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# Sequence analysis of the coding region of human methionine synthase: relevance to hyperhomocysteinaemia in neural-tube defects and vascular disease

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## Summary

Elevated homocysteine (Hcy) levels are observed in two apparently unrelated diseases: neural-tube defects (NTD) and premature vascular disease. Defective human methionine synthase (MS) could result in elevated Hcy levels. We sequenced the coding region of MS in 8 hyperhomocysteinaemic patients (4 NTD patients and 4 patients with pregnancies complicated by spiral arterial disease, SAD). We identified only one mutation resulting in an amino acid substitution: an A→G transition at bp 2756, converting an aspartic acid (D919) into a glycine (G). We screened genomic DNA for the presence of this mutation in 56 NTD patients, 69

mothers of children with NTD, 108 SAD patients and 364 controls. There was no increased prevalence of the GG and AG genotypes in NTD patients, their mothers or SAD patients. The D919G mutation does not seem to be a risk factor for NTD or vascular disease. We then examined the mean Hcy levels for each MS genotype. There was no correlation between GG- or AG-genotype and Hcy levels. The D919G mutation is thus a fairly prevalent, and probably benign polymorphism. This study, though limited, provides no evidence for a major involvement of MS in the aetiology of homocysteine-related diseases such as NTD or vascular disease.

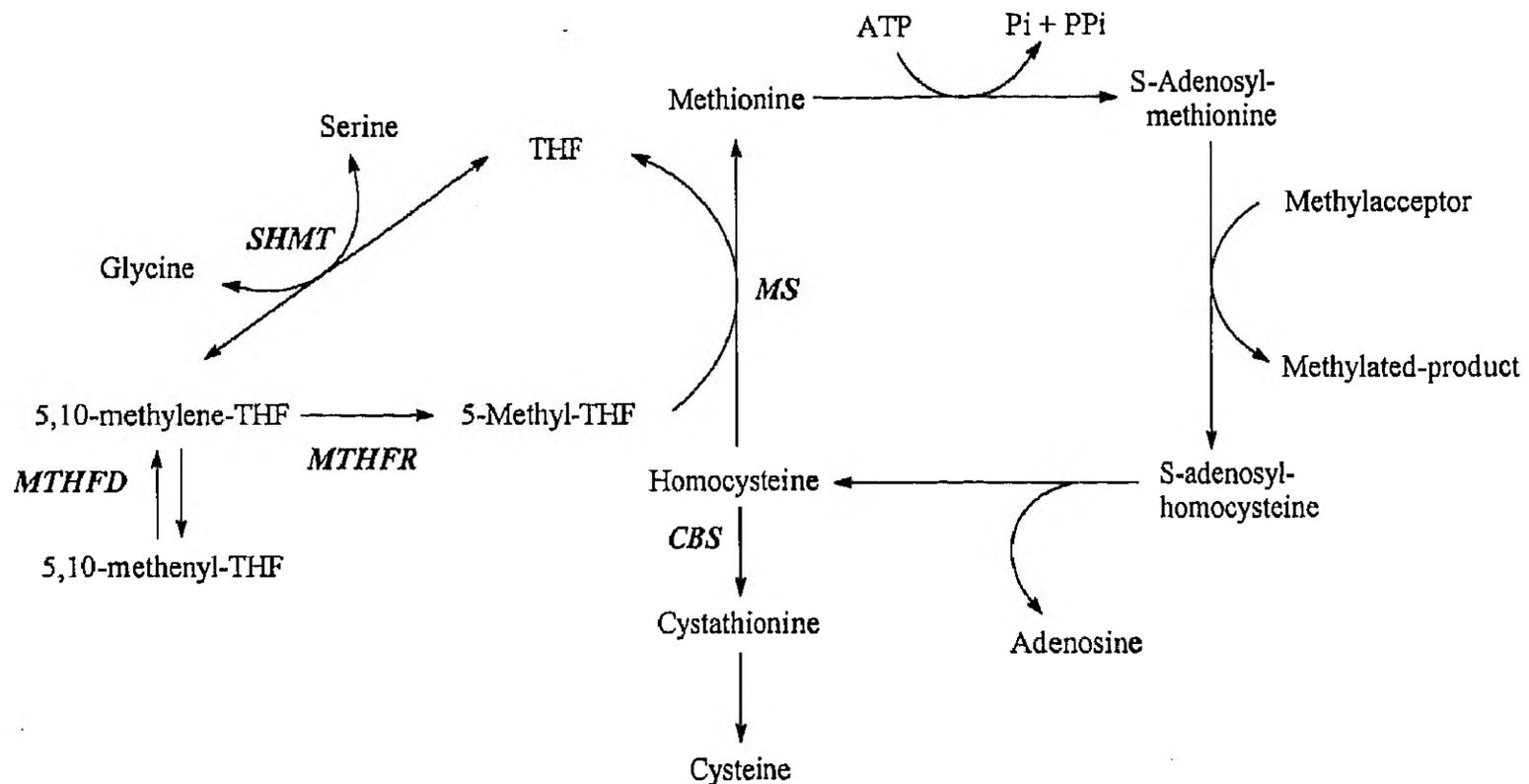
## Introduction

Two apparently unrelated diseases, neural-tube defects (NTDs) and premature vascular disease, are both associated with moderately elevated homocysteine (Hcy) levels.<sup>1-3</sup> Enzymes and cofactors involved in Hcy metabolism are currently being investigated for their contribution to these diseases. Hcy is a branch-point metabolite of methionine metabolism, and can be remethylated to methionine or catabolized in the transsulphuration pathway to cysteine, (Figure 1).

NTDs account for a significant part of present-day perinatal morbidity and mortality. Several studies showed that periconceptual folate administration

reduces the occurrence and recurrence risk of NTDs.<sup>4,5</sup> We have postulated that heterozygosity for cystathionine  $\beta$ -synthase (CS) deficiency could be a cause of moderate hyperhomocysteinaemia in NTD, but we have not found evidence for a defective CS in mothers of children with NTD.<sup>1</sup>

5,10-Methylenetetrahydrofolate reductase (MTHFR) is one of the key enzymes in the folate-dependent remethylation of Hcy to methionine. We recently showed that homozygosity for the 677C→T mutation in the MTHFR gene, causing thermolability of the enzyme,<sup>6-8</sup> can partly explain the observed elevated Hcy levels in NTD, and this has recently



**Figure 1.** A simplified scheme of folate-dependent homocysteine metabolism. Key enzymes are given. MS, methionine synthase; MTHFR, 5,10-methylenetetrahydrofolate reductase; MTHFD, 5,10-methylenetetrahydrofolate dehydrogenase; SHMT, serine hydroxymethyl transferase; CBS, cystathionine  $\beta$ -synthase.

been confirmed by others.<sup>9,10</sup> The 677C $\rightarrow$ T mutation in the MTHFR gene is the first identified genetic risk factor for NTD. However, even after exclusion of individuals homozygous for the 677C $\rightarrow$ T mutation in MTHFR, we still observe significantly elevated Hcy levels in NTD patients and their parents.<sup>11</sup> So, in addition to the 677C $\rightarrow$ T mutation in MTHFR, other mutations in genes affecting the conversion of Hcy to methionine could be present in these families.

In most Western countries, vascular disease is one of the main causes of death. Along with high blood pressure and cholesterol, increased plasma Hcy level is a risk factor for vascular disease, with a relative risk comparable to hypercholesterolaemia.<sup>3</sup> It has been postulated that the elevation of Hcy levels in patients with vascular disease could be caused by heterozygosity for CS.<sup>12,13</sup> However, we have not found any evidence for involvement of CS deficiency in vascular disease.<sup>14</sup> The 677C $\rightarrow$ T mutation in the MTHFR gene, resulting in elevated Hcy levels, can only partly explain the elevated Hcy levels in patients with vascular disease.<sup>14</sup> Several studies have reported that folate administration reduces Hcy concentrations of hyperhomocysteinaemic patients with vascular disease.<sup>15,16</sup> Therefore, a defective function of other folate-dependent enzymes may be involved in hyperhomocysteinaemia of vascular patients. However, the clinical benefits of this Hcy-lowering therapy are unproven.

The remethylation of Hcy occurs by transfer of a methyl group from 5-methyltetrahydrofolate (Me-THF) via cobalamin to Hcy. This reaction is catalysed by 5-methyltetrahydrofolate homocysteine methyltransferase (methionine synthase), which is probably present in all mammalian tissues, and

requires cobalamin as a cofactor. The enzyme, betaine-homocysteine methyltransferase, can also remethylate Hcy to methionine, but this enzyme is present only in the liver. Several studies have suggested that a defective methionine synthase (MS) could be a critical defect in folate-related NTDs.<sup>1,2,17,18</sup> MS is essential for maintaining adequate intracellular methionine and tetrahydrofolate pools, as well as for ensuring that the Hcy concentration does not reach toxic levels. Since elevated Hcy levels are observed not only in NTD patients and their parents, but also in patients with vascular diseases, both diseases could be caused by defective MS.

Recently, the human MS cDNA-sequence has been cloned<sup>19,20</sup> and mutations causing the inborn error of metabolism *cbIG* have been described.<sup>20, 21</sup> The gene has been mapped to 1q43, near the telomere of chromosome 1. The human MS gene has an open reading frame of 3768 bases which encodes a protein of 1256 amino acids with a 55% homology compared to *E. coli* MS. Although enzymic studies have indicated a higher activity for MS in kidney, the RNA expression levels of MS seem to be similar in different tissues.<sup>19</sup>

In order to study the postulated involvement of MS at the genomic level, we selected eight patients with mild hyperhomocysteinaemia: four NTD patients and four women with severe spiral arterial disease (SAD) during their pregnancies. SAD causes infarction of the placenta, resulting in a stillborn or severely growth-retarded child, and is predominantly the result of spiral artery occlusion in the myometrium or decidua. Histological examination of the spiral arteries in SAD reveals one or more signs of

vasculopathy, i.e. arteriosclerosis, narrowing, necrosis and thrombosis. SAD is an occlusive arterial disease that is associated with elevated Hcy levels,<sup>22</sup> like most vascular diseases. By direct sequencing of the complete coding region of MS from eight hyperhomocysteinaemic patients, we examined whether mutations in the MS gene were involved in these Hcy-related diseases.

## Methods

### Patients

Eight patients with mild hyperhomocysteinaemia (Hcy levels above the normal range for their sex and age category),<sup>1</sup> four NTD patients and four vascular patients who had a pregnancy complicated by SAD, were selected for direct sequencing of the cDNA of the MS gene. The elevated Hcy levels were not due to the TT genotype for the 677C→T transition in MTHFR, except patient SAD 4, who had a TT genotype but had very high Hcy levels. The age, sex, folate and Hcy data of these individuals are given in Table 1. The normal ranges of plasma folate, red-cell folate and Hcy are between 6–22 nmol/l; 213–817 nmol/l and 4.6–15 µmol/l, respectively.<sup>1</sup>

To examine whether the 2756A→G mutation was associated with mild hyperhomocysteinaemia, or was a genetic risk factor for NTD or SAD, we studied the prevalence of this mutation among NTD patients and mothers with NTD-affected offspring, and patients who had suffered from SAD. The study group consisted of 69 mothers with a child with NTD (mean age 46, SD 12.6 years) and 56 NTD patients, mostly spina bifida, (mean age 21, SD 12.3 years).<sup>6–8</sup> Furthermore, we studied 108 patients with SAD (mean age 34, SD 5.3 years). The diagnosis of SAD was established if the placenta was characterized by circumscribed areas of villous necrosis combined with a stillborn fetus or a severely growth-retarded child, i.e. having a birth weight below the 10th percentile of gestational age. The population-

based control group consisted of 364 unrelated Dutch individuals of either gender and no history of NTD or SAD (mean age 51, SD 12.4 years).<sup>8</sup>

### Laboratory methods

Total homocysteine concentrations were measured in EDTA plasma.<sup>23</sup>

Total RNA of NTD patients was extracted from EBV (Epstein Barr Virus) cell lines and the RNA of the vascular patients from lymphocytes isolated of whole blood,<sup>24</sup> and was stored as an ethanol precipitate at –80°C. A 5 µg sample of RNA was reverse-transcribed to cDNA in 1 h at 42°C with 200 units of SuperscriptII reverse transcriptase (Life Technologies), using oligo(dT) and random hexanucleotides. A 2.5 µl aliquot of this first-strand cDNA was amplified by PCR. Amplifications were carried out in a total volume of 100 µl, containing 100 ng forward and reverse primer, 200 µM each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.75–1.5 mM MgCl<sub>2</sub> and 1 unit *Taq* polymerase (Life Technologies). Amplification parameters were as follows: 35 cycles of 92 °C/60 s (denaturation), 54–64 °C/60 s (annealing) and 72 °C/90 s (extension). The cycles were preceded by an initial denaturation step of 2 min at 92 °C and were followed by a final extension of 7 min at 72 °C.

PCR and sequencing primers were designed based on the cDNA sequence.<sup>19</sup> We constructed 6 pairs of oligonucleotides (MS1–12), (Table 2). These primers were designed to generate overlapping fragments of 700–900 bp, covering the total cDNA sequence. These fragments were screened for the presence of mutations by direct sequencing; two forward and two reverse oligonucleotides were used to analyse each of the six fragments.

The sequence analysis was performed by automated sequencing (ABI Prism, model 377 version 2.1.2), using the ABI Prism *Taq* DyeDeoxy terminator cycle sequencing ready reaction kit (Perkin Elmer), according to the instructions of the manufacturer.

**Table 1** Patient profiles: sex, age, folate and fasting Hcy levels of the selected hyperhomocysteinaemic study group

Patient	Sex	Age (years)	PF (nmol/l)	RCF (nmol/l)	Hcy (µmol/l)
NTD 1	M	23	14.0	580	16.5
NTD 2	F	14	8.6	390	15.2
NTD 3	F	30	7.3	350	21.0
NTD 4	F	17	13.0	350	23.3
SAD 1	F	34	16.0	470	15.8
SAD 2	F	34	18.0	470	20.9
SAD 3	F	31	13.0	420	27.8
SAD 4	F	32	6.0	440	57.3

Hcy, homocysteine; PF, plasma folate; RCF, red-cell folate.

**Table 2** Oligonucleotide primers used for PCR and sequencing of the MS gene of hyperhomocysteinaemic patients

Name	Sequence	Location
MS1F	5'GCGTGTCTGGCTGCTAGGC	-283 to -265
MS1BF	5'CCGTCGTCACCTGTGGAGAGC	-47 to -67
MS2R	5'GTGATGTTCTATAATCCGGCC	503-482
MS3F	5'AGGTTTGTGGCAGGGGCTC	418-436
MS4R	5'GCTCCCATTCCACCTGCAC	1229-1210
MS5F	5'TTGGAGAGCGCTGTAATGTTGC	1121-1142
MS6R	5'AGTGGCCTCAGGGTCTTTATTCC	1929-1907
MS7F	5'AGTCTGGCATGGACATGGGG	1811-1830
MS8R	5'CAGGTGCACTGTATCTCGGAGC	2638-2617
MS9F	5'GACTCATCACTCCTTCCCTGG	2492-2512
MS10R	5'AACAGGCCAGGTAGTCACG	3299-3280
MS11F	5'AGGCAACAGGCTGAGAAGGAC	3196-3216
MS12R	5'CAGGTAAGTGTGAGCCAGATGC	3912-3891
MS13F	5'ATTCTGCAGAAGAGGATTATGGTG	73-96
MS14R	5'GTCCCCATCCCTCCATCCAG	116-97
MS15F	5'CACTCTACATTGGATTAATTGTGC	758-782
MS16R	5'CATTGGGATAACAGAGGACATAGGC	862-838
MS17F	5'GATGAAGAAGGACAGGCAACAG	1501-1522
MS18R	5'TTTGTGTCTGTTTCTGTTGCCTG	1535-1513
MS19F	5'CCTCAGGTTATAAAGTCAGCCCG	2191-2213
MS20R	5'ATGAAAGGAATAAGGTGGCCAAC	2252-2230
MS21F	5'TTGAAGACTATGACCTGCAGAAGC	2882-2905
MS22R	5'GCTTCCAGTCAATGTAGTCCACC	2929-2907
MS23F	5'ACCATGTGGAGACTCGCAGAC	3562-3582
MS24R	5'TGGTGAGCTTCTCGGTGTGG	3565-3546
MS28F	5'GGTGTGTTCCTCAGCTGTTAGATG	2673-2695
MS29R	5'GAGGTTTCAGACAGCCAATCC	2845-2826
MS30R	5'GACACTGAAGACCTCTGATTTGAAC	(intronic)

Numbering starts at the ATG-site as in Leclerc *et al.*<sup>20</sup>

DNA was isolated from whole blood and lymphocytes as described by Miller *et al.*<sup>25</sup> and approximately 100 ng was used for PCR amplification. When we tried to confirm the 2756A→G mutation on genomic DNA, we observed that a large intron of over a 1000 bp was present in the genomic DNA at position 2775 of the cDNA. We sequenced the intron with the oligonucleotides MS28 and MS29 and selected an intronic oligonucleotide (MS30), (Table 2), to make screening for the 2756A→G mutation on genomic DNA possible. The PCR for detection of this mutation on genomic DNA was carried out in a total volume of 50 µl, containing 50 ng of the MS28 and MS30 oligonucleotides (see Table 2), 200 µM of each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.0 mM MgCl<sub>2</sub> and 1 unit *Taq* polymerase (Life Technologies). PCR parameters were as follows: an initial denaturation step of 2 min at 92 °C, followed by 35 cycles of: 92 °C/60 s (denaturation), 56 °C/60 s (annealing) and 72 °C/90 s (extension), and a final extension for 7 min at 72 °C. The amplified PCR fragments were digested with the restriction enzyme *Hae*III to screen for the presence of the 2756A→G mutation. An AA genotype resulted in an uncut fragment of 265 bp, whereas an AG

genotype gave three fragments of 265, 180 and 85 bp, and a GG genotype showed two fragments of 180 and 85 bp.

### Statistics

Odds ratios (OR) and 95% CIs for the 2756A→G mutation were calculated to estimate the relative risk of this mutation in NTD patients, their mothers and SAD patients. The Hcy concentrations per MS genotype were expressed as means ± SD. Statistical significance was tested by a paired Wilcoxon Rank Sum test. *p* < 0.05 was considered statistically significant, all *p* values were two-tailed.

### Results

The human MS gene was examined for involvement in mild hyperhomocysteinaemia observed in NTD and vascular diseases. To look for mutations in the coding region of MS, MS cDNA from 4 NTD patients and 4 patients with SAD with mildly elevated Hcy levels, was screened by direct sequencing.

Total RNA of NTD patients was extracted from EBV cell lines, and that of the vascular patients from

isolated lymphocytes. The isolated RNA was reverse-transcribed to cDNA and amplified by PCR for direct sequencing analysis. RT-PCR of total lymphoblastoid and lymphocyte RNA yielded products of the expected size, indicating that MS is expressed in cultured lymphoblasts and lymphocytes.

By direct sequencing of the entire coding region of MS of the selected patients with mild hyperhomocysteinaemia, we observed three silent mutations in the cDNA of these individuals, see Table 3. Furthermore, we identified an A→G transition at bp 2756, converting an aspartic acid into a glycine (D919G). This mutation was present in two SAD patients and one NTD patient, in a heterozygous state in all. The observed nucleotide substitution creates a *Hae*III restriction site, which allowed the confirmation of the D919G mutation on genomic DNA.

To determine whether the 2756A→G mutation was a risk factor for NTD offspring and vascular disease, we screened for its prevalence in the genomic DNA of 56 NTD patients, 69 mothers of children with NTDs, 108 SAD patients and 364 Dutch controls. After amplification of the appropriate region of the genomic DNA by a PCR reaction using primers MS28 and MS30, the PCR fragment was digested with *Hae*III. The homozygous mutated GG genotype was present in only 1.8% of the NTD patients, 2.9% of their mothers, 2.8% of the SAD patients and 3.3% of the controls. Table 4 shows that the AG-genotype was fairly prevalent, that is in 25% of the NTD patients, 27.5% of the mothers with NTD-affected offspring, 28.7% of the SAD patients and 25.8% of the controls. The frequency in the control group of the A-allele is 0.84 and of the G-allele 0.16. According to the Hardy-Weinberg equation the following genotype distribution was expected, AA genotype 70.6%; AG genotype 26.9% and GG genotype 2.6%, which is in agreement with the observed genotype distributions among the different study populations, see Table 4.

There was at most only a slightly increased prevalence of the GG and AG genotypes in NTD patients, their mothers, and SAD patients, indicating that this amino acid change is not a major (if any) risk factor for NTD or SAD. This is reflected in the calculated ORs of 0.5–1.1 for the GG and AG genotypes in SAD patients, NTD patients and their mothers (Table 5).

**Table 4** Prevalence of the 2756A→G mutation in the MS gene of controls, SAD patients, NTD patients and their mothers

	n	Genotype (%)		
		AA	AG	GG
Controls	364	70.9	25.8	3.3
SAD patients	108	68.5	28.7	2.8
NTD patients	56	73.2	25.0	1.8
NTD mothers	69	69.6	27.5	2.9

**Table 5** Calculated odds ratios (OR) and 95% CIs for the GG and AG genotype vs. the AA genotype of MS for SAD patients, NTD patients and their mothers

	GG genotype		AG genotype	
	OR	95% CI	OR	95% CI
SAD patients	0.9	[0.3–4.0]	1.1	[0.8–1.6]
NTD patients	0.5	[0.1–4.0]	1.0	[0.6–1.6]
NTD mothers	0.9	[0.2–3.9]	1.1	[0.7–1.6]

To examine whether the 2756A→G mutation in MS was associated with mild hyperhomocysteinaemia, we determined the mean Hcy levels of the different MS genotypes. The Hcy levels of mothers with a NTD child with an AG genotype were elevated when compared with the Hcy levels of mothers with an AA or GG genotype, but not significantly so. The Hcy levels of NTD patients, patients with SAD and controls between the three MS genotypes did not differ, see Table 6.

## Discussion

By direct sequence analysis of the complete coding region of MS we observed four mutations (Table 3). Three were silent mutations, and only one mutation, an A→G substitution at bp 2756, resulted in an amino acid change, of an aspartic acid into a glycine (D919G). D919 corresponds to Q893 in *E. coli* MS. This is at the penultimate position in a long helix that leads out of the cobalamin domain to the S-adenosylmethionine-binding domain in the highly

**Table 3** Sequence differences found in the coding region of the MS gene of eight patients with mild hyperhomocysteinaemia

Mutation	Location	Amino acid substitution
Silent	2053A→T	Lysine (685)→lysine
	2127A→G	Glutamate (709)→glutamate
	3144A→G	Alanine (1048)→alanine
Polymorphism	2756A→G	Aspartic acid (D919)→glycine (G)

**Table 6** Relationship between MS genotype and plasma homocysteine levels in Dutch controls, SAD patients, NTD patients and their mothers

	n	Genotype		
		AA	AG	GG
Controls	364	13.5 ( $\pm 4.5$ )	13.0 ( $\pm 5.6$ )	12.4 ( $\pm 2.1$ )
SAD patients	108	13.9 ( $\pm 5.1$ )	13.6 ( $\pm 4.6$ )	11.9 ( $\pm 2.7$ )
NTD patients	56	13.3 ( $\pm 3.7$ )	12.4 ( $\pm 3.2$ )	15.5 (NP)
NTD mothers	69	12.8 ( $\pm 2.9$ )	15.1 ( $\pm 11.2$ )	12.4 ( $\pm 0.3$ )

Plasma homocysteine concentrations are expressed as means  $\pm$  SD, in  $\mu\text{mol/l}$ . NP, not possible, because  $n=1$ .

homologous bacterial MS.<sup>26</sup> Having the glycine residue at this position, which is a strong helix breaker, versus aspartic acid which is a moderate helix maker, could have an effect on the secondary structure of the protein and therefore have functional consequences. This 2756A $\rightarrow$ G transition has also been observed in *cbfG* patients and controls in the study of Leclerc *et al.*<sup>20</sup>

A possible involvement of the D919G mutation in the risk for NTD and SAD was investigated by analysing the prevalence of the 2756A $\rightarrow$ G mutation in the MS gene of NTD patients, mothers with NTD-affected offspring and patients that had suffered from SAD during their pregnancy. No significantly increased prevalence of GG and AG genotypes was present in NTD patients, their mothers or SAD patients when compared with the prevalence among controls, resulting in ORs of 0.5–1.1 for NTD patients, their mothers and patients with SAD (Tables 4 and 5), indicating that this amino acid change is not a major (if any) risk factor for NTD or SAD. The presence of the D919G mutation has also no effect on the Hcy levels (Table 4). This suggests that the D919G mutation in the MS gene is a fairly prevalent, but probably benign polymorphism. Expression studies with the D919G mutation will resolve whether this polymorphism has a significant effect on MS function, as might have been expected from its postulated effect on helix formation.

The folate levels of mothers with NTD offspring are not deficient,<sup>11</sup> suggesting that there are no major nutritional folate deficiencies. The 677C $\rightarrow$ T mutation in the MTHFR gene can explain at most 27.4% of the protective effect of folate.<sup>7</sup> Since folate administration reduces the risk for NTD by at least 70% or more,<sup>4,5</sup> this implies that there may be other genetic defects present in the folate-related Hcy metabolism that can be corrected by an additional folate intake.

In order to identify these candidate defective enzymes in the folate-dependent Hcy metabolic pathway, we recently analysed the plasma cobalamin, plasma and red cell folate, and plasma homocysteine levels of NTD patients and their parents.<sup>11</sup> We observed, even after exclusion of those individuals homozygous for the 677C $\rightarrow$ T mutation in the MTHFR gene, significantly elevated Hcy levels and decreased plasma folate levels in NTD patients and their parents. Plasma folate is mostly (80–90%) Me-THF, which is the circulating form of folate. Thus, a defective MS would not lead to decreased plasma folate levels, but rather increased plasma folate concentrations, since Me-THF is the substrate of MS in the remethylation of Hcy to methionine. So, the results of our vitamin and Hcy analyses of our NTD population<sup>11</sup> are in agreement with the present sequencing results. Neither indicate a direct or significant role for MS in the aetiology of NTD. It is possible that other mutations in MS may have been missed, since we screened MS in only eight hyperhomocysteinaemic patients by direct sequencing. However, if such mutations exist, they are unlikely to be present at high enough frequency to explain a significant proportion of the observed hyperhomocysteinaemia among NTD and vascular disease patients.

Since, the elevated Hcy levels in patients with vascular disease, mothers of children with NTDs, or NTD patients, are probably not due to a decreased function of CS or MS, and only partly due to the 677C $\rightarrow$ T mutation in the MTHFR gene, other enzymes involved in the folate-dependent Hcy metabolism may be compromised. In our vitamin study, we observed significantly decreased plasma folate levels in NTD families.<sup>11</sup> Decreased Me-THF levels have also been reported in patients with vascular disease,<sup>27,28</sup> suggesting the presence of other mutations present in MTHFR or other enzymes in the folate metabolism involved in the synthesis of Me-THF. Such candidate enzymes (see Figure 1), leading to mild hyperhomocysteinaemia in NTD and vascular disease, include serine hydroxymethyltransferase, methylenetetrahydrofolate dehydrogenase and the auxiliary redox proteins that regulate methionine synthase or MTHFR.

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