Genetic Polymorphisms in Methylenetetrahydrofolate Reductase and Methionine Synthase, Folate Levels in Red Blood Cells, and Risk of Neural Tube Defects

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Folic acid administration to women in the periconceptional period reduces the occurrence of neural tube defects (NTDs) in their offspring. A polymorphism in the gene encoding methylenetetrahydrofolate reductase (MTHFR), 677C→T, is the first genetic risk factor for NTDs in man identified at the molecular level. The gene encoding another folate-dependent enzyme, methionine synthase (MTR), has recently been cloned and a common variant, 2756A→G, has been identified. We assessed genotypes and folate status in 56 patients with spina bifida, 62 mothers of patients, 97 children without NTDs (controls), and 90 mothers of controls, to determine the impact of these factors on NTD risk. Twenty percent of cases and 18% of case mothers were homozygous for the MTHFR polymorphism, compared to 11% of controls and 11% of control mothers, indicating that the mutant genotype conferred an increased risk for NTDs. The risk was further increased if both mother and child had this genotype. The MTR polymorphism was associated with a decreased O.R. (O.R.); none of the cases and only 10% of controls were homozygous for this variant. Red blood cell (RBC) folate was lower in cases and in case mothers, compared to their respective controls. Having a RBC folate in the lowest quartile of the control distribution was associated with an O.R. of 2.56 (95% CI 1.28–5.13) for being a case and of 3.05 (95% CI 1.54–6.03) for being a case mother. The combination of homozygous mutant MTHFR genotype and RBC folate in the lowest quartile conferred an O.R. for being a NTD case of 13.43 (CI 2.49–72.33) and an O.R. for having a child with NTD of 3.28 (CI 0.84–12.85). We propose that the genetic-nutrient interaction—MTHFR polymorphism and low folate status—is associated with a greater risk for NTDs than either variable alone. Am. J. Med. Genet. 84:151–157, 1999.
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INTRODUCTION

Observational studies of the epidemiology of the NTDs, myelomeningoceoele and anencephaly, suggest that these malformations have a multifactorial genesis with both environmental and genetic factors. One environmental factor documented to be of major importance for the development of NTDs is the mother's periconceptional intake of folic acid; folic acid supplementation can significantly decrease the occurrence [Czeizel and Dudas, 1992] and recurrence [MRC Vita-

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min Study Research Group, 1991] of NTDs. The mechanism for this protective effect is not known. The first genetic risk factor for NTDs identified at the molecular level is a 677C→T (alanine to valine) polymorphism in the gene encoding the folate-metabolizing enzyme, methylenetetrahydrofolate reductase (MTHFR). Individuals who are homozygous for the mutation have a thermolabile enzyme with relatively low levels of MTHFR activity [Frosst et al., 1995]. Homozygosity for this mutation has been shown to be more prevalent both in individuals with NTD and in their parents [van der Put et al., 1995, 1997a; Whitehead et al., 1995, Ou et al., 1996], as compared to controls.

The enzyme methionine synthase (MTR; 5-methyltetrahydrofolate-homocysteine methyltransferase) catalyzes the remethylation of homocysteine to methionine. MTR is a cobalamin-dependent protein which uses 5-methyltetrahydrofolate (the product of the MTHFR reaction) as the carbon donor in methionine synthesis. Recently, the gene encoding MTR was cloned and the first mutations identified [Leclerc et al., 1996; Li et al., 1996; Chen et al., 1997]. One of the mutations, a 2756A→G substitution which changes an aspartic acid to a glycine residue, was found in the control population and represents a genetic polymorphism [Leclerc et al., 1996; Chen et al., 1997].

Low levels of folate [Smithells et al., 1976] and of the methionine synthase co-factor cobalamin [Kirke et al., 1993], and high levels of total homocysteine (tHcy), a marker of impaired folate and cobalamin metabolism [Steegers-Theunissen et al., 1994; Mills et al., 1995], have been observed in mothers of children with NTDs. The risk of having a child with NTD was found to be associated with early pregnancy red blood cell (RBC) folate levels in a continuous dose-response relationship [Daly et al., 1995]. RBC folate is assumed to be a better measure of folate deficiency than serum folate [Herbert, 1989], since it represents a marker of longer term folate status.

Although the influence of genetic and environmental factors on NTD risk has been evaluated separately, it is likely that these factors may interact in certain situations. The homozygous mutant genotype for MTHFR was not associated with hyperhomocysteinemia when plasma folate was above the median value; the genotype was associated with elevated homocysteine levels only when plasma folate levels were low [Jacques et al., 1996; Christensen et al., 1997]. In this regard, it has been proposed that the protective effect of folate may be due to folate-dependent stabilization of the enzyme [Rozen, 1997].

The genetic-nutrient interactive effect, rather than the genotype alone, has been proposed to increase the risk for mild hyperhomocysteinemia and vascular disease [Jacques et al., 1996]. This interactive effect has not been examined with respect to NTD risk. The aim of this study was to examine the genetic and environmental influences on the homocysteine remethylation pathway and their contributions to NTD risk in children with NTDs and in their mothers, compared to control mother-child pairs, and to assess the potential interaction between common genotypes and folate status in the determination of risk.

SUBJECTS AND METHODS

Subjects

All patients were recruited from the Montreal Children's Hospital after approval of the protocol by the Institutional Review Board. The NTD patients and mothers of patients were recruited from the ambulatory Spina Bifida Clinic. The controls were other outpatients who were having a venipuncture at the Pediatric Test Centre, Montreal Children's Hospital, and who were with their mothers. Blood samples were obtained after appropriate consent. Exclusion criteria were syndromic NTD cases, severe anemia, neoplastic disease, renal insufficiency, and immunosuppressive therapy. Individuals who were taking vitamin supplements ($n = 14$: one control, six control mothers, and seven case mothers) were also excluded.

Determination of Folate and Cobalamin

The concentrations of serum folate (S-folate), RBC-folate, and serum cobalamin (S-cobalamin) were quantitated by routine methods, using an automated system and reagents from Ciba (Ciba Corning Diagnostics Corp., Medfield, MA).

Determination of Total Homocysteine (tHcy) in Plasma

Blood samples were drawn in Becton Dickinson Vacutainers containing sodium EDTA and kept on ice until plasma was separated. Plasma was separated by centrifugation for 5 min, removed, and centrifuged again; the supernatant was collected and frozen at −20°C until analysis. tHcy in plasma was determined by high-pressure liquid chromatography as reported [Gilfix et al., 1997]. The tHcy adduct was detected by fluorescence after precolumn derivatization with the thiol-specific reagent 7-fluoro-benzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F) (Wako, TX).

Determination of MTHFR Activity

MTHFR activity was determined in mononuclear peripheral white blood cells from a subset of 45 cases, 47 case mothers, 51 controls, and 42 control mothers. The cells had been isolated in sterile vacutainers (Vacutainer® CPT Cell Preparation Tube with sodium citrate from Becton Dickinson and Company, Franklin Lakes, NJ) containing a density gradient. The cell pellets were stored at −70°C until analysis and the cells were lysed by freeze-thawing 3 times; 90 μg protein was used per assay. Enzyme activity was determined in the reverse direction by previously described protocols [Christensen et al., 1997]. Protein was determined with the Bio-Rad Protein Assay from Bio-Rad Laboratories (Hercules, CA).

Genetic Analyses

DNA was isolated from peripheral leukocytes by extraction with phenol-chloroform after cell lysis in a buffer containing Nonidet-P40 (Boehringer Mannheim, Mannheim, Germany) and stored at −20°C. The presence of the polymorphism in MTHFR was determined by polymerase chain reaction (PCR) followed by restric-

tion digestion with HinfI, as described [Frosst et al., 1995]. The polymorphism in MTR was analyzed by PCR and restriction digestion with HaeIII [Leclerc et al., 1996].

Statistical Analyses

Computer-assisted statistical analyses were carried out with SPSS for Windows. Standard summary statistics, analysis of variance, *t*-tests and calculation of O.R.'s with associated confidence limits were used where appropriate. Statistical significance was interpreted as values of $P < 0.05$.

RESULTS

Subjects

Fifty-six cases and 62 mothers of patients were included for investigation. All cases were isolated spina bifida with most at levels T10–S1. Ninety-seven controls and 90 mothers of controls participated. We were not able to recruit both the mother and child in all cases and controls (n = 50 case mother-child pairs and n = 88 control mother-child pairs). Based on inspection of surnames, case and control families appeared to have similar ethnic backgrounds (approximately 75% English or French Canadians and 25% others).

The mean ages were similar between cases and controls (11 and 10 years, respectively), as well as between case mothers and control mothers (36 and 37 years, respectively). The ranges in ages of the children and mothers were 1–19 years and 22–55 years, respectively. The miscarriage rates in case and control mothers were within the range quoted for the general population (10%–15%). Family history was noncontributory.

Genotypes

Twenty percent (11/56) of cases and 18% (11/62) of case mothers were homozygous for the 677C→T polymorphism in MTHFR, compared to 11% of controls (11/97) and 11% of control mothers (10/90) (Table I). The increased risk for being a case (O.R. = 2.2; 95% CI 0.82–5.99) or a case mother (O.R. 2.0; CI = 0.75–5.43) with this genotype was similar to that reported previously [van der Put et al., 1995, 1997a; Whitehead et al., 1995], although statistical significance was not achieved in this study.

Since the folate status of the fetus is likely to depend on both maternal and fetal influences, we assessed the MTHFR genotypes in mother-child pairs for determination of NTD risk. Fifty mother-child pairs of NTD cases and 88 pairs of mother-child controls were available from the study group. The O.R. for being a NTD pair was significantly increased to 6.0 (95% CI 1.26–28.53) if mother and child were homozygous for the polymorphism in MTHFR (Table II). This value suggests that a combined maternal and fetal mutant genotype may be a greater risk factor than either genotype alone. The risks conferred by maternal or fetal genotype alone were similar (O.R. = 2.0 or 2.2, as indicated above).

TABLE I. Prevalence of the 677C→T (A→V) Genotype in the MTHFR Gene in Case Versus Control Children and Mothers*

	A/A n (%)	A/V n (%)	V/V n (%)	% V Alleles
Cases	19/56 (34)	26/56 (46)	11/56 (20)	43
Controls	42/97 (43)	44/97 (45)	11/97 (11)	34
Case mothers	24/62 (39)	27/62 (44)	11/62 (18)	40
Control mothers	44/90 (49)	36/90 (40)	10/90 (11)	31

*O.R. for children, V/V vs. A/A = 2.2 (95% CI 0.82–5.99); O.R. for mothers, V/V vs. A/A = 2.0 (95% CI 0.75–5.43).

For the 2756A→G polymorphism in MTR, none of the cases (0/55) and 10% of controls (10/97) were homozygous (Table III). This observation results in an O.R. of 0.0 (95% CI 0.0–0.75), suggesting a reduced risk for being a NTD case with the homozygous G/G genotype. The frequencies of the three genotypes for the MTR variant were similar in case and control mothers.

Biochemical Parameters

Tables IV and V show the nutrient (S-folate, RBC folate, and S-cobalamin) and total homocysteine levels in the study group. Significantly lower values for RBC folate were found for both the cases and their mothers ($P < 0.01$), compared to controls and control mothers, respectively. The case mothers also had significantly lower serum folate ($P < 0.05$) and lower serum cobalamin ($P = 0.05$).

Mean levels of MTHFR activity in peripheral mononuclear cells are also listed in Tables IV and V. Although the mean values in cases and controls, and in their mothers, were similar, MTHFR activity differed significantly between individuals with the wild type, heterozygous, or homozygous mutant genotype for the 677C→T polymorphism (data not shown), as previously reported in studies of patients with spina bifida or vascular disease [van der Put et al., 1995; Kluijtmans et al., 1996; Christensen et al., 1997].

RBC folate levels were stratified by MTHFR genotype to determine if the genotype influenced erythrocyte folate; the values did not differ by MTHFR genotype (Table VI). Since only one control mother and one case mother were homozygous for the methionine synthase variant, the lower RBC folate levels in the case mothers cannot be due to the presence of this polymorphism. There was no significant association between the methionine synthase polymorphism and RBC folate (Table VII) or between this polymorphism and plasma homocysteine, serum folate, and serum cobalamin (Table VIII). The small numbers of homozygous mutant individuals limited the statistical power of these analyses.

Having a RBC folate value in the lowest quartile of the control distribution was associated with an O.R. of 2.56 (CI 1.28–5.13) for cases and 3.05 (CI 1.54–6.03) for case mothers (Fig. 1). A correlation was observed between the RBC folate values for mothers and their children (control pairs, r = 0.442, $P < 0.01$; case pairs, r = 0.256, $P = 0.08$) but the correlation did not reach statistical significance in the case pairs.

The MTHFR homozygous mutant genotype was associated with higher homocysteine levels in the mothers (Table IX) but not in the children. The lack of a

TABLE II. Odds Ratios With 95% Confidence Intervals for Being a NTD Family in the Presence of Different MTHFR Genotype Combinations of Mother and Child*

Genotype		Cases/case mothers		Controls/control mothers			
Child	Mother	n	%	n	%	Odds ratio	95% CI
V/V	V/V	6	12.0	3	3.4	6.00	1.26–28.53
V/V	A/V	4	8.0	6	6.8	2.00	0.47–11.54
A/V	V/V	3	6.0	7	8.0	1.29	0.27–9.62
A/V	A/V	14	28.0	18	20.5	2.33	0.86–10.88
A/V	A/A	8	16.0	13	14.8	1.85	0.59–10.10
A/A	A/V	5	10.0	11	12.5	1.36	0.38–9.28
A/A	A/A	10	20.0	30	34.1	1.00	—

*The reference group was the mother-child pairs with the A/A genotype. The study group included 50 pairs of case mothers and their children (cases), and 88 pairs of control mothers and their children (controls).

significant effect on homocysteine in children may relate to the fact that homocysteine levels are age dependent [Vilaseca et al., 1997]. Although the mean ages of case and control children were similar, the wide ranges in each group precluded comparisons at different ages.

Genotype-Nutrient Interaction

The combination of having the homozygous mutant MTHFR genotype and RBC folate in the lowest quartile conferred a significantly increased risk of being a NTD case, compared to the risk of either variable alone (Fig. 1). The O.R. for the combined variables was 13.43 (CI 2.49–72.33) for cases and 3.28 (CI 0.84–12.85) for mothers. If the low RBC folate in the cases and mothers had been due to the mutant genotype, the O.R. would not have increased when both factors were examined together. In the case and control mothers with the mutant genotype and low folate, the mean homocysteine levels were 13.9 μM, and 22.5 μM, respectively, higher than the mean values based on the homozygous mutant genotype alone (12.5 μM and 13.6 μM, respectively) (Table IX). Multiple logistic regression analysis was also performed to estimate the genetic and environmental effects independently on NTD risk. The O.R.'s for NTD risk based on genotype (adjusted for low RBC folate) were 1.95 (CI 0.67–5.64) for the children and 2.44 (CI 0.83–7.18) for the mothers. The O.R.,s for low RBC folate (adjusted for genotype) were 2.46 (CI 1.19–5.09) for the children and 2.69 (CI 1.3–5.5) for the mothers. Although our analyses were occasionally limited by the small numbers, our observations suggest independent and possibly synergistic effects between mutant MTHFR genotype and low RBC folate in increasing NTD risk.

TABLE III. Prevalence of the 2756A→G (D→G) Genotype in the MTR (Methionine Synthase) Gene in Case Versus Control Children and Mothers*

	D/D		D/G		G/G		
	n	(%)	n	(%)	n	(%)	% G Alleles
Cases	38/55	(69)	17/55	(31)	0/55	(0)	15
Controls	59/97	(61)	28/97	(29)	10/97	(10)	25
Case mothers	40/61	(66)	20/61	(33)	1/61	(2)	18
Control mothers	55/90	(61)	34/90	(38)	1/90	(1)	20

*O.R. for children, G/G vs. D/D = 0.0 (95% CI 0.0–0.75); O.R. for mothers, G/G vs. D/D = 1.4 (95% CI 0.08–22.66).

DISCUSSION

Our results confirm other reports of an increased prevalence of homozygosity for the 677C→T polymorphism in MTHFR among NTD patients and their mothers [van der Put et al., 1995; Whitehead et al., 1995; Ou et al., 1996]. The O.R. of approximately 2 for the child or the mother is in the same range as the risks previously reported. The risk for NTD was higher if both mother and child were homozygous for the mutation than if only one of them was homozygous. The O.R. in our study, for NTD risk when both mother and child were homozygous for MTHFR, was 6.0, similar to the value obtained in an earlier report (O.R. = 6.1; [van der Put et al., 1996]). Our study differs in the choice of reference group. Our controls were mother-child pairs obtained from the same institution, rather than adult controls from the general population. The frequencies from these adult controls had been used to calculate the expected genotype frequencies for control mother-child pairs [van der Put et al., 1996].

The homozygous mutant genotype for the 2756A→G polymorphism in MTR was associated with an apparent reduced risk for NTD in children. There were no case children with this genotype, compared to 10% of control children. This contrasts with two previous publications concerning NTD risk and this polymorphism [van der Put et al., 1997b; Morrison et al., 1997], where significant effects on risk were not observed. In the first study [van der Put et al., 1997a], the O.R. for the NTD patients (0.5) was in fact in the direction of a reduced risk although this value was not significant. In both previous studies [van der Put et al., 1997a; Mor-

TABLE IV. Characteristics of Cases and Control Children[a]

	Cases	n	Controls	n
S-folate (nmol/L)	17.6 ± 7.3	55	19.4 ± 9.9	94
RBC folate (nmol/L)	577 ± 256	56	726 ± 327*	95
S-Cobalamin (pmol/L)	487 ± 250	55	535 ± 339	95
tHcy (μmol/L)	8.5 ± 2.9	56	7.9 ± 3.2	97
MTHFR activity (nmol/hr/mg)	32.3 ± 15.8	45	35.3 ± 14.3	51

[a]Values shown are means ± standard deviation.
*$P < 0.01$.

TABLE V. Characteristics of Case Mothers and Control Mothers[a]

	Case mothers	n	Control mothers	n
S-Folate (nmol/L)	14.1 ± 7.9	60	17.0 ± 9.6**	90
RBC folate (nmol/L)	527 ± 236	59	694 ± 308*	90
S-Cobalamin (pmol/L)	298 ± 186	59	350 ± 135***	88
tHcy (μmol/L)	10.0 ± 3.5	61	9.9 ± 4.2	90
MTHFR activity (nmol/hr/mg)	34.6 ± 19.9	47	35.6 ± 15.8	42

[a]Values shown are means ± standard deviation.
*$P < 0.01$.
**$P < 0.05$.
***$P = 0.05$.

rison et al., 1997], however, the controls were again adult population-based controls. In a larger study of infants with spina bifida, using matched infant controls, from the California Birth Defects Monitoring Program, we also observed a decreased O.R. for the homozygous mutant genotype in methionine synthase (O.R. = 0.23; 95% CI = 0.05–1.9; [Shaw et al., 1998]). However, this was also based on a small number of cases with this genotype (1/95 cases compared to 7/160 controls). The discrepancies between various studies could relate to the low frequency of the variant, as well as to the choice of controls.

In addition to the effect of genetic polymorphisms in modifying NTD risk, we assessed the levels of serum and RBC folate in the children and their mothers, since folate responsiveness is an important aspect of this birth defect. RBC folate is generally considered to be a more useful indicator of folate status than serum folate, since it reflects levels of intracellular folate and folate turnover during the past 120 days [Herbert, 1989]. RBC folate is probably influenced by nutritional as well as by genetic factors [Mitchell et al., 1997]. In contrast with the numerous studies of serum folate in mothers of NTD cases, relatively few studies of RBC folate levels in mothers or cases with NTD have been performed. In a study of women who had had two NTD pregnancies, no difference was seen in serum or RBC folate among women with different pregnancy outcomes, but an altered relation to dietary intake of folate was observed between the groups [Yates et al., 1987]. In another study, the finding of a negative correlation between the RBC folate in mothers and the number of infants affected with NTD could not be adequately accounted for by the dietary intake [Wild et al., 1994]. Daly et al. [1995] have suggested that maternal RBC folate in early pregnancy is a marker of NTD risk, in a continuous dose-response relationship. In this study, we found decreased levels of RBC folate

TABLE VI. RBC Folate Levels (nmol/L) in Different MTHFR Genotypes*

	A/A	n	A/V	n	V/V	n
Cases	560 ± 276	19	611 ± 270	26	526 ± 185	11
Controls	675 ± 278	41	749 ± 331	43	836 ± 466	11
Case mothers	575 ± 231	22	489 ± 248	26	520 ± 224	11
Control mothers	708 ± 350	44	700 ± 288	36	612 ± 154	10

*Values shown are mean ± standard deviation.

TABLE VII. RBC Folate Levels (nmol/L) in Different Methionine Synthase Genotypes*

	D/D	n	D/G	n	G/G	n
Cases	569 ± 214	38	611 ± 336	17	—	0
Controls	735 ± 335	58	748 ± 363	27	623 ± 139	10
Case mothers	498 ± 200	39	610 ± 295	18	239	1
Control mothers	713 ± 330	55	666 ± 276	34	568	1

*Values shown are mean ± standard deviation.

in NTD cases and in case mothers, compared to their respective controls. This observation was seen approximately 10 years after the birth of the NTD child, consistent with a strong genetic influence on RBC folate levels, as hypothesized previously [Mitchell et al., 1997]. The correlation in RBC folate between mother and child is also consistent with a genetic determinant. On the other hand, similar dietary patterns over long periods of time could also explain these findings.

Individuals who were homozygous for the MTHFR polymorphism did not have a lower mean level of RBC folate. Although one study recently reported lower RBC folate levels due to this genetic variant [Molloy et al., 1997], our results are consistent with other reports [van der Put et al., 1995; Molloy et al., 1998a; Bagley and Selhub, 1998] which did not show a decrease in mutant individuals. A recent study has shown that the mutant MTHFR genotype results in an altered distribution of RBC folates, but total folate is not affected [Bagley and Selhub, 1998]. The polymorphism in methionine synthase was not common enough in the mothers to influence the mean RBC folate in this group. Consequently, we suggest that the decreased levels of RBC folate are largely due to other factors,

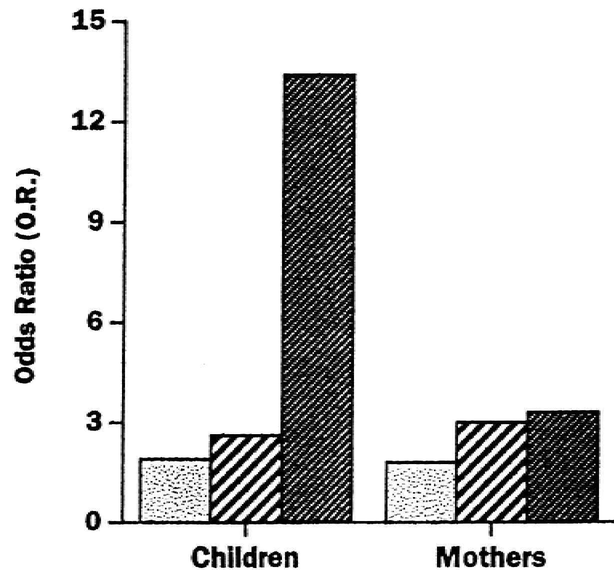

Fig. 1. Interaction between MTHFR genotype and low RBC folate. The figure shows the O.R.'s for having a neural tube defect (children) or for having a child with a neural tube defect (mothers) for individuals who are homozygous for the 677C→T polymorphism in MTHFR (shaded bar), who have RBC folate in the lowest quartile (bar with thick stripes), and for individuals who are homozygous for the 677 C→T polymorphism in MTHFR and have RBC folate in the lowest quartile (bar with thin stripes). The reference groups were, respectively: individuals without the mutant genotype, individuals in the other three quartiles for RBC folate, or individuals without the combination.

TABLE VIII. Homocysteine (μM), Serum Folate (nmol/L) and Serum Cobalamin (pmol/L) in Different Methionine Synthase Genotypes*

	D/D	n	D/G	n	G/G	n
Homocysteine						
Cases	8.5 ± 2.9	38	8.0 ± 2.9	17	—	0
Controls	8.0 ± 3.5	59	7.7 ± 3.0	28	8.3 ± 1.9	10
Case mothers	10.7 ± 4.0	39	8.8 ± 2.3	20	8.1	1
Control mothers	10.3 ± 4.7	55	9.4 ± 3.3	34	7.6	1
Serum folate						
Cases	17 ± 6	37	19 ± 9	17	—	0
Controls	20 ± 11	58	18 ± 10	26	16 ± 4	10
Case mothers	14 ± 8	40	14 ± 7	18	12	1
Control mothers	16 ± 9	55	19 ± 11	34	11	1
Serum cobalamin						
Cases	462 ± 232	37	564 ± 270	17	—	0
Controls	520 ± 240	58	582 ± 507	28	489 ± 247	9
Case mothers	275 ± 112	39	359 ± 288	18	169	1
Control mothers	328 ± 118	53	375 ± 145	34	700	1

*Values shown are mean ± standard deviation.

such as decreased dietary folate intake or a different genetic determinant. Our results for mothers are consistent with those of Molloy et al. [1998b] who reported that maternal folate status may be a stronger risk factor than the MTHFR genotype.

Several studies on the MTHFR variant have reported an interaction between the 677C→T polymorphism and plasma/serum folate in the development of hyperhomocysteinemia, a risk factor for vascular disease [Jacques et al., 1996; Harmon et al., 1996; Christensen et al., 1997], but this interaction has not been examined in NTDs. In this report, we evaluated the interaction between the variant and folate status in a study of NTD risk. The results of our study suggest that the combination of mutant genotype and folate status may be a greater risk factor than either variable alone, suggesting a genetic-nutrient interactive effect. However, it is important to note that the nutritional status at the time of the study may not reflect nutrition during organogenesis.

The mechanism by which the genotype or folate status increases NTD risk is not clear. Hyperhomocysteinemia, a marker for both the mutant genotype and low folate, was clearly seen in the mothers with the MTHFR mutant genotype. The mean homocysteine level was higher in the mothers who had the mutant genotype in combination with a RBC folate level in the lowest quartile, compared to the mothers with only the mutant genotype. Our finding of normal homocysteine levels in NTD cases, even when stratified by genotype, is in keeping with other reports [Graf et al., 1996; van der Put et al., 1997a]. Differences in homocysteine levels among cases and control children may be difficult to detect since homocysteine levels are age dependent [Vilaseca et al., 1997]. Although homocysteine itself may be teratogenic [Rosenquist et al., 1996], other possibilities include deficient synthesis of methionine or S-adenosylmethionine [Essien and Wannberg, 1993], an important component in many methylation reactions.

TABLE IX. Mean Levels of tHcy in Plasma (μM) in Case Versus Control Children and Mothers

	A/A (n)	A/V (n)	V/V (n)	V/V + RBC folate in lowest quartile (n)
Cases	7.8 (19)	8.9 (26)	8.6 (11)	8.0 (7)
Controls	7.4 (42)	8.0 (44)	9.7 (11)	24.4* (1)
Case mothers	8.6 (23)	10.1 (27)	12.5* (11)	13.9* (6)
Control mothers	9.1 (44)	10.0 (36)	13.6* (10)	22.5* (2)

*$P < 0.01$ compared to A/A genotype.

Most of the NTD risk studies examining MTHFR or methionine synthase variants have involved living children and, therefore, intrauterine loss of mutant fetuses cannot be ruled out. In light of reports showing an association of the MTHFR genotype with recurrent early pregnancy loss [Nelen et al., 1997], differences in fertility related to MTHFR genotypes [Weitkamp et al., 1998], and decreased numbers of female newborns with the mutant MTHFR genotype [Rozen et al., 1999], large prospective studies with assessment of viable and nonviable fetuses are required to address the issue of genetic variation and NTD risk.

In summary, our results suggest that the MTHFR mutant genotype and/or low RBC folate in children and in their mothers increase the risk for giving birth to an NTD offspring. A combination of these two variables increases the risk even further, particularly for being an NTD case. Additional studies on methionine synthase variants are clearly warranted, since the low frequency of this variant has precluded an unequivocal assessment of NTD risk.

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