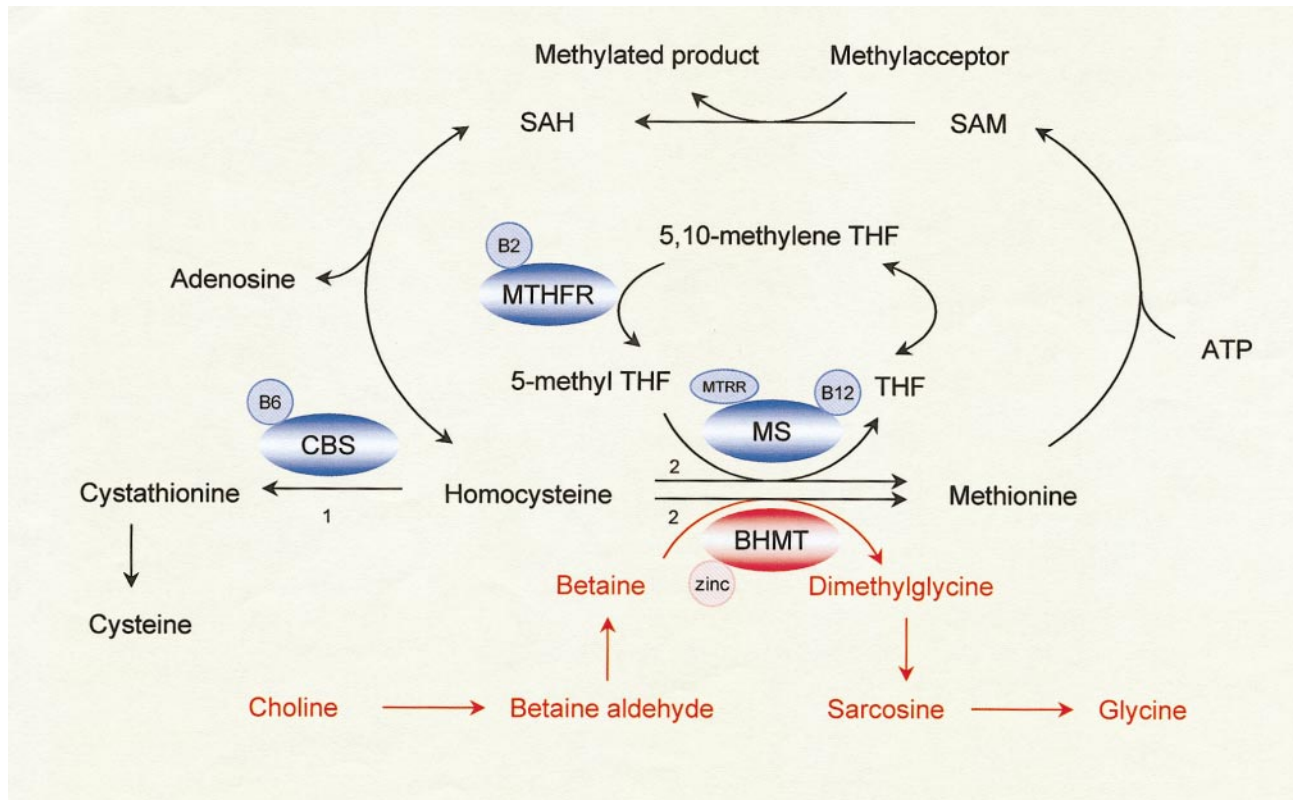


FIG. 1. Simplified scheme of methionine/homocysteine metabolism. Main regulating enzymes are depicted and cofactors are encircled; BHMT, betaine-homocysteine methyltransferase; CBS, cystathionine β -synthase; MTHFR, methylenetetrahydrofolate reductase; MS, methionine synthase; MTRR, methionine synthase reductase; SAM, *S*-adenosylmethionine and SAH, *S*-adenosylhomocysteine; 1, trans-sulfuration; 2, remethylation.

metabolism are expected to contribute to HHcy. Genes coding for CBS and MS have been investigated, but whether mutations in these genes lead to mild HHcy remains questionable (5–8).

Betaine-homocysteine methyltransferase (BHMT; EC 2.1.1.5) is the enzyme, along with methionine synthase, which remethylates Hcy to methionine. Altered functioning of BHMT may result in elevated Hcy levels and could therefore contribute to the risk for vascular disease. BHMT is also involved in choline metabolism, where it converts betaine into dimethylglycine, which is then transferred to sarcosine to form glycine (Fig. 1) (9). BHMT is primarily expressed in liver and kidney. In liver, BHMT is responsible for 50% of Hcy remethylation. Oral betaine supplementation lowers Hcy concentrations in severe hyperhomocysteinemic patients, but this effect is poorly studied in other forms of HHcy (10). This Hcy lowering effect of betaine is accomplished via two pathways (11). First, via donation of methyl groups for direct remethylation of Hcy to methionine

by BHMT. Secondly, the oxidation of dimethylglycine to glycine introduces one-carbon units into the folate pool that will stimulate the folate-dependent Hcy remethylation, resulting in decreased Hcy levels.

Recently, it was indicated that BHMT is a zinc metalloenzyme (12,13). Cysteine residues of BHMT protein function as zinc-binding domains, which in turn are required for the binding of Hcy to BHMT. Replacement of three highly conserved cysteine residues with either alanine or serine resulted in complete loss of activity (12). These results suggest that mutated BHMT may lead to decreased binding of Hcy to BHMT, resulting in accumulated Hcy levels.

Enzymatic measurement of BHMT activity cannot be performed in fibroblasts or lymphocytes, which explains in part why defects of BHMT have not been observed previously. Molecular analysis enables screening for BHMT defects and could answer the question: is mutated BHMT involved in HHcy and subsequently in vascular disease? Recently, the

TABLE 1
Characteristics of Study Group

	Cases (<i>n</i> = 190) mean (95% CI)	Controls (<i>n</i> = 601) mean (95% CI)	<i>P</i> value
Age (years)	49.0 (47.5–50.4)	50.6 (49.5–51.6)	0.08
Gender (% male)	75.3	47.1	<0.001
Fasting homocysteine ($\mu\text{mol/L}$) ^a	14.3 (13.7–15.0)	13.1 (12.7–13.4)	0.005 ^b
Postload homocysteine ($\mu\text{mol/L}$) ^a	41.3 (39.1–43.2)	40.0 (39.1–41.1)	0.10 ^b

^a Geometric mean.

^b Adjusted for age and gender differences.

gene coding for betaine-homocysteine methyltransferase has been cloned (11,14,15). The BHMT gene has been localized to chromosome 5q13.1–q15 and consists of 8 exons and 7 introns. In the present study, we report the molecular genetic analysis of the BHMT gene and investigate the influence of the three mutations discovered in this gene on Hcy metabolism in a study group of 190 vascular cases and 601 controls.

MATERIALS AND METHODS

Patient Material

A patient group, with diagnosed vascular disease and mildly elevated Hcy levels, was preselected according to availability of fibroblasts. This enabled the use of this patient group for different strategies of mutation analysis. In total 16 patients were selected and their material was used for genomic sequencing. Fibroblasts were cultured according to standard procedures and DNA was isolated with the Puregene DNA extraction kit (Biozym, The Netherlands), essentially as indicated by the manufacturer. To investigate whether mutations of the BHMT gene lead to elevated Hcy levels and may therefore contribute to the risk for vascular disease, we used a second group of cases and a large group of controls, which we will call the study group (Table 1). One part consists of 130 cases with severe coronary occlusions and 101 population-based controls as described by Verhoef *et al.* (16). Another group of 60 cases with documented premature cardiovascular disease as described by Kluijtmans *et al.* was also used (17). Furthermore, we used 500 additional controls, which were recruited from a general practice in The Hague, The Netherlands (18). Finally, 13 patients with severe HHcy (Hcy > 100 $\mu\text{mol/L}$) were also screened for the three mutations.

Homocysteine Measurements

Hcy concentrations were measured in EDTA plasma after an overnight fast and 6 h after a methionine load (19). The Hcy values of cases and controls described by Verhoef *et al.* (16) were remeasured at our laboratory in order to make a proper comparison with other Hcy values of the study group.

Molecular Analysis

Molecular analysis was performed by genomic sequencing of the different exons. Intronic primers were developed based on the published genomic sequence (GenBank Accession Number AF118371, Table 2 and Fig. 2).

PCR of the different exons was carried out in a total volume of 50 μl on a Perkin-Elmer 9600 thermocycler (PE Biosystems, The Netherlands), containing 50 ng of the forward and reverse primers (Table 2), 200 μM dNTPs, 10 mM Tris-HCl buffer (pH 8.3), 1.0 mM MgCl_2 , and 0.5 unit *Taq* polymerase (all from Life Technologies, The Netherlands). PCR parameters were as follows: 92°C/120 s (initial denaturation) followed by 35 cycles of 92°C/30 s (denaturation), 47–58°C/30 s (annealing), 72°C/30 s (elongation) followed by a final elongation step of 7 min at 72°C. The obtained PCR products were separated on a 2% agarose gel and subsequently sequenced on the ABI Prism 377 automated sequencer using the ABI Prism BigDye Terminator cycle sequencing kit according to the instructions of the manufacturer (PE Biosystems, The Netherlands).

To determine whether mutations of the BHMT gene lead to elevated Hcy levels and therefore are possible risk factors for vascular disease, a detection method was developed to screen for these mutations in the total study group. The first mutation in the BHMT gene, the 595 G > A mutation was screened using restriction enzyme analysis. This mutation,

TABLE 2
Primer Sequences Used for Molecular Analysis of the BHMT Gene

Primer	Sequence (5' → 3') ^a	T _m (°C)	Product size (bp)
1 forward	gggctcgctagtcggtc	58	188
1 reverse	cggcagggtcgagggc		
2 forward	cctccctcatctgtaatttag	51	226
2 reverse	gctgggattacagcatgag		
3 forward	tctgctgtttatctgagagcc	58	304
3 reverse	caaactctgaagaggttgaacc		
4 forward	tagatgtgaatggttgataataat	55	300
4 reverse	tttatcttcaccacatcttc		
5 forward	agtactctaaccttaactgattccag	47	235
5 reverse	ggcactagtggttctttaag		
6 forward	tgccctgctggttctgg	50	313
6 reverse	ggtatagatttacttgagtttatag		
7 forward	ctacttgtagtacttatcttg	50	347
7 reverse	ctaatacataaattagtttggg		
8 forward	gagtcgtgtgctggtgcatg	47	270
8 reverse	gtgacccaacacctagaattc		
MB-PCR ¹ forward	CTTGACCCACCATTAG	59	110
MB-PCR ¹ reverse	CAGGAGTGTGGTAAGCC		
MB-WT ²	GCGACGGCTGCCGACTGAGTCGC	59	
MB-MUT ²	GCGACAGGCTGCCAACTGAGTCGC		
8 reverse ³ PIRA	CATCAAAAATAGCTTCTATCGAGGGT	58	248

^a Exonic sequences are indicated by uppercase and intronic sequences are indicated by lowercase.

¹ Used for PCR in molecular beacon analysis to screen for the 716 G > A polymorphism.

² Molecular beacons, which hybridize to wild-type (WT) or mutant (MUT) alleles, 716 G or A are indicated in bold.

³ Used for screening of the 1218 G > T mutation with PCR-PIRA, altered nucleotide is depicted in bold.

which is present in exon 5 of the BHMT gene, resulted in an abolishment of a *NciI* restriction site. A 235-bp region containing exon 5 was amplified using

PCR with exon 5 forward and reverse primers (Table 2). *NciI* digestion of wild-type sequences (595 AA) then leads to two fragments of 143 and 92 bp, re-

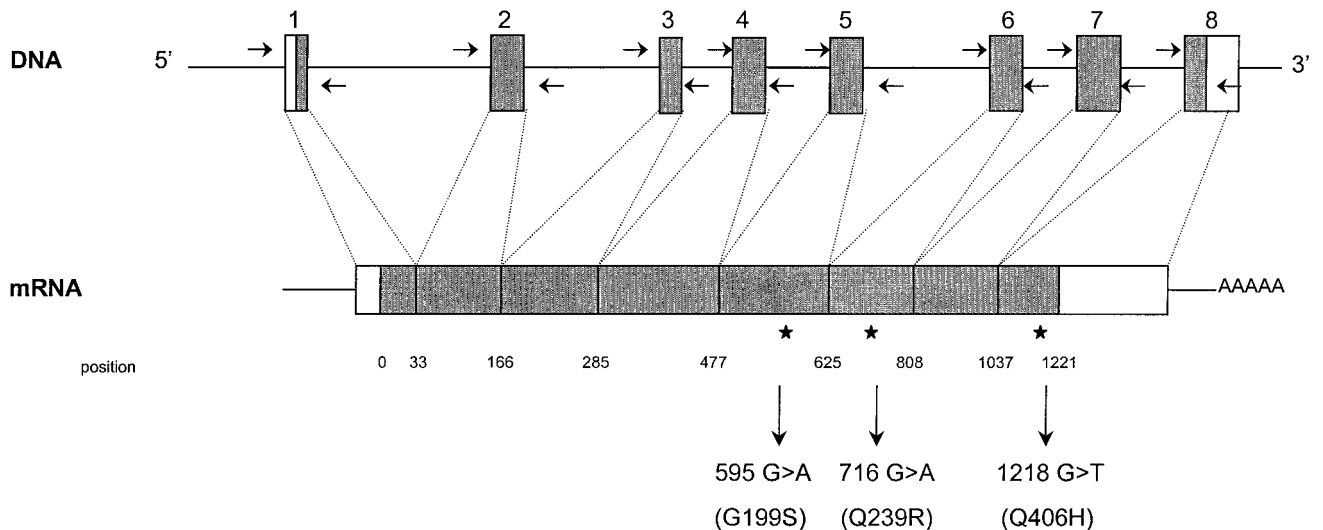


FIG. 2. Location of human BHMT mutations. Gene structure of BHMT gene shows schematically the different exons and corresponding intronic primers used for sequencing analysis. Exons are indicated by shaded areas. The mutations are depicted (*) with their position on mRNA level and corresponding amino acid substitution.

spectively. The products were analyzed on a 2% agarose gel. The second mutation, which was found in exon 6 of the BHMT gene, did not lead to a formation or abolishment of a restriction site. To screen for this mutation we first tried primer-introduced restriction analysis (PCR-PIRA) but the results were not satisfactory. Subsequently, we decided to use molecular beacons for the detection of this mutation (20,21). Two new primers were developed, which created 110-bp PCR products (Table 2). Two molecular beacons were designed, one to detect the G allele (MB-WT) and one that detects the A allele (MB-MUT). PCR was carried out in a total volume of 50 μ l containing 50 ng of the forward and reverse primers, 150 ng of the wild-type TET beacon, and 250 ng of the mutant HEX beacon, 200 μ M dNTPs, 10 mM *Taq* gold amplification buffer, 4 mM MgCl₂ and 1.5 unit of AmpliTaq Gold (PE Biosystems, The Netherlands). PCR conditions were as follows: cycling was preceded by 10 min 95°C (activation AmpliTaq Gold) followed by 40 cycles of 95°C/30 s (denaturation), 59°C/30 s (annealing), and 72°C/30 s (elongation). PCR was performed on the ABI-Prism 7700 (PE Biosystems, The Netherlands) and fluorescence was measured during the second step of stage 2 (annealing). Results were analyzed by Sequence Detection System software (PE Biosystems, The Netherlands). The third mutation found in exon 8 of the BHMT gene, namely the 1218 G > T substitution, does not result into an abolishment or formation of a restriction site. Therefore, PCR-PIRA was used to induce an alternative restriction site. For the 1218 G > T substitution, guanine at position 1221 was substituted with cytosine by the reverse PIRA primer, thereby creating a restriction site for *Rsa*I (Table 2). This allows *Rsa*I to cut the PCR-PIRA fragment of 248 bp into two fragments of 222 and 26 bp in the case of the wild-type sequence (1218GG). These fragments were analyzed on a 3% agarose gel.

Statistics

Differences between cases and controls were calculated using Student's *t* test for continuous variables and Pearson χ^2 for frequencies. *P* values were age- and sex adjusted by linear regression analysis. To determine if mutated BHMT contributes to elevated Hcy levels, means, confidence intervals, and *P* values were calculated. Homocysteine showed positive skewness, and therefore in all cases geometric means were calculated instead of means. Statistical significance between geometric means of different

genotypes was estimated by one-way analysis of variance (ANOVA) and Student's *t* test. *P* values were age- and sex-adjusted by linear regression analysis. In addition, *P* values were adjusted for MTHFR 677 C > T mutation, which is evidently associated with increased Hcy levels (3,4,16,17). *P* < 0.05 was considered statistically different; all *P* values were two tailed. To estimate the possible risk of different genotypes on vascular disease, odds ratios (OR) and 95% confidence intervals (95% CI) were calculated.

RESULTS

The relevance of BHMT to HHcy and vascular disease was tested by genomic sequencing of the BHMT gene. Sixteen preselected patients with mildly elevated Hcy levels were subjected to mutation analysis. Genomic sequencing of all 8 exons of the BHMT gene revealed three mutations, which were located in different exons (Fig. 2). The first mutation detected, was the 595 G > A transition in exon 5 of the BHMT gene, which leads to a substitution of glycine to serine at amino acid position 199 (G199S). This mutation was found in one patient in a heterozygous state. A second mutation was found in exon 6 of the BHMT gene at nucleotide position 716, which was already reported by Park and Garrow (14). This 716 G > A mutation leads to a substitution of glutamine to arginine at amino acid position 239 (Q239R). Two of the 16 patients were homozygous mutant (716 AA) and 4 patients were heterozygous (716 GA) for this mutation. A third mutation was found in exon 8 of the BHMT gene at nucleotide position 1218 (1218 G > T). This mutation leads to an amino acid substitution of glutamine to histidine at position 406 (Q406H). Only one patient was heterozygous for this mutation. The relevance of these mutations to HHcy and subsequently to vascular disease was tested in a study group. The characteristics of this study group are depicted in Table 1. Screening of the total study group revealed the presence of the 595 G > A mutation only in a heterozygous state in 2.8% of the cases and in 1.9% of the controls, which did not lead to an increased risk for vascular disease (OR 1.5, 95% CI (0.5–4.5)) (Table 3). Moreover, the effect of this mutation on Hcy levels was investigated (Table 4). Among cases as well as controls, Hcy levels for those with a GA genotype did not differ from those with a GG genotype (Table 4). Additionally, 13 vascular patients with severe HHcy (fasting Hcy > 100 μ mol/L) were

TABLE 3
Prevalence of the 595 G > A and 716 G > A
Mutations among Cases and Controls

Mutation	Cases	Controls	Odds ratio (95% CI)
595 G > A			
595 GG	97.2% (n = 171)	98.1% (n = 461)	1.0 ^a
595 GA	2.8% (n = 5)	1.9% (n = 9)	1.5 (0.5–4.5)
595 AA	— ^b	—	
716 G > A			
716 GG	52.6% (n = 90)	47.7% (n = 248)	1.0 ^a
716 GA	40.4% (n = 69)	42.5% (n = 221)	0.9 (0.6–1.2)
716 AA	7.0% (n = 12)	9.8% (n = 51)	0.6 (0.3–1.3)

^a Reference category.

^b Not observed.

screened for the 595 G > A mutation (data not shown), in order to find a possible 595 AA allele. The mutation was not detected in any of these patients.

The second mutation, the 716 G > A mutation, was found homozygously mutant (AA) in 7% of the cases compared to 9.8% of the controls (Table 3), which was not significantly different. In addition, the effect of this mutation on Hcy levels was tested. This mutation did not lead to significant divergent Hcy levels neither fasting nor postload (Table 4).

Three individuals were heterozygous for both the 595 G > A and the 716 G > A mutation and also had increased Hcy levels (fasting Hcy 18.7 $\mu\text{mol/L}$ and postload Hcy 50.4 $\mu\text{mol/L}$) when compared to the wild-type (Table 5). However, after statistical analysis by ANOVA and after adjustment for confounders this increase in Hcy was not significant ($P = 0.09$ for fasting Hcy and $P = 0.19$ for postload Hcy). Although, one genotype combination did result in significantly elevated postload Hcy levels (Table 5, 595 GA/716 GA vs 595 GA/716 GG; $P = 0.03$).

Screening of the total study group for the 1218 G > T mutation resulted in the detection of one heterozygous control. The presence of this heterozygous mutation did not lead to higher Hcy levels (data not shown).

BHMT protein sequence of various species was compared to determine if amino acid substitutions of the BHMT gene can be found in a conserved region between different species. Only the 595 G > A mu-

tation (G199S) is conserved between different species (human, mouse, rat, and pig). The other two mutations are not in any conserved region of the gene (data not shown). Furthermore, possible secondary structure influences due to different mutations were studied by Chou & Fasman analysis (Omega 2.0, Oxford Molecular Ltd, England). Only the G199S substitution was predicted to result in a different secondary structure (data not shown).

DISCUSSION

Deficiency of BHMT can theoretically result in elevated Hcy levels and may therefore contribute to the risk for vascular disease. Until now, no defects of BHMT have been described. Recently, the gene coding for BHMT has been published enabling molecular analysis, which facilitates the detection of mutants with possible BHMT deficiency in patients. In this study, 16 vascular patients with HHcy were subjected to genomic sequencing of the BHMT gene and three mutations were detected in the coding region of the BHMT gene.

The first mutation is a 595 G > A transition, which results in an amino acid substitution of glycine to serine at position 199 of the BHMT protein (G199S). This mutation seems to be a rare polymorphism with no relevance to HHcy or vascular disease, at least in the heterozygous state. As indicated in Table 4, Hcy levels (fasting as well as postload) of cases and controls are slightly elevated when the 595 GA allele is present, although this increase is not significant. Presence of the homozygous mutated allele (595AA) may theoretically result in significant elevated Hcy levels. Screening of 170 cases and 460 controls did not reveal any homozygous mutated alleles. Thus, 595 AA alleles seem rare and will hardly contribute to mild HHcy or subsequently to the risk for vascular disease. Additionally, 13 patients with severe HHcy (fasting Hcy > 100 $\mu\text{mol/L}$) were screened for this mutation. None of them carried the possible 595 AA allele (data not shown). Comparison of the BHMT amino acid sequence of different species indicated that glycine at position 199 is conserved between human, mouse, rat, and pig (data not shown). Recently, Breska *et al.* (12) showed that replacement of conserved cysteine residues (Cys217, Cys299, and Cys300) in human, mouse, rat, and pig BHMT already resulted in complete loss of activity, which suggested that glycine at position 199 could also be a functional amino acid. Secondary structure prediction by Chou & Fasman

TABLE 4
Geometric Means of Homocysteine Levels of BHMT 595 G > A and
BHMT 716 G > A Genotypes among Study Group

	Cases mean ^a (95% CI)	Controls mean ^a (95% CI)	All subjects mean ^a (95% CI)
BHMT 595 G > A			
Fasting Hcy (μmol/L)			
GG	14.2 (13.6–14.9) ⁿ⁼¹⁶⁴	13.1 (12.7–13.4) ⁿ⁼⁴⁵⁴	13.4 (13.0–13.7) ⁿ⁼⁶¹⁸
GA	11.3 (7.1–18.0) ⁿ⁼⁴	16.0 ¹ (12.1–21.3) ⁿ⁼⁹	14.4 ² (11.5–18.1) ⁿ⁼¹³
AA	—	—	—
Postload Hcy (μmol/L)			
GG	40.5 (38.4–42.6) ⁿ⁼¹⁵³	39.8 (38.7–40.9) ⁿ⁼⁴³⁸	40.0 (39.0–41.0) ⁿ⁼⁵⁹¹
GA	37.1 (24.1–57.1) ⁿ⁼⁵	43.3 ³ (33.4–56.3) ⁿ⁼⁸	40.8 ⁴ (33.6–49.6) ⁿ⁼¹³
AA	—	—	—
BHMT 716 G > A			
Fasting Hcy (μmol/L)			
GG	14.3 (13.4–15.4) ⁿ⁼⁸⁶	12.8 (12.3–13.3) ⁿ⁼²⁴⁷	13.2 (12.7–13.6) ⁿ⁼³³³
GA	13.8 (12.9–14.8) ⁿ⁼⁶⁶	13.3 (12.7–13.8) ⁿ⁼²¹⁵	13.4 (12.9–13.9) ⁿ⁼²⁸¹
AA	15.6 ⁵ (12.8–19.1) ⁿ⁼¹¹	13.2 (12.1–14.4) ⁿ⁼⁶⁰	13.6 (12.6–14.7) ⁿ⁼⁶¹
Postload Hcy (μmol/L)			
GG	41.7 (38.9–45.2) ⁿ⁼⁸¹	40.1 (38.6–43.3) ⁿ⁼²⁴⁰	40.5 (39.1–41.9) ⁿ⁼³²¹
GA	39.7 (37.1–42.5) ⁿ⁼⁶³	41.4 (39.6–43.3) ⁿ⁼²⁰⁶	41.0 (39.5–42.6) ⁿ⁼²⁶⁹
AA	43.3 ⁶ (34.0–55.2) ⁿ⁼¹¹	39.5 (36.7–42.5) ⁿ⁼⁴⁷	40.2 (37.4–43.2) ⁿ⁼⁵⁸

^a Geometric mean; no significant differences were observed before and after adjustment for confounders.

^b Not observed.

¹ $P = 0.42$, ² $P = 0.79$, ³ $P = 0.83$, ⁴ $P = 0.60$, ⁵ $P = 0.83$, ⁶ $P = 0.60$ (after adjustment for age, sex, and MTHFR 677 genotype).

analysis indicated that the protein, which normally has a turn structure, changes into a coil structure when serine is introduced. Furthermore, the substitution at amino acid position 199 (G199S) converts the nonpolar amino acid glycine, which is commonly present on the surface of proteins, into the polar amino acid serine, which is frequently located in the interior part of the protein. Therefore, this substitution could result in secondary structure changes, which supports the findings by Chou & Fasman analysis. Based on these findings we hypothesize that the G199S mutation may result in altered functioning of BHMT, although further studies are required to confirm this hypothesis.

The second mutation found in the BHMT gene (716 G > A transition) results in a substitution of glutamine to arginine at position 239 (Q239R). Screening of 171 cases and 520 controls did not show a significant difference on allele frequencies between cases and controls. Neither did the mutation influence Hcy concentrations, fasting nor postload (Table 4). This polymorphism was also noted by Park and Garrow (14), which suggests that this polymorphism is not unique to the Dutch population. Comparing the BHMT protein of different species (human, mouse, rat, and pig) indicated the presence of either

glutamine or arginine at position 239, suggesting that this polymorphism is also present in species other than humans. Therefore, the presence of arginine instead of glutamine at position 239 probably does not seem to have a major impact on folding of the protein and subsequently on protein functioning. Furthermore, secondary structure prediction by Chou & Fasman analysis does not lead to divergent structures. In summary, we have no evidence that this common 716 G > T mutation influences Hcy metabolism and therefore we consider this as a benign polymorphism.

The third mutation found in exon 8 of the BHMT gene, the 1218 G > T (Q406H), was only found in the heterozygous state in one of the preselected patients and one of the controls of the study group. Therefore, this mutation seems to be a very rare polymorphism, which is not assumed to be a risk factor for HHcy or vascular disease. Glutamine at position 406 is not conserved, assuming no effect of this mutation on protein functioning. The presence of this mutation on both alleles (1218 TT) is therefore not expected to have a major impact on protein functioning.

A combination of two mutations of the BHMT gene may have an additive effect on the phenotype. Therefore, we examined the combined effect of the

TABLE 5
Geometric Means of Fasting and Postload Homocysteine Values for Combinations of BHMT 595 G > A and 716 G > A Genotypes

716 G > A genotype	595 G > A genotype		
	595 GG mean ^a (95% CI)	595 GA mean ^a (95% CI)	595 AA mean ^a (95% CI)
Fasting homocysteine ($\mu\text{mol/L}$)			
716 GG	13.2 (12.7–13.7) (<i>n</i> = 283)	13.8 (11.4–16.7) (<i>n</i> = 9)	— ^b
716 GA	13.3 (12.8–13.8) (<i>n</i> = 244)	18.7 ^{1,2} (3.8–91.7) (<i>n</i> = 3)	—
716 AA	13.3 (12.2–13.6) (<i>n</i> = 52)	—	—
Postload homocysteine ($\mu\text{mol/L}$)			
716 GG	40.0 (38.5–41.5) (<i>n</i> = 272)	39.1 (30.5–50.2) (<i>n</i> = 8)	—
716 GA	40.5 (38.9–42.2) (<i>n</i> = 252)	50.4 ^{3,4} (34.2–74.2) (<i>n</i> = 4)	—
716 AA	39.7 (36.7–43.1) (<i>n</i> = 50)	—	—

^a Geometric mean.

^b Not observed.

¹ $P = 0.09$ 595 GA/716 GA vs 595 GG/716 GG, ² $P = 0.06$ 595 GA/716 GA vs 595 GG/716 GA, ³ $P = 0.19$ 595 GA/716 GA vs 595 GG/716 GG, and ⁴ $P = 0.03$ 595 GA/716 GA vs 595 GA/716 GG.

595 G > A mutation and the 716 G > A mutation on Hcy levels. This combination does not lead to significantly elevated Hcy levels, when compared to the wild-type. However, elevated postload Hcy levels were observed in one other genotype combination ($P = 0.03$, 595GA/716GA vs 595 GA/716GG). To exclude a possible role of genotype combinations on Hcy levels, extension of the study group is necessary. In addition, a combination of mutated BHMT with mutated genes coding for other enzymes (MS, MTHFR, CBS, methionine synthase reductase (MTRR)) of the Hcy metabolism also could influence Hcy concentrations (Fig. 1).

Choline is the precursor of betaine (Fig. 1). When choline intake is low, less betaine is formed and subsequently less Hcy is remethylated to methionine, which may result in elevated Hcy concentrations. Choline is present in both animal and vegetable fats, but predominates in animal fats (22). Thus, when mutations are present in the BHMT gene, we hypothesize that the availability of betaine may become limiting, and therefore a stronger effect of mutations could be expected in populations with low choline intake. Based on fat-intake, the Dutch population is presumed to have normal to high choline levels; therefore, mutations of the BHMT gene may

have less effect on Hcy in this population. An increased effect of these mutations could be expected in populations with less animal fat intake, e.g., the Chinese population.

Mutated BHMT may contribute to other disorders related to a disturbed Hcy or folate metabolism such as neural tube defects or habitual abortions, due to different interactions of genetic and nutritional factors (23,24). Therefore, it would be interesting to investigate the role of mutated BHMT in relation to these disorders.

In this study three mutations were found in the BHMT gene, one of these three mutations (G199S) is conserved and could probably have an effect on BHMT protein functioning. To investigate whether mutations of the BHMT gene are pathogenic, mutations could be expressed in a bacterial expression system (12). Using such a system would evaluate any possible effects of the homozygous mutated allele (595 AA). Mutations in the BHMT gene could result in altered functioning of this gene and could therefore lead to HHcy. This is the first study in which molecular genetic analysis of the BHMT gene is performed in relation to HHcy and vascular disease. The results of this study indicate that mutated BHMT is not a major cause of mild HHcy or vascular disease. Further molecular genetic and

enzymatic studies on BHMT are required to answer the question if defective BHMT functioning contributes to HHcy and vascular disease.

ACKNOWLEDGMENTS

The authors thank Dr. J. Vet and M. Smit for excellent assistance with molecular beacon technology and Dr. N. van der Put for valuable discussions. Furthermore, the technical assistance of A. de Graaf-Hess, H. van Lith-Zanders, D. van Oppenraaij-Emmerzaal, M. Te Poele-Pothoff, and S. Vloet is gratefully acknowledged. Grants D 97.021 and 97.071 of the Netherlands Heart Foundation supported this study.

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