

A Second Common Mutation in the Methylenetetrahydrofolate Reductase Gene: An Additional Risk Factor for Neural-Tube Defects?

Nathalie M. J. van der Put,¹ Fons Gabreëls,² Erik M. B. Stevens,¹ Jan A. M. Smeitink,¹ Frans J. M. Trijbels,¹ Tom K. A. B. Eskes,³ Lambert P. van den Heuvel,¹ and Henk J. Blom¹

Departments of ¹Pediatrics, ²Child Neurology, and ³Obstetrics and Gynecology, University Hospital Nijmegen, Nijmegen, The Netherlands

Summary

Recently, we showed that homozygosity for the common 677(C→T) mutation in the methylenetetrahydrofolate reductase (MTHFR) gene, causing thermolability of the enzyme, is a risk factor for neural-tube defects (NTDs). We now report on another mutation in the same gene, the 1298(A→C) mutation, which changes a glutamate into an alanine residue. This mutation destroys an MboII recognition site and has an allele frequency of .33. This 1298(A→C) mutation results in decreased MTHFR activity (one-way analysis of variance [ANOVA] $P < .0001$), which is more pronounced in the homozygous than heterozygous state. Neither the homozygous nor the heterozygous state is associated with higher plasma homocysteine (Hcy) or a lower plasma folate concentration—phenomena that are evident with homozygosity for the 677(C→T) mutation. However, there appears to be an interaction between these two common mutations. When compared with heterozygosity for either the 677(C→T) or 1298(A→C) mutations, the combined heterozygosity for the 1298(A→C) and 677(C→T) mutations was associated with reduced MTHFR specific activity (ANOVA $P < .0001$), higher Hcy, and decreased plasma folate levels (ANOVA $P < .03$). Thus, combined heterozygosity for both MTHFR mutations results in similar features as observed in homozygotes for the 677(C→T) mutation. This combined heterozygosity was observed in 28% ($n = 86$) of the NTD patients compared with 20% ($n = 403$) among controls, resulting in an odds ratio of 2.04 (95% confidence interval: .9–4.7). These data suggest that the combined heterozygosity for the two MTHFR common mutations accounts for a proportion of folate-related NTDs, which is not explained by homozygosity for the 677(C→T) mutation, and can be an additional genetic risk factor for NTDs.

Introduction

Meningo(myelo)cele, encephalocele, and anencephaly are the most common severe congenital malformations. These malformations arise because of failure of closure of the neural tube and are designated as neural-tube defects (NTDs). Periconceptional folate administration reduces the occurrence, as well as the recurrence risk, of NTDs (Medical Research Council [MRC] Vitamin Study Group 1991; Czeizel and Dudas 1992). Both genetic and environmental factors, such as maternal vitamin status, have been proposed to affect the risk for NTDs (Copp et al. 1990).

Homocysteine (Hcy) is a sulphur amino acid formed by demethylation of the essential amino acid methionine, and it can be irreversibly degraded by cystathionine β -synthase (CBS). Alternatively, Hcy may be remethylated to conserve methionine, a process requiring an adequate function of several enzymes. Methionine synthase (MS) remethylates Hcy in the presence of methyl-cobalamin (Me-Cbl) as a cofactor and the cosubstrate 5-methyltetrahydrofolate (Me-THF). Production of Me-THF requires both an adequate supply of reduced folate and proper function of the enzyme methylenetetrahydrofolate reductase (MTHFR). Dysfunctional enzymes or inadequate amounts of cofactors may therefore result in elevated concentrations of Hcy.

We and others have demonstrated that elevated plasma Hcy levels are present in mothers of children with NTDs (Steeegers-Theunissen et al. 1994; Mills et al. 1995). Several studies have shown that folate levels in mothers of children with NTDs are not deficient but are in the lower range of control levels (Kirke et al. 1993; Steegers-Theunissen et al. 1994; van der Put et al. 1997*b*). Therefore, we have no indication for an overt nutritional folate shortage but rather an indication for an inadequate folate metabolism, pointing to reduced function of enzymes involved in the Hcy metabolism such as MS, MTHFR, or CBS. Complete sequencing of the coding region of MS in NTD patients with elevated Hcy levels gave no evidence for an involvement of MS in NTD (van der Put et al. 1997*c*). Also, enzymatic and molecular genetic studies on CBS did not implicate a

Received November 4, 1997; accepted for publication March 3, 1998; electronically published April 10, 1998.

Address for correspondence and reprints: Dr. Henk J. Blom, Department of Pediatrics, University Hospital Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands. E-mail: H.Blom@ckslkn.azn.nl

© 1998 by The American Society of Human Genetics. All rights reserved. 0002-9297/98/6205-0008\$02.00

major involvement of CBS in NTDs (Stegers-Theunissen et al. 1994; Ramsbottom et al. 1997). Recently, we discovered that a common genetic defect in the MTHFR gene, the 677(C→T) mutation, resulting in reduced but not abolished enzyme activity, is a genetic risk factor for spina bifida (SB) (van der Put et al. 1995, 1996b, 1997a). The 677(C→T) mutation is associated with a 2–4-fold increased risk if the NTD patient or the mother is homozygous for this mutation (Ou et al. 1995; van der Put et al. 1995, 1996b, 1997a; Whitehead et al. 1995). We observed that the risk is mostly associated with sporadic SB offspring and less with inherited SB offspring (van der Put et al. 1996a). This mutation is associated with even a sevenfold increased risk of sporadic SB offspring if both the mother and her child are homozygous for this mutation (van der Put et al. 1996a). The 677(C→T) mutation results in elevated plasma Hcy levels and lowered plasma folate levels because of a redistribution of folates and explains a substantial part of the observed elevated Hcy levels in mothers of children with SB (van der Put et al. 1995, 1996b). This risk factor is likely modulated by folate levels in the body.

In order to locate other possible genetic risk factors of NTDs, we examined, after exclusion of individuals homozygous for the 677(C→T) mutation, the Hcy and vitamin values of NTD patients and their parents. We still observed elevated Hcy and decreased plasma folate levels in NTD patients and their parents when compared with controls (van der Put et al. 1997b), which indicates the possible presence of other mutations in the MTHFR gene. Here we present a second mutation in the MTHFR gene, an A→C transition at position 1298. To investigate the impact of this mutation on the folate/Hcy metabolism and its potential role as a risk factor for NTD, we examined the prevalences of the 1298(A→C) mutation, the activities of MTHFR in isolated lymphocytes, and the Hcy and folate levels in NTD patients, their parents, and controls. The 677(C→T) mutation was included in this study because it affects the MTHFR activity and the metabolites converted by this enzyme. Therefore, we examined the effect of these common MTHFR mutations separately and in combination with each other on plasma Hcy, vitamin B12, red cell folate (RCF), and plasma folate levels of families with NTD offspring.

Patients, Materials, and Methods

Patients and Controls

Patients with NTD and their parents were recruited by the participation of the BOSK, a Dutch society for patients with CNS defects and their parents (van der Put et al. 1995, 1996b). This study group was extended by a group from the pediatric neurology department of our hospital, the University Hospital of Nijmegen. The total

study group consisted of families with mostly sporadic SB offspring: 122 mothers (mean age 46.3 ± 12.7 years); 103 fathers (mean age 47.4 ± 11.7 years); and 109 children with NTDs (mean age 20.4 ± 12.4 years).

The control group was recruited from a general practice in The Hague; all were volunteers (den Heijer et al. 1995). These individuals, aged 20–90 years, were invited to participate in a health survey of risk factors for cardiovascular disease. The 403 unrelated persons that agreed to take part were used as control group in the present study (mean age 51.2 ± 13.7 years). None of the controls suffered from NTDs nor delivered an NTD child.

The protocol of this study was approved by the local ethics committee, and a written informed consent of the NTD patients, their parents, and controls was obtained.

Mutation Detection

By complete sequencing of the coding region of the MTHFR gene (GenBank accession number U09806; http://www2.ncbi.nlm.nih.gov/irx/cgi-bin/birx_doc?genbank+20556) from severe MTHFR patients (authors' unpublished results) we observed a common mutation in this gene, an A→C change at base pair 1298, resulting in the substitution of glutamate by an alanine residue. This 1298(A→C) mutation abolishes an *Mbo*II restriction site and was confirmed by PCR on genomic DNA followed by restriction enzyme analysis with *Mbo*II. The PCR was carried out in a total volume of 50 μ l, containing 50 ng of the forward primer 5'-CTT TGG GGA GCT GAA GGA CTA CTA C and 50 ng of the reverse primer 5'-CAC TTT GTG ACC ATT CCG GTT TG, 200 μ M each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3.0 mM MgCl₂ 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), 51°C/60 s (annealing), and 72 °C/30 s (extension), and a final extension for 7 min at 72°C to ensure a complete extension of all PCR products. The amplified PCR fragment of 163 bp was digested with the restriction enzyme *Mbo*II, followed by gel electrophoresis analyses on a 20% polyacrylamide (PAA).

The 677(C→T) mutation alters an alanine into a valine residue, creating a *Hinf*I site. The presence of this mutation was analyzed according to established procedures (Frosst et al. 1995). In order to investigate if these two common mutations appear in *cis* or *trans* in the DNA, we performed an allele-specific PCR for the 677C allele on cDNA of NTD patients and examined the 1298 genotype by direct sequencing. We analyzed the cDNA of those NTD patients for whom RNA was available and were heterozygous for both MTHFR mutations (677CT/1298AC); in total, we were able to analyze 10 patients.

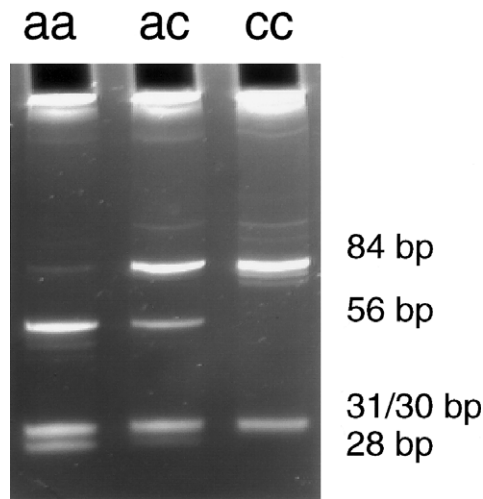


Figure 1 RFLP analysis for the 1298(A→C) mutation on a 163-bp PCR fragment with *Mbo*II. The 1298(A→C) mutation abolishes an *Mbo*II restriction site. Digestion of the 163-bp fragment of the 1298AA genotype gives five fragments, of 56, 31, 30, 28, and 18 bp, whereas the 1298CC genotype results in four fragments, of, namely, 84, 31, 30, and 18 bp. The figure depicts the three possible genotypes. The 18-bp fragment has been run off the gel.

The RNA extraction and cDNA synthesis were performed as described earlier (van der Put et al. 1997c). We used a forward primer specific for the 677C allele 5'-AGA AGG TGT CTG CGG GAG C and reverse primer 5'-CAC TTT GTG ACC ATT CCG GTT TG. The PCR was performed by a 59°C/60 s (annealing) and 72°C/90 s (extension), the other PCR parameters were not changed. The sequence analysis of this PCR fragment 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 using the forward primer 5'-CCA GGC CTC CAC TTC TAC ACC and the above-mentioned reverse primer.

Enzymatic Analysis

MTHFR activities were determined in lymphocytes, which were isolated from heparinized blood, by a radiochemical assay in its physiological reverse direction. Activities were determined after incubation at 37°C and after heat inactivation of the homogenate for 5 min at 46°C (Engbersen et al. 1995; van der Put et al. 1995) and were expressed in nmol formaldehyde/mg protein.h. In this study, we designated MTHFR activity as thermolabile in case the residual activity after heat inactivation was below 37% of the specific activity (van der Put et al. 1995). Lymphocytes for MTHFR activities

measurements were available for 134 of the controls, 55 patients, 71 mothers, and 60 fathers.

Hcy and Vitamin Analysis

Hcy concentrations were determined in EDTA plasma by high performance liquid chromatography with fluorescence detection as described earlier (TePoele-Pothoff et al. 1995). Folate and vitamin B12 levels of heparinized plasma and folate and vitamin B6 levels of red cells were determined by using the Dualcount Solid Phase Boil Radioassay (Diagnostic Products). Since the NTD patients had a lower mean age and were often taking medicine, possibly affecting the folate/Hcy metabolism, we have no appropriate control values for their Hcy and vitamin values. Therefore, their Hcy and vitamin data were not taken into account in this study. The Hcy and vitamin data of controls or parents who were taking vitamin supplements were also excluded.

Statistics

Odds ratios (Morris and Gardner 1989) and 95% confidence intervals (95% CI) were calculated to estimate the relative risk of the different genotype combinations. Results are expressed as the mean value \pm SD. One-way analysis of variance (ANOVA) was used to estimate the statistical significant differences between the mean values of the different genotypes, followed by pairwise Wilcoxon Rank Sum tests. *P* values were two tailed, and *P* < .05 was considered statistically different.

Results

Characteristics of the 1298(A→C) Mutation in the MTHFR Gene

By direct sequencing of the coding region of severely MTHFR deficient patients (authors' unpublished results), we observed a common base pair change at position 1298: an A was altered into a C leading to an amino acid substitution of a glutamate into an alanine. This 1298(A→C) mutation in the MTHFR gene abolishes an *Mbo*II restriction site and has very recently also been observed in patients with ovarian carcinomas (Viel et al. 1997). After restriction enzyme analysis of the 163-bp PCR fragment with *Mbo*II, we expected and observed the following: the 1298CC genotype results in 4 fragments, namely 84, 31, 30, and 18 bp, whereas the 1298AA genotype gives 5 fragments. The 84-bp fragment is cut in a 56 and 28-bp fragment, and thus base pair lengths of 56, 31, 30, 28, and 18 can be observed. These fragments that resulted from the digestion were screened on a 20% PAA-gel (fig. 1). The observed frequency of the C allele of the 1298(A→C) mutation is 0.33 for NTD patients, 0.28 for mothers, 0.34 for fa-

Table 1

Prevalence and Calculated Odds Ratios with 95% CI of the MTHFR Mutations Investigated by Restriction Enzyme Analysis with *HinfI* and *MbolI* in Controls, Patients with NTD, and Their Parents^a

Genotype	677CC	677CT	677TT
NTD patients:			
1298AA	1 ^b (9)	1.53 [95% CI: 0.64–3.64] (18)	1.92 [95% CI: 0.71–5.15] (10)
1298AC	1.12 [95% CI: 0.47–2.65] (17)	2.04 [95% CI: 0.89–4.70] (24)	NO ^c
1298CC	1.45 [95% CI: 0.52–4.08] (8)	NO	NO
Mothers:			
1298AA	1 ^b (13)	1.18 [95% CI: 0.54–2.55] (20)	2.38 [95% CI: 1.05–5.53] (18)
1298AC	1.14 [95% CI: 0.54–2.38] (25)	1.06 [95% CI: 0.48–2.33] (18)	NO
1298CC	0.63 [95% CI: 0.21–1.90] (6)	NO	NO
Fathers:			
1298AA	1 ^b (9)	1.70 [95% CI: 0.73–3.99] (20)	1.53 [95% CI: 0.54–4.32] (8)
1298AC	1.25 [95% CI: 0.53–2.93] (19)	1.70 [95% CI: 0.73–3.99] (20)	NO
1298CC	1.81 [95% CI: 0.68–4.86] (10)	NO	NO
Controls:			
1298AA	(62)	(81)	(36)
1298AC	(105)	(81)	NO
1298CC	(38)	NO	NO

^a The number of individuals is indicated in parentheses.

^b Reference category.

^c NO = not observed.

thers, and 0.33 for controls. Thus, we observed no increased frequency of the mutated allele in the NTD patients and their parents when compared with controls.

Prevalence of the Two Common MTHFR Mutations

The observed prevalences of 677(C→T) genotypes in combination with the 1298(A→C) genotypes in controls versus mothers, fathers, and affected children are given in table 1. The determined crude odds ratios and 95% CI showed an increased risk of 1.53–2.38 for the prevalence of the 677TT genotype in the parents and their affected children, respectively, versus controls (table 1). Comparable odds ratios were calculated for the 1298CC genotype (1.45–1.81) in the fathers and the affected children. There was no increased risk observed for this genotype in the mother. Furthermore, in NTD patients there is a trend toward an increased risk for individuals that have a 677CT/1298AC genotype (table 1).

We observed that an individual with a 677TT genotype always had a 1298AA genotype and vice versa. To investigate whether these two common MTHFR mutations appear in *cis* or *trans* we analyzed the cDNA of 10 NTD patients, who were heterozygous for both

MTHFR mutations, and performed an allele-specific PCR for the 677C allele. The amplified 677C allele was screened for the 1298 genotype by direct sequencing. We observed that the 677T and 1298C allele are always in *trans* and the 1298C never occurred on the 677C allele (data not shown). Furthermore, we studied the transfer of both mutations from the parents to their offspring and observed in four different families that the mutations had to occur in *trans*. We observed that, for example, a mother with a 677CT/1298AC genotype gave birth to a child with a 677TT/1298AA genotype. If the MTHFR mutations would have occurred in *cis* then her affected child would have been heterozygous for the 1298(A→C) mutation, which was not observed. Obviously, these two common mutations arose on different alleles, and because of their small distance on the chromosome there has never occurred a crossover thus far in the studied families and controls. Finally, we looked at the transfer of the 677T and 1298C allele from the parents to their NTD child and found a genotype frequency distribution for both MTHFR mutations among parents and patients that was as expected according to the Hardy-Weinberg equilibrium (data not shown).

MTHFR Activities

To examine the effect of this new common mutation on the MTHFR activity, we determined the activities of this enzyme in isolated lymphocytes. The measured MTHFR activities are independent of age and sex (van der Put et al. 1995); therefore, all of the data of the NTD family members and controls could be pooled. Table 2

Table 2

Relationship between MTHFR Genotype, Specific Enzyme Activity and Residual Activity Percentage in Controls and NTD Patients and Their Parents

Genotype	677CC	677CT	677TT
Specific MTHFR activity:			
1298AA	26.2 (± 6.7) ^a	17.5 (± 5.3) ^a	6.5 (± 2.6) ^a
1298AC	21.8 (± 5.1) ^a	12.5 (± 3.7) ^a	NO
1298CC	16.0 (± 4.2) ^a	NO	NO
Residual MTHFR activity (%):			
1298AA	66.0 (± 8.2)	56.1 (± 10.5) ^b	17.6 (± 14.4) ^a
1298AC	65.8 (± 8.8)	51.9 (± 9.4) ^b	NO
1298CC	61.0 (± 8.5) ^c	NO	NO

MTHFR activities were determined in lymphocytes of 320 individuals. The mean specific MTHFR activity (± SD) is given in nmol CH₂O/mg protein**h*. The residual MTHFR activity (%) is the residual activity (activity after a preincubation for 5 min by 46°C) divided by the specific activity multiplied by 100%.

^a *P* < .0001 for 677TT vs. TC or CC; 677CT vs. CC; 1298CC vs. AC or AA; and 1298AC vs. AA.

^b *P* < .003 for the 677CT vs. 677CC, irrespective of the 1298 genotype; and 677CT/1298AC vs. 677CT/1298AA.

^c *P* < .03 for the 1298CC vs. AC or AA; NO = not observed.

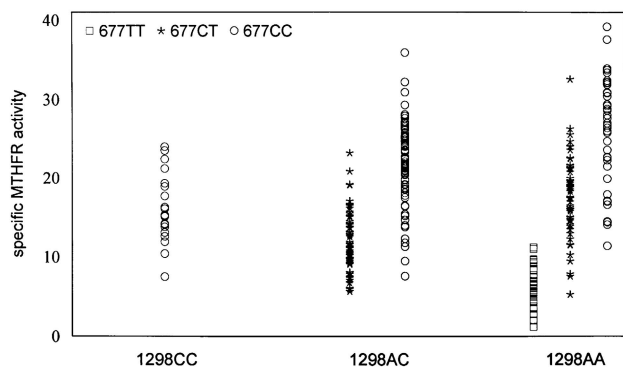


Figure 2 Specific MTHFR activities in the different observed genotype combinations for the 677(C→T) and 1298(A→C) mutations in this gene.

documents the separate and combined effects of the 677(C→T) and 1298(A→C) mutations on the MTHFR activity. The mean specific and residual MTHFR activities are significantly lower in individuals with the 677(C→T) mutation in homozygous (677TT) and even in heterozygous (677CT) state (ANOVA $P < .0001$; significant difference between all three genotypes $P < .0001$). A significant decreasing effect of the 1298(A→C) mutation on the MTHFR activity is also observed in the homozygous 1298CC as well as in the heterozygous 1298AC state (table 2). Furthermore, we observed significantly decreased activities in individuals heterozygous for both mutations, namely, 677CT/1298AC, when compared with individuals that have a 677CT/1298AA or 677CC/1298AC genotype ($P < .0001$). Figure 2 shows the correlation between phenotype (MTHFR activity) and genotype for both MTHFR mutations.

Although the residual enzyme activity of individuals with a 677CC/1298CC, 677CT/1298AA, or 677CT/1298AC genotype is significantly decreased ($P < .03$), only the 677TT genotype results in thermolability of the enzyme (table 2).

Hcy Levels

Individuals with a 677TT genotype had, as reported previously (van der Put et al. 1995, 1996b), significantly elevated plasma Hcy levels when compared with individuals with a 677CT or 677CC genotype (table 3). In our previous studies on NTD, we observed no significant differences in Hcy levels between the 677CT and 677CC genotype, probably because of the smaller sample size. The 1298(A→C) mutation on itself, when the 677 genotype was not taken into account, had no effect on the Hcy value of an individual (table 3). This study shows that combined heterozygosity for both MTHFR mutations, the 677CT/1298AC genotype, does result in significantly elevated Hcy levels ($14.2 \pm 3.1 \mu\text{mol/liter}$)

when compared with individuals that have a 677CT/1298AA genotype ($12.8 \pm 3.1 \mu\text{mol/liter}$; $P < .05$), demonstrating that the 1298(A→C) mutation can affect the Hcy concentration. There were no significant differences observed between the Hcy values of individuals that had a 677CC/1298AC or 677CC/1298CC genotype, when compared with individuals with a 677CC/1298AA genotype (table 3).

Vitamin Levels

The mean vitamin B12 or B6 levels in NTD family members were not affected by the two MTHFR mutations (data not shown). The mean RCF levels of individuals with a 677TT genotype were significantly higher ($630 \pm 192 \text{ nmol/liter}$) than those with a 677CT genotype ($526 \pm 157 \text{ nmol/liter}$) or a 677CC genotype ($530 \pm 125 \text{ nmol/liter}$) (ANOVA $P < .02$; significant difference between 677TT genotype when compared with the 677CT or the 677CC genotype $P < .001$; see table 3). If the 677 genotype was not taken into account, then the RCF did not differ between individuals with the different 1298(A→C) genotypes (table 3). However, we observed significantly higher RCF levels in individuals with a 677CT/1298AC genotype when compared with individuals with a 677CT/1298AA genotype ($P < .03$). Furthermore, RCF levels in individuals with a 677CC/1298CC genotype were significantly elevated when compared with individuals with a 677CC/1298AC or 677CC/1298AA genotype ($P < .03$) (table 3).

The mean plasma folate levels in individuals with the 677TT genotype for the 677(C→T) mutation ($11.2 \pm 4.3 \text{ nmol/liter}$) were significantly decreased in comparison with 677CT ($13.0 \pm 4.2 \text{ nmol/liter}$) and 677CC genotypes ($14.4 \pm 4.8 \text{ nmol/liter}$; ANOVA $P < .002$; significant difference between 677TT genotypes when compared with the 677CT and 677CC genotype $P < .001$; see table 3). There were no significant differences between individuals with the different 1298(A→C) genotypes if the 677 genotype was not taken into account (table 3). The plasma folate levels of individuals with a 677CT/1298AC genotype ($11.7 \pm 4.4 \text{ nmol/liter}$) were significantly decreased when compared with individuals with a 677CT/1298AA genotype ($13.3 \pm 5.8 \text{ nmol/liter}$; $P < .04$; see table 3).

Discussion

Recently, we showed that homozygosity for the common 677(C→T) mutation in the MTHFR gene is a genetic risk factor for NTD in man (van der Put et al. 1995, 1996b, 1997a), which was confirmed by other studies (Ou et al. 1995; Whitehead et al. 1995). We analyzed the MTHFR gene by sequencing the coding region and identified another common mutation in the

MTHFR gene, namely, an A→C change at position 1298. This mutation has very recently also been observed by SSCP in patients with ovarian carcinomas; however, no additional data concerning this mutation was given (Viel et al. 1997). This mutation causes a glutamate to alanine substitution in the MTHFR protein, abolishes an *Mbo*II recognition site, and has an allele frequency of 0.33. This A→C mutation at position 1298 results like the 677(C→T) mutation in a decreased MTHFR activity (ANOVA $P < .0001$), which is more pronounced in the homozygous than the heterozygous state but did not result in a thermolabile protein (table 2). The 677(C→T) lies in the catalytic domain of the protein, whereas the 1298(A→C) is located in the presumed regulatory domain.

We examined the prevalence of this mutation and the already known 677(C→T) mutation among NTD patients, their parents, and controls (table 1). We observed that homozygote subjects for either one of the MTHFR mutations always had a wild type for the other mutation. By direct sequencing of cDNA of the amplified 677C allele of NTD patients heterozygous for both MTHFR mutations, we observed that the two MTHFR mutations were never present on the same allele in the studied individuals. It is likely that the 677T and 1298A mutations evolved on different alleles and a crossover has not occurred in the studied individuals. Yet, it is possible that if both mutations would occur in *cis* this could result in a selection against these individuals because of a severe clinical phenotype. Theoretically, the effects of both mutations in *cis* on the MTHFR activity could be studied in an expression system. However, the known *Escherichia coli* expression system for the MTHFR gene does not hold the region of the 1298(A→C) mutation (Frosst et al. 1995), so we are not able to study the effects of both mutations in *cis* on the function of the MTHFR protein.

Although the 1298(A→C) mutation has a significant effect on the MTHFR activity (table 2), neither the homozygous or heterozygous states for the 1298(A→C) mutation are associated with higher Hcy or a lowered plasma folate concentration (table 3), phenomena that are evident with homozygosity for the 677(C→T) mutation (table 3). However, there appears to be an interaction between the two common mutations in the MTHFR gene. Heterozygosity for both mutations resulted in an even lower MTHFR activity than heterozygosity for either of the MTHFR mutations separately (table 2), resulting in significantly elevated Hcy and decreased plasma folate levels (table 3).

In general, the 1298(A→C) mutation influenced specific enzyme activity and Hcy and folate concentrations, but to a lesser extent than the 677(C→T) mutation. It may be expected that the 1298(A→C) mutation is also a risk factor for NTD, but with a smaller relative risk

than the 677(C→T) mutation. Indeed, the frequency of the 1298(A→C) mutation in NTD-affected children tended to be increased, which also suggests that this mutation is a genetic risk factor for this developmental anomaly (table 1). Especially under conditions of low intake of folates or during high requirements of folate, like pregnancy, this mutation could become of clinical importance. The observed odds ratios for the 1298(A→C) are not significant, possibly because of the small sample sizes in the different categories of the different genotypes. Therefore, the prevalence of this mutation needs to be determined in large groups of NTD populations.

The product of MTHFR, Me-THF, is the predominant circulatory form of folate, whereas the noncirculating forms of folate such as the substrate of MTHFR, 5,10-methylenetetrahydrofolate, are mainly inside the cell. The effect of decreased MTHFR activity on folate metabolism, because of homozygosity for the 677(C→T) mutation and heterozygosity for both mutations, results in a redistribution of the different folates that is reflected by the decreased plasma folate levels (table 3). Very recently, Molloy et al. (1997) observed decreased RCF in individuals that were homozygous for the 677(C→T) mutation. In this study, we excluded from our vitamin and Hcy analyses the data of NTD patients and individuals that were taking vitamins. Again we did not observe decreased RCF levels in individuals that are homozygous for the thermolabile mutation, but rather we observed increased RCF levels. This discrepancy may be due to the different folate assays used (Molloy et al. 1998). We applied the radioassay, whereas Molloy et al. used the microbiological assay. Different assays have different affinities for different kinds of folate metabolites.

Since the effect of homozygosity for the 677(C→T) mutation on Hcy levels can be compensated by additional folic acid intake (Kang et al. 1988; Malinow et al. 1997), it is very likely that the effect of compound heterozygosity for both common MTHFR mutations may also be overcome by folate administration. By analyzing the frequency distribution of the 677TT and 677CT/1298AC genotypes among NTD patients and their mothers, our findings provide an explanation for the protective role of folate in the etiology of NTD for maximally 36%–50% (table 1). In order to obtain high enough Me-THF levels for an adequate Hcy metabolism, individuals with a decreased MTHFR activity obviously need a higher dietary intake of folate. Several studies even pointed out that folate intake high enough to prevent NTDs cannot be achieved by a diet of folate-rich nutrition (Verhoef 1996). Only intake of folate supplements or fortified foods such as flour and cereals can achieve these daily recommended values.

In conclusion, next to the 677(C→T), mutation we identified a second common mutation in the MTHFR

Table 3**Relationship between MTHFR Genotype and the Homocysteine and Folate Levels in Parents with NTD Offspring**

	Genotype		
	677CC (<i>n</i> = 82)	677CT (<i>n</i> = 78)	677TT (<i>n</i> = 26)
Homocysteine ($\mu\text{mol/L}$)			
L	13.1 (± 3.4)	12.9 (± 3.4)	18.4 (± 8.3) ^a
Red cell folate (nmol/L)	530 (± 125)	526 (± 157)	630 (± 192) ^a
Plasma folate (nmol/L)	14.4 (± 4.8)	13.0 (± 4.2)	11.2 (± 4.3) ^a
	1298AA (<i>n</i> = 88)	1298AC (<i>n</i> = 82)	1298CC (<i>n</i> = 16)
Homocysteine ($\mu\text{mol/L}$)			
L	14.1 (± 6.6)	13.3 (± 3.7)	12.9 (± 3.0)
Red cell folate (nmol/L)	531 (± 154)	540 (± 150)	575 (± 154)
Plasma folate (nmol/L)	12.6 (± 5.5)	12.5 (± 4.5)	13.5 (± 4.2)
	677CC	677CT	677TT
Homocysteine ($\mu\text{mol/L}$):			
1298AA	12.9 (± 2.8) (<i>n</i> = 22)	12.8 (± 3.1) (<i>n</i> = 40)	19.0 (± 12.5) ^a (<i>n</i> = 26)
1298AC	13.6 (± 4.0) (<i>n</i> = 44)	14.2 (± 3.1) ^b (<i>n</i> = 38)	NO ^c
1298CC	13.9 (± 3.9) (<i>n</i> = 16)	NO	NO
Red cell folate (nmol/L):			
1298AA	493 (± 150) (<i>n</i> = 22)	483 (± 119) (<i>n</i> = 40)	628 (± 167) ^a (<i>n</i> = 26)
1298AC	527 (± 126) (<i>n</i> = 44)	553 (± 173) ^b (<i>n</i> = 38)	NO
1298CC	585 (± 143) ^d (<i>n</i> = 16)	NO	NO
Plasma folate (nmol/L):			
1298AA	14.3 (± 6.0) (<i>n</i> = 22)	13.3 (± 5.8) (<i>n</i> = 40)	10.3 (± 3.6) ^a (<i>n</i> = 26)
1298AC	13.2 (± 4.5) (<i>n</i> = 44)	11.7 (± 4.4) ^b (<i>n</i> = 38)	NO
1298CC	13.7 (± 4.2) (<i>n</i> = 16)	NO	NO

^a *P* < .05 for 677TT vs. TC or CC; 677CT vs. CC; 1298CC vs. AC or AA; and 1298AC vs. AA.

^b *P* < .05 for 677CT/1298AC vs. 677CT/1298AA.

^c NO = not observed.

^d *P* < .05 for 677CC/1298CC vs. 677CC/1298AC or 677CC/1298AA.

gene, the 1298(A→C) mutation, which, like the 677(C→T) mutation, affects enzyme activity and concentrations of Hcy and folate in the plasma and the cell. Although the observed odds ratios in patients and their parents are modest and did not reach significance, this 1298(A→C) mutation is likely a genetic risk factor for NTDs, with a lower relative risk than that of the 677(C→T) mutation. Large groups of NTD patients and their parents need to be studied to confirm our tentative conclusion. From the point of view of general health care, the observed risk factor is of great importance because of the high prevalence of homozygous and heterozygous individuals for both mutations in the general population. Homozygosity of the mothers and NTD children for the 677(C→T) mutation and heterozygosity for both MTHFR mutations can at the very most explain 36%–50% of the observed protective effect of folate. Since periconceptional folate administration reduces the risk for NTD for $\geq 72\%$ (MRC Vitamin Study Group

1991; Czeizel and Dudas 1992) there may still be other defective genes present in the folate, vitamin B12, or Hcy metabolism associated with an increased risk of NTD offspring.

Acknowledgments

We thank C. Thomas, M. Segers, S. Heil, S. Vloet, A. de Graaf-Hess, M. te Poele-Pothoff, D. Oppenraaij, H. van Lith-Zanders, E. Steegers, R. Steegers-Theunissen, M. den Heyer, and E. Mariman, for excellent support. This study was supported by grant 93-1104 from the Beatrix Fonds, The Netherlands.

References

- Copp AJ, Brook FA, Estibeiro JP, Shum ASW, Cockroft DL (1990) The embryonic development of mammalian neural tube defects. *Progr Neurobiol* 35:363–403
- Czeizel AE, Dudas I (1992) Prevention of the first occurrence

- of neural-tube defects by periconceptional vitamin supplementation. *N Engl J Med* 327:1832–1835
- den Heijer M, Blom HJ, Gerrits WBJ, Rosendaal FR, Haak HL, Wijermans PW, Bos GMJ (1995) Is hyperhomocysteinemia a risk factor for recurrent venous thrombosis? *Lancet* 345: 882–885
- Engbersen AMT, Franken DG, Boers GHJ, Stevens EMB, Trijbels FJM, Blom HJ (1995) Thermolabile 5,10-methylenetetrahydrofolate reductase as a cause of mild hyperhomocysteinemia. *Am J Hum Genet* 56:142–150
- Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJ, et al (1995) A candidate genetic risk factor for vascular disease: a common mutation methylenetetrahydrofolate reductase. *Nat Genet* 10:111–113
- Kang S-S, Zhou J, Wong PWK, Kowalysyn J, Strokosch G (1988) Intermediate homocysteinemia: a thermolabile variant of methylenetetrahydrofolate reductase. *Am J Hum Genet* 43:414–421
- Kirke PN, Molloy AM, Daly LE, Burke H, Weir DG, Scott JM (1993) Maternal plasma folate and vitamin B12 are independent risk factors for neural tube defects. *Q J Med* 86: 703–708
- Malinow MR, Nieto FJ, Kruger WD, Duell PB, Hess DL, Gluckman RA, Block PC, et al (1997) The effects of folic acid supplementation on plasma total homocysteine are modulated by multivitamin use and methylenetetrahydrofolate reductase genotypes. *Arterioscler Thromb Vasc Biol* 17:1157–1162
- Mills JL, McPartlin JM, Kirke PN, Lee YJ, Conley MR, Weir DG, Scott JM (1995) Homocysteine metabolism in pregnancies complicated by neural-tube defects. *Lancet* 345: 149–151
- Molloy AM, Daly S, Mills JL, Kirke PN, Whitehead AS, Ramsbottom D, Conley MR et al (1997) Thermolabile variant of 5,10-methylenetetrahydrofolate reductase associated with low red-cell folates: implications for folate intake recommendations. *Lancet* 349:1591–1593
- Molloy AM, Mills JL, Kirke PN, Whitehead AS, Weir DG, Scott JM (1998) Whole-blood folate values in subjects with different methylenetetrahydrofolate reductase genotypes: differences between the radioassay and microbiological assays. *Clin Chem* 44:186–188
- Morris JA, Gardner MJ (1989) Calculating confidence for relative risks, odds, and standardised ratios and rates. In: Gardner MJ, Altman DG (eds) *Statistics with confidence*. British Medical Journal, London. pp 50–63
- MRC Vitamin Study Research Group (1991) Prevention of neural tube defects: results of the medical research council vitamin study. *Lancet* 338:131–137
- Ou CY, Stevenson RE, Brown VK, Schwartz CE, Allen WP, Khoury M, Oakley GP, et al (1995) C677T homozygosity associated with thermolabile 5,10-methylenetetrahydrofolate reductase as a risk factor for neural tube defects. *Am J Hum Genet Suppl* 57:A223
- Ramsbottom D, Scott JM, Weir DG, Kirke PN, Mills JL, Gallagher PM, Whitehead AS (1997) Are common mutations of cystathionine β -synthase involved in the aetiology of neural tube defects? *Clin Genet* 51:39–42
- Stegers-Theunissen RPM, Boers GHJ, Trijbels FJM, Finkelstein JD, Blom HJ, Thomas CMG, Borm GF, et al (1994) Maternal hyperhomocysteinemia: a risk factor for neural-tube defects? *Metabolism* 43:1475–1480
- TePoele-Pothoff MTWB, VanDenBerg M, Franken DG, Boers GHJ, Jakobs C, DeKroon IFI, Eskes TKAB, et al (1995) Three different methods for the determination of total homocysteine in plasma. *Ann Clin Biochem* 32:218–220
- van der Put NMJ, Eskes TKAB, Blom HJ (1997a) Is the common 677CT mutation in the methylenetetrahydrofolate reductase gene a risk factor for neural tube defects? a meta-analysis *Q J Med* 90:111–115
- van der Put NMJ, Steegers-Theunissen RPM, Frosst P, Trijbels FJM, Eskes TKAB, van den Heuvel LP, Mariman ECM, et al (1995) Mutated methylenetetrahydrofolate reductase as a risk factor for spina bifida. *Lancet* 346:1071–1072
- van der Put NMJ, Thomas CMG, Eskes TKAB, Trijbels FJM, Steegers-Theunissen RPM, Mariman ECM, de Graaf-Hess A, et al (1997b) Altered folate and vitamin B12 metabolism in families with spina bifida offspring. *Q J Med* 90: 505–510
- van der Put NMJ, Trijbels FJM, Hol F, Eskes TKAB, Steegers-Theunissen RPM, van den Heuvel LPWJ, Mariman ECM et al (1996a) Mutated methylenetetrahydrofolate reductase in sporadic and hereditary spina bifida offspring. In: Mato JM, Caballero A (eds) *Proceedings of III Workshop on Methionine Metabolism, Molecular Mechanism and Clinical Implications*. Consejo Superior de Investigaciones Cientificas, Madrid, pp 186–191
- van der Put NMJ, van den Heuvel LP, Steegers-Theunissen RPM, Trijbels FJM, Eskes TKAB, Mariman ECM, den Heyer M, et al (1996b) Decreased methylenetetrahydrofolate reductase function due to the 677CT mutation in families with spina bifida offspring. *J Mol Med* 74:691–694
- van der Put NMJ, van der Molen EF, Kluijtmans LAJ, Heil SG, Trijbels FJM, Eskes TKAB, van Oppenraaij-Emmerzaal D, et al (1997c) Sequence analysis of the coding region of human methionine synthase: relevance to hyperhomocysteinemia in neural-tube defects and vascular disease. *Q J Med* 90:511–517
- Verhoef P (1996). Homocysteine, B-vitamins and cardiovascular disease: epidemiological evidence. PhD thesis, Wageningen Agricultural University, The Netherlands
- Viel A, Dall-Agnese L, Simone F, Canzonieri V, Capozzi E, Visentin MC, Valle R, et al et al (1997) Loss of heterozygosity at the 5,10-methylenetetrahydrofolate reductase locus in human ovarian carcinomas. *Br J Cancer* 75:1105–1110
- Whitehead AS, Gallagher P, Mills JL, Kirke PN, Burke H, Molloy AM, Weir DG, et al (1995). A genetic defect in the 5,10-methylenetetrahydrofolate reductase in neural tube defects. *Q J Med* 88:763–766