Maternal One-Carbon Metabolism, *MTHFR* and *TCN2* Genotypes and Neural Tube Defects in India

Koumudi Godbole,[1] Panjalingam Gayathri,[2] Smita Ghule,[1] Batchu Venkatanaga Sasirekha,[2] Amruta Kanitkar-Damle,[1] Nilam Memane,[1] Seshadri Suresh,[3] Jayesh Sheth,[4] Giriraj Ratan Chandak,[2]* and Chittaranjan S. Yajnik[1]*

[1]Diabetes Unit, KEM Hospital Research Center, Rasta Peth, Pune, India, PIN: 411011
[2]Centre for Cellular and Molecular Biology (CCMB), Council of Scientific and Industrial Research (CSIR), Uppal Road, Hyderabad, India, PIN: 500 007
[3]Fetal Care Research Foundation, 203 Avvai Shanmugam Road, Royapettah, Chennai, India, PIN: 600014
[4]Foundation for Research in Genetics & Endocrinology (FRIGE), 20/1 Bimannagar, Opp Umiyavijay, Satellite Road, Ahmedabad, India, PIN: 380015

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BACKGROUND: Neural tube defects (NTDs) are among the most common severe congenital malformations, representing a long-term public health burden in India. A deranged one-carbon metabolism and genes regulating this metabolism have been linked to NTDs. Vitamin B_{12} deficiency is reported to be more prevalent than folate deficiency in the Indian population. We investigated the role of maternal nutritional and genetic markers related to one-carbon metabolism in the etiology of NTDs. **METHODS:** We conducted a multicenter case-control study to compare plasma folate, vitamin B_{12}, homocysteine and holo-transcobalamin levels, and polymorphisms in methylenetetrahydrofolate reductase (*MTHFR*, 677C>T, 1298A>C, 1781G>A and 236+724A>G) and transcobalamin (*TCN2*, 776C>G) genes, in 318 women with NTD-affected offspring (cases) and 702 women with apparently healthy offspring (controls). The samples were collected at diagnosis in cases and at delivery in controls. **RESULTS:** We observed a significant association of high maternal plasma homocysteine concentrations with NTDs in the offspring ($p = 0.026$). There was no association of maternal folate or B_{12} levels with NTDs ($p > 0.05$) but low maternal holo-transcobalamin predicted strong risk of NTDs in the offspring ($p = 0.003$). The commonly associated maternal polymorphism 677C>T in the *MTHFR* gene did not predict risk of NTDs in the offspring ($p > 0.05$) and 1298A>C and 1781G>A polymorphisms in *MTHFR* were protective ($p = 0.024$ and 0.0004 respectively). Maternal 776C>G polymorphism in *TCN2* was strongly predictive of NTD in the offspring ($p = 0.006$). **CONCLUSION:** Our study has demonstrated a possible role for maternal B_{12} deficiency in the etiology of NTDs in India over and above the well-established role of folate deficiency. *Birth Defects Research (Part A) 00:000–000, 2011.* © 2011 Wiley-Liss, Inc.

Key words: neural tube defects; homocysteine; holo-transcobalamin; one-carbon metabolism, *MTHFR* polymorphisms, *TCN2* polymorphism; gene-nutrient interaction; predictive risk

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*Correspondence to: Giriraj Ratan Chandak, Centre for Cellular and Molecular Biology (CCMB), Council of Scientific and Industrial Research (CSIR), Uppal Road, Hyderabad, India, PIN: 500 007. E-mail: chandakgrc@ccmb.res.in or Chittaranjan S Yajnik, Director, Diabetes Unit, KEM Hospital Research Center, Rasta Peth, Pune, India, PIN: 411011. E-mail: csyajnik@hotmail.com
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INTRODUCTION

Neural tube defects (NTDs) are among the most common severe congenital malformations, representing a long-term public health burden in both social and economic terms. It is well established that maternal hyperhomocysteinemia and low folate and vitamin B_{12} status increases the risk of NTDs in the offspring (Kirke et al., 1993; Blom et al., 2006). A series of studies from different parts of the world have clearly demonstrated that periconceptional folic acid supplements prevent NTD in the offspring (MRC Vitamin Study Research Group, 1991; Czeizel and Dudas, 1992). The role of maternal B_{12} deficiency in the etiology of NTDs was suggested by low levels in the amniotic fluid (Gardiki-Kouidou and Seller, 1988; Steen et al., 1998) and in the blood of mothers with NTD-affected offspring: in pre-fortification (Kirke et al., 1993; Suarez et al., 2003; Molloy et al., 2009) and also in post-fortification era (Ray et al., 2007). Low maternal holo-transcobalamin (holo-TC), representing the bioavailable fraction of circulating vitamin B_{12} was reported to increase the risk of NTD in their children (Afman et al., 2001; Ray et al., 2007). It is proposed that holo-TC is a more reliable marker of vitamin B_{12} deficiency compared to circulating B_{12} levels during pregnancy, because it is more stable through gestation (Obeid et al., 2006; Morkbak et al., 2007).

The first genetic defect in folate metabolism linked to NTDs was the 677C>T polymorphism in methylenetetrahydrofolate reductase (*MTHFR*) gene that reduced the enzymatic activity by 60 to 70% in homozygotes for the risk allele 'T' (van der Put et al., 1995; Whitehead et al., 1995). In TT homozygotes, hyperhomocysteinemia occurs primarily under conditions of mild to moderate folate deficiency (Jacques et al., 1996; Kluijtmans et al., 2003). Although many studies have confirmed an elevated risk of NTD for both maternal (van der Put et al., 1995; Volcik et al., 2000) and offspring TT genotype (Ou et al., 1996; van der Put et al., 1997), several others have failed to replicate these observations (Koch et al., 1998; Relton et al., 2004, Behunova et al., 2010). The role of a second polymorphism within the *MTHFR* coding region, 1298A>C in NTD susceptibility has been controversial (Koch et al., 1998; van der Put et al., 1998). Several other polymorphisms in the *MTHFR* gene have been reported to influence the homocysteine levels and thus likely associated with NTD and other related disorders such as cleft lip and palate (Botto and Yang, 2000; Bufalino et al., 2010). Transcobalamin (*TCN2*) is another gene of interest based on findings of low vitamin B_{12} and holo-TC concentrations in women with NTD-affected fetuses. The association of *TCN2* polymorphism, 776C>G with NTDs has been studied earlier with variable results (Candito et al., 2008).

Studies on prevalence of NTDs in India are scarce (Godbole et al., 2009). Small hospital-based surveys have reported high incidence of NTDs up to 11.4 per 1000 in certain regions of India (Verma, 1978; Kulkarni et al., 1987) while a recent population-based study from Uttar Pradesh in North India reported the prevalence to be 6 to 8/1000 (Cherian et al., 2005). Very few studies in India have measured circulating folate and vitamin B_{12} concentrations in mothers with NTD-affected fetuses. High prevalence of B_{12} deficiency has been reported in young Indians (Refsum et al., 2001) including pregnant women, which is associated with intrauterine growth retardation and other unfavorable outcomes in the offspring (Muthayya et al., 2006; Yajnik et al., 2008). The only Indian trial to investigate the role of high-dose folic acid in the prevention of recurrent NTDs was terminated prematurely after the publication of the MRC trial (MRC Vitamin Study Research Group, 1991), and showed a non-significant reduction (Central Technical Co-coordinating Unit, Indian Council of Medical Research, 2000).

We designed a multicenter case-control study to investigate the role of maternal nutrition and genetic markers of folate and B_{12} metabolism in the etiology of NTDs. We measured circulating concentrations of homocysteine (a summative marker of one-carbon metabolism), folate, B_{12}, and holo-TC. We genotyped four common single nucleotide polymorphisms (SNPs) in the *MTHFR* gene and 776C>G polymorphism in *TCN2* gene that are reported to be associated with NTD risk in western populations.

MATERIALS AND METHODS

Recruitment of Cases and Controls

This study was conducted between January 2007 to December 2009, at four centers from Ahmedabad and Pune in Western India & Hyderabad and Chennai in South India, which provide expert facilities for diagnosis and/or management of fetal abnormalities. Most of these centers are also members of the Birth Defects Registry of India run by a non-governmental organization (http://www.mediscansystems.org/bdr). The staff of the referring hospitals were trained about inclusion/exclusion criteria and sample handling. Cases were women having offspring affected with isolated NTDs, which was diagnosed by antenatal ultrasound examination. In some cases, the diagnosis of NTD was made by clinical examination within 6 weeks of birth. Controls were women with healthy full-term newborns and no family history of NTDs or other midline birth defects in the family. In the subsequent sections, mothers having NTD-affected offspring will be referred as cases while those having normal offspring will be referred as controls. We excluded women with history of diabetes, those on antiepileptic medication, and those having fetuses with associated birth defects in addition to NTDs. All centers used a common protocol for data collection and processing of biologic samples. The following information was collected by trained assistants: maternal age, gestational age, weight, height, family income, consanguinity, dietary history (vegetarian/non-vegetarian), history of consumption of alcohol and tobacco (smoking and chewing), and intake of vitamin supplements during pregnancy. Details of NTDs including site (upper or lower), type (anencephaly, encephalocele, einencephaly, meningocele, meningomyelocele, and complete spinal dysraphism) and mode of diagnosis were also recorded.

Collection of Samples and Estimation of Biochemical Parameters

Tissue samples from NTD-affected fetuses were collected from the thigh or from the site of defect in cases of pregnancy terminations. Peripheral blood was obtained from affected newborns when NTD was diagnosed after birth and cord blood from control newborns at birth. Blood was collected from case mothers at the time of

diagnosis of NTDs (at variable gestation) and at delivery from control mothers. All blood samples were collected in EDTA vacutainers, processed within 2 hours of collection and plasma stored in aliquots at −20°C. The samples were transported in dry ice from all the centers to Pune and Hyderabad for biochemical and molecular analysis, respectively, where they were stored at −80°C until further use.

The following measurements were done on maternal plasma samples: vitamin B_{12} and folate were measured by microbial assays (Kelleher et al., 1987; Horne and Patterson, 1988), homocysteine (tHcy) by fluorescence polarization immunoassay (Abbott Laboratories, Abbott Park, IL), holo-TC by Micro Particle Enzyme Immunoassay (AxSYM active B_{12} assay, Abbott Laboratories) and creatinine by automated biochemical analyzer (ALCYON 300). The coefficient of variation for tHcy was <3% while it was 8% for the rest of the measurements. Maternal hemoglobin concentration and various erythrocyte indices were estimated where fresh blood was available (mainly Pune and Ahmedabad; Beckman Coulter $A^{C\cdot}T$ diff Analyzer, Miami, FL).

Genetic Analysis

Genomic DNA was isolated from all mothers and their offspring using the salt precipitation method and samples were plated in a uniform concentration. We used Sequenom-based Mass ARRAY assay to genotype four SNPs (rs1801133 [c.665C>T, earlier known as 677C>T; p.Arg222Val], rs1801131 [c.1286A>C, earlier known as 1298A>C; p.Glu429Ala], rs2274976 [c.1781G>A; p.Arg594Gln] and rs9651118 [c.236+724A>G]) in the *MTHFR* gene and the 776C>G polymorphism (rs1801198) in the *TCN2* gene as part of a 54 SNP pool collated from 20 genes in the folate-B_{12} pathway. We confirmed the genotypes for ~10% of the samples by a combination of restriction fragment length polymorphism and sequencing and inconsistency of only 0.007% (3/416) was observed.

Statistical Analysis

Data are presented as median (25th, 75th centile) and as n or % as appropriate. Plasma folate concentrations were square rooted while B_{12}, tHcy, and holo-TC concentrations were natural log-transformed to satisfy the assumption of normality. The difference in maternal concentrations of plasma B_{12}, folate, tHcy, and holo-TC between cases and controls was assessed using ANOVA after adjusting for confounders such as maternal age, diet (vegetarian/nonvegetarian), income (<Rs 5000>Rs 5000), history of antenatal supplementation, and center of collection. We did not adjust for gestational age while studying differences in nutritional markers between cases and controls as there was little overlap between the two groups. Categorical data was analyzed by a chi-square test. The genotype distribution for all the SNPs was analyzed for deviation from Hardy–Weinberg equilibrium (HWE) using $\chi 2$ analysis. Association between maternal or fetal SNPs and risk of NTDs was determined by logistic regression analysis. The odds ratios (ORs) with 95% confidence intervals (CIs) are presented with respect to the risk allele and the risk genotype. The reported '*p*' values for genetic associations have not been corrected by Bonferroni method since it usually overcorrects, and also because of the fact that a strict correction is not critical given the biologic plausibility implicating these genes in NTDs. However, we have performed permutation analysis by shuffling genotype assignment 10,000 times and calculated significant empiric *p* values.

The cut-off points for defining deficiencies were 7.0 nM for plasma folate (Clarke et al., 2004), 150 pM for vitamin B_{12} concentration (Refsum et al., 2001), 10 μM for tHcy (Refsum et al., 2004), and 35 pM for holo-TC concentrations (Abbott Laboratories, manufacturer's literature). We also used 20 pM as low-level cut-off for holo-TC because a recent study established the 95% reference interval as 19 to 134 pM using Abbott AxSYM kit (Brady et al., 2008). We considered plasma B_{12} concentration above 1000 pM and folate concentration >100 nM as indicative of high-dose supplementation and removed them from further analysis. An informed consent following the Indian Council of Medical Research guidelines was obtained from all participants. The institutional ethics committees of each center approved the study.

RESULTS

Of a total of 331 NTD families and 722 controls, 318 NTD cases and 702 control families fulfilled the inclusion criteria. We excluded pregnant women with pre-existing diabetes (n = 21), treatment with antiepileptic drugs (n = 4), and presence of associated malformations (n = 8). Center-wise distribution of the subjects has been provided in Supplementary Table 1. Of 318 cases, 82% (n = 260) were diagnosed by prenatal ultrasound scan while the remaining were diagnosed at birth. NTD type was known in 305 of 318: upper NTDs such as anencephaly, encephalocele were seen in 124 infants (40.7%) while 194 infants (66.5%) had lower NTDs such as spina bifida and spinal dysraphism. Multiple site NTDs were seen in 19 infants (6.2%).

Socio-demographic Profile

Characteristics of case and control mothers are listed in Table 1. Case and control mothers had similar age, parity, history of consanguinity, and monthly income. None of the women smoked, few chewed tobacco, while only one woman consumed alcohol. There was a significant difference in the gestational age at sample collection between cases and controls. Vitamin supplementation history was difficult to obtain. Only six case mothers and 15 control mothers reported pre-conceptional vitamin supplementation. Forty-four women had received no supplementation while 144 women could not specify if any vitamin supplementation was consumed. The rest (n = 811) had received supplements beginning at their first medical consultation at variable gestational ages, but not peri-conceptionally. The history of supplementation was significantly higher in controls than in cases ($p = 0.001$).

Biochemical Analysis

Circulating concentrations of nutritional and biochemical parameters varied among the centers (Supplementary Fig. 1), therefore, the statistical analysis was adjusted for the center of study. Nutritional and biochemical measurements were available on varying numbers due to technical reasons.

Table 1
Characteristics of Neural Tube Defect Cases and Control Mothers

	Cases		Controls		
	n	Value[a]	n	Value[a]	p value[b]
Age (years)	313	25.0 (22.0–28.0)	683	24.0 (22.0–27.0)	0.27
Income (< = Rs5000/pM) (%)	155/301	51.5	302/668	45.2	0.07
Vegetarian (%)	115/311	37.0	257/693	37.1	0.97
Smokers	0	0	0	0	
Alcohol (%)	0	0	1	0.1	0.50
Tobacco chewers (%)	1/308	0.3	5/686	0.7	0.45
Coffee drinker (%)	114/309	36.9	228/688	32.7	0.20
Consanguinity (%)	55/308	17.9	108/683	15.8	0.42
Parity (%)					
1	103/160	64.4	278/459	60.6	
2	41/160	25.6	148/459	32.2	0.21
>2	16/160	10.0	33/459	7.2	
Gestational age (in weeks)	283	23.0 (19.0–32.0)	651	37.3 (36.0–39.0)	**0.001**
Antenatal supplementation (%)	204/235	86.8	628/641	98.0	**0.001**

'n' represents the number of women on whom observations are available.
[a]Values are median (25–75th centile) or percentage.
[b]The p values are calculated using ANOVA and chi-square test for categorical variables.

Nutritional and Biochemical Parameters in Case and Control Mothers and Risk of Neural Tube Defects in offspring

Hematologic parameters and plasma creatinine concentrations were comparable in cases and controls. The median plasma homocysteine concentration was marginally higher in cases than in controls (10.5 and 10.2 mM, respectively, $p = 0.026$) but the prevalence of hyperhomocysteinemia was similar in cases and controls (53.4 vs. 52%, respectively, $p > 0.05$; Table 2). We did not observe any significant difference in plasma folate concentrations between the two groups, although controls had higher circulating folate concentrations than cases (cases: 27.1 nM and controls: 35.0 nM, $p > 0.05$). Median plasma vitamin B_{12} concentrations were low in both cases and controls and there was no statistical difference between the two groups (cases: 168.0 pM and controls: 159.7 pM, $p > 0.05$). Different groups have suggested cut-offs ranging from 20 to 40 pM for holo-TC deficiency (Brady et al., 2008, Bamonti et al., 2010). Using the commonly used cut-off point of 35 pM, many cases were holo-TC deficient compared to controls (cases: 65.3% and controls: 55.2%, $p = 0.003$). However, using 20 pM as the cut-off point, 32.7% cases and 27.7% controls were holo-TC deficient but the difference was not statistically significant ($p > 0.05$). Because the majority of studies have used 35 pM as the cut-off point for holo-TC deficiency, hence we have presented the data using this cut-off point. However, given the usual problem of using ''cut-off points'', an analysis of difference between groups (continuous variable) is more sensitive to comparison between the two groups, and with this approach, cases had significantly lower holo-TC concentrations than controls (25.4 vs. 31.7 pM, respectively, $p < 0.001$).

We further analyzed the differences between cases and controls based on history of antenatal supplementation. Eight hundred thirty-two women had received some supplementation at sometime in their pregnancy and 44 were never supplemented. The circulating median folate concentrations were significantly higher in the supplemented group (37.0 vs. 12.3 nM, $p = 0.001$) and tHcy concentrations were significantly lower in the supplemented compared to the nonsupplemented group (10.0 vs. 12.2 μM, $p = 0.01$; Supplementary Table 2). However, there was no significant difference in the median vitamin B_{12} or holo-TC concentrations between supplement users and nonusers ($p > 0.05$). This may imply folic acid as the predominant antenatal supplement received by the women in this population.

Genetic Analysis

We did not observe any significant heterogeneity between centers in the allelic and genotypic frequency of any of the polymorphisms in *MTHFR* and *TCN2* genes in mothers and offspring ($p > 0.05$ for Cochran's Q-statistics), hence, we performed the association analysis by combining their genotype data. Genotype frequencies at all the SNPs did not deviate from HWE.

Association of Maternal Methylenetetrahydrofolate Reductase Genotypes with Homocysteine Concentration and Neural Tube Defect Risk in the offspring

Maternal 'T' allele at 677C>T SNP in the *MTHFR* gene predisposes to NTDs in Europeans. In our study, the allele frequency of this risk allele was much lower compared to Whites (0.12 vs. 0.40). However, both allelic as well as genotypic frequency at 677C>T was similar between case and control mothers ($p > 0.05$; Table 3). On the other hand, the frequency of 1298A>C polymorphism in the *MTHFR* gene was higher in our population compared to Whites (0.40 vs. 0.30). The frequency of the minor allele at 1298A>C was higher in the controls and there was enrichment of CC and AC genotypes in control mothers compared to case mothers, suggesting a protective role against NTDs (OR = 0.80; 95% CI = 0.65-0.97;

Table 2
Biochemical Parameters in Neural Tube Defect Cases and Control Mothers

	Cases		Controls		
Marker	n	Value[a]	n	Value[a]	*p* value[b]
B_{12} (pM)	291	168.0 (113.0–233.0)	640	159.7 (115.3–221.5)	0.37
B_{12} deficiency <150 pM (%)		42.7		45.3	0.45
Folate (nM)	303	27.1 (14.1–56.0)	676	35.0 (17.1–58.0)	0.68
Folate deficiency <7 nM (%)		9.4		6.7	0.13
Homocysteine (μM)	309	10.5 (7.3–14.6)	689	10.2 (7.7–14.1)	**0.026**
Hyperhomocysteinemia >10 μM (%)		53.4		52.0	0.68
Holo-TC (pM)	308	25.4 (17.8–42.6)	686	31.7 (19.3–51.3)	**0.000**
Holo-TC deficiency <35 pM (%)		65.3		55.2	**0.003**
Plasma creatinine	309	0.63 (0.57–0.72)	691	0.70 (0.60–0.72)	0.56
Hemoglobin (g/dL)	189	11.0 (9.8–12.0)	508	11.1 (9.5–12.1)	0.08
Anemia (Hb <11 g/dL) (%)	92/189	48.7	235/508	46.3	0.57
MCH (pg/cell) (%)					
<27	77/162	47.5	216/483	44.7	0.82
27–31	69/162	42.6	218/483	45.1	
>31	16/162	9.9	49/483	10.2	
MCHC (gm) (%)					
<33	54/162	33.3	171/483	35.4	0.65
33–37	92/162	56.8	275/483	56.9	
>37	16/162	9.9	37/483	7.7	
MCV (fL) (%)					
<80	73/162	45.1	214/483	44.3	0.79
80–99.9	87/162	53.7	259/483	53.6	
>99.9	2/162	1.2	10/483	2.1	

'n' represents the number of women on whom observations are available.
[a]Values are median (25–75th centile) or percentage.
[b]The *p* values are calculated using ANOVA adjusted for maternal age, income, supplementation, diet type, site, and using chi-square test for categorical variables.
Holo-TC, holo-transcobalamin.

$p = 0.024$). Similar observation was seen with 1781G>A polymorphism (OR = 0.62; 95% CI = 0.47-0.81; $p = 0.0004$) but not with 236 + 724A>G polymorphism in the *MTHFR* gene ($p > 0.05$). Genotypes with at least one copy of the minor allele at 1298A>C (AC+CC) predicted lower homocysteine levels compared to the wild type, while the other three polymorphisms including 677C>T had no influence on tHcy levels after adjusting for confounders such as diet, vitamin supplementation, and center (Table 4).

Table 3
Maternal *MTHFR* Polymorphisms and Neural Tube Defect in the Offspring

	Cases		Controls				
Maternal genotype	n	(%)	n	(%)	OR	(95% CI)[a]	*p* value
1298A>C (rs1801131)	300		675		0.80	(0.65–0.97)	**0.024**
AA	114	38.0	215	31.9			
AC	144	48.0	333	49.3			
CC	42	14.0	127	18.8			
Minor allele frequency		C-0.38		C-0.43			
677C>T (rs1801133)	305		684		0.96	(0.71–1.28)	0.76
CC	238	78.0	521	76.2			
CT	62	20.3	158	23.1			
TT	5	1.6	5	0.7			
Minor allele frequency		T-0.12		T-0.12			
1781G>A (rs2274976)	301		681		0.62	(0.47–0.81)	**0.00042**
GG	227	75.4	439	64.5			
GA	68	22.6	213	31.2			
AA	6	2.0	29	4.3			
Minor allele frequency		A-0.13		A-0.20			
236+724A>G (rs9651118)	296		663		1.08	(0.88–1.34)	0.44
AA	149	50.3	348	52.5			
AG	119	40.2	261	39.4			
GG	28	9.5	54	8.1			
Minor allele frequency		G-0.30		G-0.28			

[a]OR (95% CI) is presented with reference to the minor allele. MTHFR, methylentetrahydrofolate reductase; OR, odds ratio; CI, confidence interval.

Table 4
Maternal *MTHFR* Polymorphisms and Homocysteine Concentration

Maternal genotype	n	Homocysteine (μM)	*p* value
1298A>C (rs1801131)			
AA	324	11.01 (8.35–14.35)	**0.01**
AC+CC	637	9.80 (7.37–14.19)	
677C>T (rs1801133)			
CC	747	10.36 (7.68–14.36)	0.92
CT+TT	227	10.16 (7.66–14.00)	
1781G>A (rs2274976)			
GG	650	10.48 (7.89–14.3)	0.37
GA+AA	306	9.61 (7.35–13.58)	
236+724 A>G (rs9651118)			
AA	485	10.30 (7.55–14.32)	0.53
AG+GG	450	10.34 (7.74–13.91)	

MTHFR, methylentetrahydrofolate reductase.

Association of Fetal Methylenetetrahydrofolate Reductase Genotypes with Neural Tube Defect Risk

The 'T' allele frequency at 677C>T was comparable in the offspring with and without NTDs ($p > 0.05$; Table 5). On the other hand, 1298A>C polymorphism was more prevalent in offspring without NTDs (OR = 0.82; 95% CI = 0.67-0.99; $p = 0.04$), suggesting a protective role. We did not find any allelic or genotypic differences at the other *MTHFR* polymorphisms, namely 1781G>A and 236+724A>G between the NTD-affected or normal offspring (Table 5).

Compound Heterozygosity for Two Common Maternal Methylenetetrahydrofolate Reductase Mutations and Risk of Neural Tube Defects in the offspring

Compound heterozygosity for 677C>T and 1298A>C mutations (CT+AC) which is known to result in decreased *MTHFR* activity (van der Put et al., 1998), was not differently distributed in cases and control mothers (10.3 vs. 9.8%) as well as in the affected and unaffected offspring (10 vs. 12.1%).

Association of Maternal TCN2 776C>G Genotype with Biochemical Parameters and Neural Tube Defect Risk in the Offspring

The 776C>G polymorphism in *TCN2* gene was studied to further substantiate the association of low holo-TC concentrations in cases compared with controls. Recent studies have shown the G allele to be associated with low holo-TC concentrations (Hazra et al., 2009). In this study, the association between maternal *TCN2* 776C>G polymorphism and plasma holo-TC concentration was borderline significant ($\beta = -0.08$; $p = 0.08$), while plasma B_{12} concentration ($\beta = 0.03$; $p = 0.53$) and plasma tHcy concentration ($\beta = 0.025$; $p = 0.59$) were not significant. However, the risk allele 'G' was more prevalent in case mothers compared to control mothers (minor allele frequency = 0.65 and 0.58, respectively) and was strongly predictive of risk of NTDs in the offspring (OR = 1.34; 95% CI = 1.08-1.64; $p = 0.006$; Table 6).

Association of Fetal *TCN2* Polymorphism 776C>G with Holo-transcobalamin Levels and Neural Tube Defects Risk

The offspring's own genotype at *TCN2* 776C>G variant had no influence on the holo-TC levels or on risk of NTD (OR = 1.20; 95% CI = 0.97-1.48; $p = 0.10$; Table 6).

DISCUSSION

Our study showed that mothers with NTD-affected offspring had higher homocysteine and lower holo-TC concentrations compared to mothers with unaffected

Table 5
MTHFR Polymorphisms in Offspring and Risk of Neural Tube Defect

	Cases		Controls				
Offspring genotype	n	(%)	n	(%)	OR	(95% CI)[a]	*p* value
1298A>C (rs1801131)	297		675				
AA	113	38.0	220	32.6	0.82	(0.67–0.99)	**0.04**
AC	144	48.5	335	49.6			
CC	40	13.5	120	17.8			
Minor allele frequency		C-0.38		C-0.43			
677C>T (rs1801133)	297		680				
CC	207	69.7	489	71.9	1.19	(0.92–1.55)	0.18
CT	78	26.3	181	26.6			
TT	12	4.0	10	1.5			
Minor allele frequency		T-0.17		T-0.15			
1781G>A (rs2274976)	295		673		0.86	(0.66–1.13)	0.27
GG	216	73.2	470	69.8			
GA	71	24.1	181	26.9			
AA	8	2.7	22	3.3			
Minor allele frequency		A-0.14		A-0.17			
236+724A>G (rs9651118)	280		616		1.07	(0.86–1.34)	0.53
AA	134	47.9	310	50.3			
AG	123	43.9	258	41.9			
GG	23	8.2	48	7.8			
Minor allele frequency		G-0.30		G-0.29			

[a]OR (95% CI) is presented with reference to the minor allele. MTHFR, methylentetrahydrofolate reductase; OR, odds ratio; CI, confidence interval.

Table 6
Maternal and Offspring *TCN2* 776C>G Polymorphism and Neural Tube Defect in the Offspring

	Cases		Controls				
Genotype	n	(%)	n	(%)	OR	(95% CI)[a]	*p* value
Mothers	262		693		1.34	(1.08–1.64)	**0.006**
CC	33	12.6	130	18.8			
CG	120	45.8	327	47.2			
GG	109	41.6	236	34.0			
Risk allele frequency		G-0.65		G-0.58			
Offspring	255		668		1.20	(0.97–1.48)	0.10
CC	34	13.3	105	15.7			
CG	109	42.7	309	46.3			
GG	112	43.9	254	38.0			
Risk allele frequency		G-0.65		G-0.61			

[a]OR (95% CI) is presented with reference to the risk allele. OR, odds ratio; CI, confidence interval.

offspring. There was no association of maternal plasma folate and vitamin B_{12} concentration with NTDs in the offspring. Maternal *MTHFR* 677C>T polymorphism was not associated with NTDs in offspring and maternal 1298A>C and 1781G>A polymorphisms in the *MTHFR* gene were protective. Maternal 776C>G polymorphism in the *TCN2* gene significantly predicted the risk of NTDs in the offspring. Thus, nutritional and genetic factors contributing to NTDs seem to be different in our population compared to the western populations. The distribution of the types of NTDs (upper vs. lower) was also different in this study (i.e., there were more fetuses with lower than upper NTDs, unlike in the western populations where the distribution is almost similar; Verma, 1978; Cherian et al., 2005). The majority of NTD cases were detected by prenatal ultrasound scan and the remaining by medical examination in the stillborn and liveborn, thus making underreporting of upper NTDs less likely.

Maternal one carbon metabolism has a prominent role in the etiology of NTDs. In white populations who are predominantly non-vegetarian and, therefore, B_{12} sufficient, folate deficiency is a major determinant of NTD risk as shown in clinical, epidemiologic, and intervention studies (Centers for Disease Control, 2004; Beaudin and Stover, 2007). In these populations, *MTHFR* polymorphisms producing ''relative'' folate deficiency predispose to NTDs. Peri-conceptional folic acid supplementation and/or universal flour fortification (as in North America) has significantly reduced the risk of NTDs (Williams et al., 2002; Centers for Disease Control, 2004). Vitamin B_{12} deficiency has emerged as an important factor contributing to the risk of NTDs in the post-folate fortification era (Ray et al., 2007). The role of B_{12} deficiency in the etiology of NTDs was initially suggested by the findings of low B_{12} levels in the amniotic fluid of NTD-affected pregnancies (Gardiki-Kouidou and Seller, 1988; Steen et al., 1998) and later in the maternal blood (Suarez et al., 2003; Molloy et al., 2009). A recent study has substantiated the protective role of vitamin B_{12} in NTDs in a nonsupplemented Irish population (Molloy et al., 2009). We and others have demonstrated that low vitamin B_{12} status is common in the Indian population, which is predominantly vegetarian (Refsum et al., 2001). In this study, we did not find any significant difference in vitamin B_{12} concentrations between cases and controls, but plasma holo-TC concentrations were significantly lower in cases compared to those in controls. Holo-TC is considered to be a better marker of B_{12} status during pregnancy compared to circulating B_{12} concentration because it remains stable throughout the pregnancy (Morkbak et al., 2007). We additionally studied the common 776C>G polymorphism in maternal *TCN2* gene and found it to be strongly associated with NTDs in the offspring. The risk allele 'G' that codes for arginine in place of proline is known to influence the transcription leading to lower intracellular concentration of transcobalamin (Namour et al., 2001). The risk allele is also known to influence the tHcy levels in the presence of low B_{12} concentrations. This is important in view of the findings of a genome-wide association study (Hazra et al., 2009) that identified new biologic candidates for plasma homocysteine and vitamin B_{12}. In this study, maternal plasma tHcy concentrations were higher in cases compared to controls, similar to the observations in previous studies (Steegers-Theunissen et al., 1994; Candito et al., 2008). However, this may have been confounded by the differences in the gestational age at which sampling was performed in cases and controls.

A majority of studies have reported a strong association of fetal genotype 'TT' at 677C>T polymorphism in the *MTHFR* gene with NTDs and a modest association of maternal TT genotype with the risk of NTDs in their children (van der Put et al., 1997; Botto and Yang, 2000; Blom et al., 2006). The risk allele or TT genotype at 677C>T polymorphism in mothers as well as offspring was not found to be associated with NTDs in this study. One reason may be extremely low frequency of T allele (0.12 in both cases and controls) in our population and an even lower frequency of 677TT homozygotes (1.6% in cases and 0.7% in controls). Given this low prevalence, it is rather unlikely that this *MTHFR* polymorphism will contribute substantially to the etiology of NTDs in this population. Interestingly, González-Herrera et al. (2002) have similarly reported a lack of association between *MTHFR* 677C>T polymorphism and NTDs in the State of Yucatan, Mexico, which has the highest reported T allele frequency of 54%. Two other polymorphisms in *MTHFR*, 1298A>C and 1781G>A were found to be protective against NTDs. Additionally, fetal 1298C allele was also overrepresented in control offspring. Interestingly, the ''C'' allele at 1298A>C SNP was associated with lower plasma tHcy concentration, thus providing a plausible mechanism for its protective role against NTD. Some studies have reported maternal 1298A>C polymorphism to be associated with NTDs along with 677C>T polymorphism (van der Put et al., 1998; González-Herrera et al., 2002) while several others have failed to demonstrate any

association of 1298A>C with NTDs (Parle-McDermott et al., 2003; Félix et al., 2004). Candito et al. (2008) reported a significantly higher frequency of the risk allele 'C' in controls than in cases (36.9 vs. 23.4%; $p = 0.02$) and reduced odds of having NTDs in the presence of C allele. Relton et al. (2004) have also reported a protective role of maternal 1298A>C polymorphism. Another *MTHFR* SNP, 1781G>A, in mothers but not in the offspring was protective against NTDs in our study and had no influence on the tHcy concentration. It is difficult to explain how 1298A>C polymorphism protects against both hyperhomocysteinemia and the risk of NTDs. One possibility is an excess of nucleotide synthesis as a result of shunting of 5,10-methylenetetrahydrofolate along thymidylate synthase pathway instead of the methylation pathway (Lucock et al., 2000), in the presence of adequate folate but deficient B_{12} status of our population.

The main strength of this study was the large sample size; it is probably the largest study to date with over 300 cases and over 700 controls providing comprehensive evaluation of maternal biochemical and genetic determinants of NTDs. We are aware of only one larger study in which 471 cases were recruited over a period of 9 years through various national resources such as the Irish Association for Spina Bifida and Hydrocephalus, but the maternal biochemical measurements were performed many years after delivery and not at the time of diagnosis of NTDs (O'Leary et al., 2005).

Difference in the gestational ages between cases and controls at the time of sampling is one of the major constraints while analyzing and interpreting nutritional markers in this study. Ideally, we should have measured nutritional markers before conception; this is not feasible in practice. In fact, several studies have made such measurements many years after an NTD pregnancy (Ubbink, 1999, Martínez deVillarrreal et al., 2001). We measured nutrient levels as close as possible to the time of diagnosis of the NTDs. The difference in the gestation age of cases and controls is expected to affect circulating homocysteine and vitamin B_{12} levels, which progressively fall with advancing gestation (Morkbak et al., 2007). In another study in Pune, we did not find a difference in B_{12} concentrations between 17 and 34 weeks of gestation, in non-B_{12} supplemented women (Katre et al., 2010). However, plasma holo-TC is stable during pregnancy (Morkbak et al., 2007) and thus supports our interpretation that low B_{12} status in the mothers is associated with the offspring's NTD risk in our population. Results of *TCN2* polymorphism provide a further support to this interpretation.

Another limitation of this study is an inadequate history of antenatal vitamin supplementation that may have confounded the association between circulating markers of one-Carbon metabolism and the risk of NTDs. Higher folate concentrations in the supplemented group but similar vitamin B_{12} and holo-TC concentrations in supplemented and nonsupplemented groups, suggests that the supplements contained little vitamin B_{12}, thus, increasing confidence in our finding of low holo-TC in the cases than in the controls. This was further supported by the association of 776C>G polymorphism in the *TCN2* gene with NTDs.

In summary, this study has demonstrated the association of high maternal homocysteine concentration and a possible role for maternal B_{12} deficiency in the etiology of NTDs in India over and above the well-established role of folate deficiency. The strongest known maternal *MTHFR* risk variant, 677C>T, did not predispose to NTDs while other variants, 1298A>C and 1781G>A, were protective. Association of maternal 776C>G polymorphism *TCN2* gene with NTDs in the offspring lends further support to the role of vitamin B_{12}. Studies exploring role of other genes involved in one-carbon metabolism and epigenetic modifications will provide further insight in the etiopathogenesis of NTDs in India.

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