Original Article

Low maternal folate concentrations and maternal MTHFR C677T polymorphism are associated with an increased risk for neural tube defects in offspring: a case-control study among Pakistani case and control mothers

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Background and Objectives: There is considerable evidence that periconceptional maternal folate deficiency and coding variants in maternal genes coding for critical enzymes in the folate pathway are associated with neural tube defects (NTDs) in offspring. In a case-control study we investigated C677T polymorphism in the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene in case and control mothers of Pakistani origin, and compared these with the respective maternal folate concentrations measured at the time of delivery. **Methods and Study Design:** A case-control study was conducted among 109 case and 100 control mothers identified through the Holy Family Hospital Rawalpindi, Quaid-i-Azam University, Islamabad, Pakistan. Red blood cell (RBC) and serum folate concentrations and MTHFRC677T polymorphism were compared between case and control mothers. **Results:** Mean RBC folate and serum folate concentrations were significantly lower in cases compared with control mothers ($p<0.0001$). Maternal MTHFR 677CT and 677TT genotypes were more common among cases compared with control mothers (CC vs TT $p<0.0393$ and CC/CT vs TT $p<0.021$). T-allele frequency was higher in cases compared with control mothers (C vs T $p<0.017$). Case mothers with 677CT or 677TT genotypes had significantly lower serum ($p<0.0001$) and RBC folate concentrations ($p<0.0001$) compared with control mothers. **Conclusions:** The present study provides further evidence that maternal folate deficiency and MTHFRC677T polymorphism might be associated with an increased risk for NTDs in offspring. Our results are limited by the fact that maternal folate concentrations were not obtained during the periconceptional period, but at delivery. Further analyses, including maternal folate levels during the periconceptional period, are warranted.

Key Words: folate, blood folate concentrations, neural tube defects, MTHFR, case-control study

INTRODUCTION

Neural tube defects (NTDs) are congenital malformations of the central nervous system, resulting from failure of the neural tube to close during early embryogenesis.[1] NTDs are associated with life-long neurologic, cognitive, urologic, and gastrointestinal co-morbidities. Especially varying degrees of limb paralysis and urinary and bowel incontinence constitute severe functional impairments.[2,3] Sub-phenotypes of NTDs comprise spina bifida, anencephaly and encephaloceles.[4,5]

NTDs have a complex etiology with involvement of genetic and environmental factors.[5-7] Besides a lack of folate substitution during the periconceptional period,[8-12] the development of NTDs has been associated with lower

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socio-economic status,[13] maternal diabetes,[14] maternal hyperthermia,[15,16] maternal obesity,[17,18] maternal age, maternal alcohol abuse, excessive maternal use of Vitamin A, maternal exposure to lead, and a high intake of tea during the first trimester of pregnancy.[19,20] Furthermore, a history of abortion and higher parity have been implicated as risk factors.[21]

So far the only preventive treatment known constitutes the enrichment of maternal diets with folate during the periconceptional period.[9-12] The Medical Research Council [MRC] Vitamin Study Group concluded that a daily dosage of 4,000 mcg of folic acid during the periconceptional period is required, to considerably reduce the risk of NTDs in offspring.[8]

Besides maternal folate deficiency during the periconceptional period, deficient folate conversion into an active coenzyme might confer an additional risk factor. Methylenetetrahydrofolate reductase (MTHFR) is an important enzyme in folate metabolism. Its position in the folate pathway regulates the distribution of one-carbon units in nucleotide synthesis and other methylation reactions in the cell. MTHFR catalyses the conversion of 5,10-methylenetetrahydrofolate, a carbon donor for nucleotide synthesis, to 5-methylenetetrahydrofolate by using NAD(P)H as a reducing agent.[22] 5-methylenetetrahydrofolate is essential for the re-methylation of homocysteine to methionine with generation of the universal methyl donor, S–adenosyl-L-methionine. In the *MTHFR* gene, several single nucleotide polymorphisms have been characterized, but the most widely studied is the *C677T* polymorphism.[23,24] In exon 4, the C>T transition at nucleotide 677 leads to an amino acid substitution of alanine to valine (c.C677T, p.Ala222Val, rs1801133). The nucleotide transition creates a Hinf I site, which allows for screening of this polymorphism by restriction analysis of polymerase chain reaction amplified products (PCR-RFLP).[25,26] The p.Ala222Val polymorphism results in a reduction of enzyme activity with decreased folate concentrations in serum, plasma, and red blood cells, and an increase in plasma homocysteine levels.[27]

Low concentrations of folate, together with the *MTHFR* T-allele, have been associated with higher concentrations of homocysteine. This association between the *MTHFR* polymorphism and folate concentrations is the hypothesized link between the *C677T* polymorphism and NTDs.[28,29] Homozygosity for the T-allele (*677TT*) has been associated with a 7.2 fold increase in the risk for NTDs.[29-37] Previous studies have suggested that not only the fetal genotype, but also the maternal genotype, might have an impact on fetal development.[33,38,39] Based on these studies, we carried out a case-control study of case and control mothers of Pakistani origin. We genotyped the *C677TMTHFR* polymorphism by PCR-RFLP in case and control mothers, and compared these results with the folate blood concentrations measured in both cohorts at the time of delivery.

MATERIALS AND METHODS

Subjects

This study was conducted at Holy Family Hospital Rawalpindi, Quaid-i-Azam University, Islamabad, Pakistan and the Institute of Human Genetics, Life and Brain-Center, University of Bonn, Germany. Holy Family Hospital is a tertiary care teaching hospital of Rawalpindi Medical College, Rawalpindi, Pakistan. The study was approved by the institutional ethic committee of the Quaid-i-Azam University, Faculty of Biological Sciences, Department of Animal Sciences, Islamabad.Pakistan (Ref No: DAS-2007/13) and was conducted according to the principles of the Declaration of Helsinki. Written informed consent was obtained from all subjects.

Case mothers

Case mothers were identified at the Department of Gynecology and Obstetrics, Holy Family Hospital. Case mothers with prenatal diagnosis of fetal NTD on prenatal ultrasound or after delivery of newborns with NTDs were included. The latter were referred from nearby rural areas or small towns, where health facilities are either not available or cannot manage complicated pregnancies. NTDs included anencephaly, encephalocele and spina bifida. The latter comprised meningocele, myelomeningocele and spina bifida aperta. All phenotypes were re-assessed by pediatricians or neurosurgeons. Altogether, we were able to include 109 case mothers.

Control mothers

For the control group we included women who had delivered a healthy newborn at the Department of Gynecology and Obstetrics at the Rawalpindi Medical College. Altogether, we were able to include 100 control mothers.

Demographic data

This included the residential area of case and control mothers, whether from rural or urban areas. Maternal age was recorded. History was taken regarding smoking habits or partner's smoking habits (or anybody else smoking in the household). Breathing in "second-hand smoke" or "passive smoke" is equally hazardous as active smoking.[40] The educational status of the mothers was recorded and categorized as those who did not receive school education, or received college or university education. Economic status was based on the monthly income of the husband and was divided into four classes: (i) between Rs 5,000-10,000, (ii) between Rs 11,000-15,000, (iii) between Rs 16,000-20,000 and (iv) >20,000. Holy Family Hospital is a public sector hospital and the majority of patients seeking medical treatment fall in the lower socio-economic class, with no schooling or poor educational status'. Nutritional status was assessed by a semi-quantitative food intake questionnaire on the average intake of fresh fruits/nuts, eggs, milk and vegetables. Mothers were divided in two groups. Those who had an adequate intake of fruits and vegetables per week, and those whose diet was deficient in fruits and vegetables. The diet history questionnaire from Laurence et al[41] was used in a modified form. Meat, except liver, does not contain folic acid, and hence was not included for assessment of folate rich foods. A lack of knowledge and low awareness of the importance of folate were the two most common reasons reported for not consuming a folate rich diet, especially in the periconceptional period.

Collection of blood samples

Blood samples were collected after delivery, following informed consent from the subjects. Blood samples were collected in the morning following an 8 hour fasting period, to obtain basal folate concentrations. For serum and red blood cell (RBC) folate analysis, blood samples were collected aseptically in k3EDTA-Vacutainer tubes and red cap serum separator tubes (Becton Dickinson, Franklin Lakes, and NJ), and were centrifuged within 1 hour of collection. 10 mL blood was drawn from the anti-cubital vein and divided into three tubes: 5 mL was placed in EDTA (ethylenediaminetetraacetic acid) lined tubes for DNA analysis, 2.5 mL was placed in another EDTA lined tube for RBC folate analysis, and 2.5 mL was placed in a red cap serum separator tube for serum folate estimation and kept at -20ºC until analysis.

Serum specimens

After complete clot formation, each sample was centrifuged (centrifuge 3000/Rev/min; Rotofix 32 A, Hettich Lab Technology, Germany). Serum was separated from the clots within 24 hours after blood drawing. Serum specimens were stored at -20°C until testing.

Whole blood specimens

Blood samples for DNA analysis were permanently stored at -20ºC prior to analysis. Whole blood specimens collected in tri potassium EDTA tubes for RBC folate analysis were stored at -20°C until testing. A complete blood picture was performed on a Sysmex XP-300™ Automated Hematology Analyzer for hemoglobin and hematocrit before storage. RBC folate and serum folate analysis was carried out on an immunoassay analyzer (AxSYM, Abbot Laboratories, Abbot Park, Ill), according to the manufacturer's protocol (Abbot Diagnostic Division, 2010).

DNA extraction

DNA extractions were performed from whole blood by using magnetic bead technology with the Chemagic Magnetic Separation Module I and the Chemagic DNA Kit (Chemagen, Baesweiler, Germany), according to manufacturers' protocol.

PCR-reaction and conditions

To analyze the *MTHFR* polymorphism *C677T*, amplification of exon four using PCR was carried out using standard conditions and modified primers (4F:5'-TCTTCATCCCTCGCCTTGAAC-3'; 4R:5'-AGGACGG TGCGGTGAGAGTG-3'). The *MTHFR* polymorphism was determined by enzymatic digestion of the initial PCR product with HinfI enzyme (Thermoscientific, MA; USA) using standard conditions.

Genotyping of the MTHFR C677T polymorphism by PCR-RFLP

MTHFRC677T polymorphism analysis was carried out using PCR and Hinf I as previously described.[25] The RFLP digestion products were run on agarose gel electrophoresis to identify whether an individual was homozygous for the major allele, heterozygous or homozygous for the minor allele.

Statistical analysis

Differences in the genotype and allele frequencies of the *C677T* polymorphism were assessed by χ^2-analysis. Student's t-test was used to compare means, and the Pearson chi-square test was used to compare categorical variables. Analysis of covariance was conducted to compare means adjusted for appropriate potential confounding variables. Multivariate logistic regression analysis was used to identify risk factors for serum and RBC folate deficiency. Risk of NTD, related to Folate vitamin deficiency in serum and RBCs, was assessed using binary logistic regression while adjusting for covariates. All significance tests were two sided and significant at $p<0.05$.

RESULTS

For this study, 109 case and 100 control mothers were recruited from January 2010 to January 2013. Demographic variables and obstetric data are given in Table 1. The mean age of case mothers was 27.17±0.50 years and of control mothers 27.73±0.53 years. The majority of case mothers had a rural background (59%) and 45% had never attended school. None of the mothers in our study population was a cigarette smoker or indulged in drinking alcohol. However, 41.05% of case mothers were exposed to passive smoke as compared with 25% of control mothers. A diet not adequate in fruits and vegetables was reported by 60% of the case mothers ($p<0.0073$). For 32% of case mothers it was the first pregnancy, whereas 67% were multigravida. As outlined in other studies, we found a tendency of lower socio-economic status ($p<0.0025$) and less adequate diet among case compared with control mothers.[13-16]

Folate status of study population

RBC folate concentrations

The mean RBC folate concentrations in control mothers (n=100) were 337.2±18.42 ng/mL and 104.1±9.17 ng/mL in case mothers (n=109). In case mothers RBC folate concentrations were significantly lower ($p<0.0001$) compared with control mothers (Table 2). RBC folate concentrations were arranged in different groups, starting from the lowest to the highest concentrations, in both case and control mothers (Table 2). RBC folate concentrations in case mothers were significantly lower in the lowest group of concentrations (0-150 ng/mL) compared with control mothers ($p<0.0001$). In the following two groups (151-300 ng/mL; 301-450 ng/mL) with higher RBC folate concentrations, there was no significant difference between case and control mothers. Most of the case mothers were in the lowest concentration group (n=77; 70.64%). None of the case mothers had RBC folate concentrations above 440 ng/mL. In control mothers there were 16% who had concentrations in the 0-150 ng/mL group displaying folate deficiency and 84% had concentrations above 150 ng/mL.

Serum folate concentrations

Overall, mean serum folate concentrations were 6.75±0.42 ng/mL in case mothers (n=109) and 10.83±0.56 ng/mL in control mothers (n=100) ($p<0.0001$). According to the RBC folate concentrations, we divided case and control mothers into four different groups (Table

Table 1. Demographic characteristics of the both study cohorts (case and control mothers)

Demographic variable	Control mothers n (%)	Case mothers n (%)	*p* values
Age at presentation (years)			
15-19	5 (5)	5 (4.6)	
20-24	24 (24)	30 (27.6)	
25-29	36 (36)	29 (26.6)	
31-34	27 (27)	27 (24.7)	
>35	8 (8)	18 (16.5)	
Mean age (years)	27.17±0.50	27.73±0.53	
Range (years)	16-40	16-40	
Residential location			
Rural	32 (32)	62 (59.6)	
Urban	68 (68)	47 (43.1)	
Level of education			
No schooling	45 (45)	50 (45,9)	
School	28 (28)	40 (36.7)	
College	16 (16)	16 (14.7)	
University	11 (11)	3 (2.7)	
Socio-economic status (ruppees)			
5,000-10,000	40 (40)	43 (39.5)	
10,000-15,000	14 (14)	31 (28.40)	
16,000-20,000	-	29 (26.6)	χ^2 (1) = 9.108;
>20,000	46 (46)	6 (5.5)	p<0.0025
Nutritional status (weekly intake of meat, vegetables and fruits)			
Adequate	58 (58)	43 (39.5)	χ^2 (1) = 7.187;
Not adequate	42 (42)	66 (60.5)	p<0.0073
Obstetric history			
Primigravida	29 (29)	35 (32.1)	
Multigravida	71 (71)	74 (67.9)	

Table 2. Mean RBC folate concentrations (ng/mL) in case and control mothers

Mean RBC folate groups (ng/mL)	Mean RBC folate concentrations (ng/mL)		*p* values
	Control mothers (n=100; %)	Case mothers (n=109; %)	
0-150	105.4±6.31 (n=16; 16%)	52.13±4.29 n=77 (70.6%)	$t_{(91)}$=5.40 p<0.0001
151-300	238.3±8.14 (n=34; 34%)	205.2±7.38 n=28 (25.7%)	$t_{(63)}$=2.95 p<0.0045
301-450	352.5±8.43 (n=23; 23%)	395.5±23.51 n=4 (3.7%)	$t_{(25)}$=1.92 p<0.0658^{ns}
451-600+	586.2±23.95 (n=27; 27%)	-	

t-test was applied to assess the statistical difference between the means of the two groups.
^{ns} not significant.

3). Here, 27 (24.77%) case mothers showed concentrations below 3 ng/mL compared with 8% control mothers with severe deficiency.

Results of PCR-RFLP genotyping

The frequency of *MTHFRC677T* alleles and genotypes is shown in Table 4. The frequency of *CC*, *CT*, and *TT* genotypes in case mothers was 61%, 28% and 10%, respectively, while in control mothers it was 72%, 26% and 2%, respectively. The frequency of the C and T allele was 76% and 24% in case mothers and 85% and 15% in control mothers, respectively (p<0.017).

Folate analysis and MTHFR genotypes

The distribution of mean serum folate concentrations and mean RBC folate concentrations among case and control mothers corresponded with the *MTHFR* genotype. As outlined in Table 5, RBC folate concentrations in the TT homozygous groups in case mothers were significantly lower compared with control mothers (p<0.0001). In case mothers, those mothers with *677C/T* and *677TT* genotypes showed significantly lower serum (p<0.0001) and RBC folate concentrations (p<0.0001) compared with control mothers.

Risk of NTDs in offspring of Case- and Control mothers depending on maternal genotypes adjusted for covariates

For homozygous maternal *677TT* genotypes, the risk of NTD in offspring increased significantly (OR=5.5, 95% CI=1.19-25.46) (Table 6). When adjusted for covariates using logistic regression analysis the OR was slightly lower with is OR=4.05 (95% CI 0.75-21.73) (Table 6). However, adjusting maternal heterozygous CT genotype or the CT/TT additive model did not increase the OR for the occurrence of NTDs in offspring. This suggests that

Table 3. Mean serum folate concentrations (ng/mL) in case and control mothers

Mean serum folate groups (ng/mL)	Mean serum folate concentrations (ng/mL)		*p* values
	Control mothers (n=100; %)	Case mothers (n=109; %)	
0-2.9	1.98±0.24 (n=8; 8%)	2.18±0.1 (n=27; 24.8%)	$t_{(33)}$=0.13 *p*<08935^{ns}
3-4.9	4.30±0.25 (n=4; 4%)	4.04±0.14 (n=18; 16.5%)	$t_{(23)}$=0.79 *p*<0.4341^{ns}
5-6.9	5.92±0.14 (n=22 (22%)	5.88±0.14 (n=20; 18.3%)	$t_{(40)}$=0.89 *p*<0.9297^{ns}
7-8.9	7.79±0.14 (n=19; 19%)	7.94±0.12 (n=18; 16.5%)	$t_{(35)}$=1.36 *p*=0.1819^{ns}
>9	15.23±0.6 (n=47; 47%)	13.93±0.65 (n=26; 23.9%)	$t_{(71)}$=2.16 *p*<0.0343

t-test was applied to assess the statistical difference between the means of the two groups.
^{ns} not significant.

Table 4. Genotype and allelic frequencies of the *MTHFR* C677T polymorphism in Case- and Control mothers

	Genotype frequencies			*p* values	Allele frequencies		*p* value
	CC	CT	TT		C	T	
Case mothers (n=109)	67	31	11	CC vs TT <0.0393	165	53	
Control mothers (n=100)	72	26	2	CC vs CT vs TT <0.021	170	30	<0.017

CC vs TT (χ^2 (2) = 6.31); CC vs. CT vs. TT (χ^2 (2) = 6.47);C vs. T (χ^2 (1)= 5.68)

Table 5. *MTHFRC677T* genotypes in Case- and Control mothers with mean RBC and mean serum folate concentrations (ng/ml)

	MTHFR Genotypes	Mean RBC folate concentrations (ng/mL)	Mean serum folate concentrations (ng/mL)
Control mothers (n=100)	CC	328.6±20.81	9.94±0.64
	CT	378.6±39.78	11.55±1.19
	TT	111.4±12.47	5.60±3.10
Case mothers (n=109)	CC	112.5±12.40	7.25±0.58
	CT	100.5±16.87	6.36±0.72
	TT	62.6±13.70	4.86±0.83
Control vs Case mothers CC/TT		Serum folate concentrations	*p*<0.0001
		RBC folate concentrations	*p*<0.0001
Control vs Case mothers CT/TT		Serum folate concentrations	*p*<0.0001
		RBC folate concentrations	*p*<0.0001

the maternal TT homozygous genotype represents an independent risk factor for development of NTDs in offspring. When looking at the dietary history of case and control mothers it shows that even those control mothers with an inadequate history of dietary intake of fruits, vegetables and meat had higher mean serum and RBC folate concentrations than the respective case mothers (Table 7).

DISCUSSION

In this study case and control mothers differed significantly in regards to their socio-economic status and nutritional intake, and also to their exposure to passive smoking. For the development of NTDs, it is known that a low folic acid diet predisposes to the development of NTDs[8]. Folate status is determined by measurement of RBC folate and plasma/serum concentrations. While RBC folate concentrations represent a measurement of dietary folate intake over the previous 180 days, serum folate reflects the recent dietary intake.[42] Folate deficiency is defined as RBC folate concentrations below 315 nmol/L (140 ng/mL) and serum folate concentrations below 7 nmol/L (3 ng/mL).[43] Maternal periconceptional folic acid deficiency due to deficient maternal dietary intake and disturbed maternal folate metabolism due to reduced MTHFR activity has been associated with the occurrence of NTDs in offspring.[33]

In the present study the mean RBC folate concentrations in case mothers was significantly lower compared with control mothers (*p*<0.0001). In accordance, the number of case mothers in whom RBC folate concentrations were less than 150 ng/mL (folate deficiency cohort) was significantly higher compared with control mothers. The results are consistent with those of Martinez de Villareal et al[33] in Mexican women and earlier studies.[44-46] In a large case-control study of Irish mothers, Kirke et al determined folate concentrations in women on their first prenatal visit with their obstetrician.[44] They found an increased risk of NTDs to be associated with decreased RBC concentrations. With RCB folate concentrations below <150 ng/mL, the risk of NTDs was 6,6 per 1.000 newborns, whereas maternal RBC folate concentrations above 400 ng/mL decreased the risk to 0,8 per 1.000

Table 6. Risk of NTDs in offspring of case and control mothers depending on maternal genotypes adjusted for covariates using logistic regression analysis

Genotypes	Case mothers n	Control mothers n	χ^2 value (*df*)	*p* value	Odds ratio (95% CI)	Odds ratio (95% CI
CC	67	72	2.597 (*1*)	0.107	0.62 (0.35 – 1.11)	0.50 (0.24 - 1.06)
CT	31	26	0.157 (*1*)	0.692	1.13 (0.61 - 2.08)	1.47 (0.67 - 3.23)
TT	11	2	5.854 (*1*)	0.016	5.5 (1.19 - 25.46)	4.05 (0.75 - 21.73)
CT & TT	42	28	2.597 (*1*)	0.107	1.61 (0.9 - 2.89)	1.98 (0.94 - 4.14)

The covariates adjusted for in the logistic regression analysis comprised (see also Table 1) residency (urban vs rural); dietary status (inadequate vs adequate); educational level (Illiterate, primary, middle, matric, graduate, post-graduate); husband's salary (in 1000PKR 5-10=0, 11-15=1, 16-20=3, >20=4).

Table 7. Dietary information for case and control mothers and the corresponding folate concentrations according to reported diet history

Reporte ddietary history	Mean folate concentrations (ng/mL)					
	Control mothers (n=100)			Case mothers (n=109)		
	n	Serum	RBC	n	Serum	RBC
Adequate diet in fruits, vegetables and meat	58	11.16±0.72	382.8±25.51	43	8.26±0.81	143.2±16.71
Inadequate diet in fruits, vegetables and meat	42	8.75±0.82	274.3±23.57	66	5.53±0.40	79.86±9.74
Control vs Case mothers						
Adequate diet	Mean serum folate concentrations			$t_{(99)}=2.88$; $p=0.0049$		
	Mean RBC folate concentration			$t_{(99)}=7.36$; $p<0.0001$		
Inadequate diet	Mean serum folate concentration			$t_{(106)}=3.28$; $p=0.0014$		
	Mean RBC folate concentration			$t_{(106)}=8.69$; $p<0.0001$		

newborns. In the present study, none of the case mothers had RBC folate concentrations above 400 ng/mL, a level needed for protection against the occurrence of NTDs in the offspring.[45]

By looking at the maternal *MTHFRC677T* polymorphism, we not only focused on the maternal folate status but also on the maternal ability to metabolize folate. Here, case mothers were significantly more often hetero- or homozygous for the T-allele, compared with control mothers ($p<0.0393$). Among persons, hetero- or homozygous for the T-allele, MTHFR enzyme activity decreases to 65% or 30%, respectively.[46,47] Accordingly, Martinez de Villarreal et al,[33] in a Mexican case-control-study, found the homozygous *677TT* genotype to be significantly more prevalent in case mothers compared with control mothers ($p<0.05$). In the present investigation, we found NDT to be associated with low RBC and serum folate concentrations, as well as with the TT genotype in the case mothers. Christensen et al,[48] investigating Canadian families with NTDs, found similar results by showing that the *MTHFR677TT* genotype and low maternal folate status were both associated with NTDs in the offspring.

Nevertheless, case-control studies among case and control mothers of Italian and Irish backgrounds found no difference between both cohorts and the frequency of the *677TT* genotype.[49,50] Another study by Yan et al[51] found an association of NTDs among Asian and Caucasian case mothers with the *677CT* and *677TT* genotypes, but not among African case mothers. However, independent of the *MTHFR* genotype and across different populations, low maternal folate status was associated with an increased risk for NTDs.[29]

Our study has several important limitations. First, the present study is limited by the fact that folate concentrations were not obtained during the periconceptional period, but at the time of delivery, which does not reflect the vulnerable time for NTD formation. Hence, it can only be speculated from the RBC and serum folate concentrations, measured here at the time of delivery in case and control mothers, that these concentrations might also reflect the folate concentrations during the periconceptional period. Second, our study comprised rather smaller cohorts of case and control mothers, which might have limited the power of our analysis. Third, our study did not analyze newborns', only the mothers' genotypes for the *MTHFRC677T* polymorphism, allowing only for analysis about the maternally conferred risk to their fetuses, but not for the risk the fetuses carry in their own *MTHFR* genotype.

In conclusion, more studies are warranted to elucidate the complex network of pre-disposing gene-nutrient interactions in NTDs. We would also like to stress the importance of public health intervention programs to create awareness and promote use of folic acid supplementation and food fortification in women of reproductive age to prevent NTDs.

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AUTHOR DISCLOSURES

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