Molecular Human Reproduction Vol.13, No.6 pp. 419–424, 2007
Advance Access publication April 16, 2007 doi:10.1093/molehr/gam017

Methylenetetrahydrofolate reductase gene polymorphisms and the risk of anencephaly in Mexico

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The precise etiology of neural tube defects (NTDs) is not known. There is some evidence that mutations in *MTHFR* gene provide susceptibility to NTDs in some populations; however, other studies have not found this association. One of the problems with previous studies is that they treat NTDs as a homogeneous group, when specific defects could have different etiologies. We conducted a case–control study specifically for anencephaly, based on the Mexican Epidemiological Surveillance System of Neural Tube Defects to evaluate its association with maternal *MTHFR 677C > T* and *1298A > C* polymorphisms, in three states with high frequencies of NTDs: Puebla, Estado de México and Guerrero. We interviewed and collected blood samples from 118 case mothers and 112 control mothers. The questionnaire included information on their reproductive history, socioeconomic characteristics, prenatal care, tobacco and alcohol use, presence of chronic diseases, acute illnesses and fever, consumption of multivitamins and drugs during the periconceptional period. After adjusting for potential confounders, the risk from the mutated homozygous mothers (*677TT* genotype) was significantly higher than that from mothers with *677CC* genotype (OR 3.16, 95% CI 1.29–7.73); in the case of the heterozygous mothers, an increased risk of anencephaly was observed, even though this was not statistically significant (OR 1.81 95% CI 0.78–4.25). The association found between maternal *677TT* genotype and anencephaly and the elevated presence of the *677T* allele among Mexican women of fertile age urges intensifying folic acid supplementation which has proved to modify this genetic risk in other populations

Keywords: anencephaly/Mexico/*MTHFR* polymorphisms

Introduction

Congenital malformations constitute one of the principal causes of mortality and morbidity in childhood. Failures of neural tube closure designated as neural tube defects (NTDs) constitute one of the main congenital malformations.

In recent years, various clinical and experimental studies have demonstrated that folic acid supplementation during the periconceptional period can prevent the occurrence and recurrence of NTDs (Czeizel and Dudas, 1992). This helped, on the one hand to establish folic acid supplements for women of fertile age, as a fundamental strategy for reducing the prevalence of NTDs, and on the other to generate hypothesis concerning the role played by the mechanisms involved in the transport and metabolism of folic acid, in the origins of these malformations.

Methylenetetrahydrofolate reductase (MTHFR) is an enzyme involved in the metabolism of folic acid through the conversion of 5,10-methylenetetrahydrofolate to 5 methyltetrahydrofolate, the methyl donor for methionine synthesis from homocysteine. This reaction is important in one-carbon metabolism because methionine is the precursor of S-adenosylmethionine, the methyl group donor in more than 100 reactions (Bagley and Selhub, 1998). Two common polymorphisms exist (*677C > T* and *1298A > C*) in the *MTHFR* gene that reduces the enzyme activity in *in vitro* assays. The *677C > T* mutation causes an alanine to valine substitution in the predicted catalytic domain of MTHFR, rendering the enzyme thermolabile; homozygosity for the 677T allele is associated with an increase in homocysteine (Hcy) levels and decreased methyltetrahydrofolate pool, predominantly in states of folate deficiency (Frosst *et al.*, 1995; Weisberg *et al.*, 1998). The *1298 A > C* mutation results in a glutamate to alanine substitution and decreased enzyme activity; however, in both heterozygotes and mutated homozygotes, it does not appear to cause elevations in the plasmatic homocysteine. Individuals, who are compound heterozygotes for both *MTHFR* mutations (*677CT/1298AC* genotype), have a biochemical profile similar to that seen among 677T homozygotes (van der Put *et al.*, 1998; Weisberg *et al.*, 1998).

Some studies have shown that those who are affected by NTDs, their mothers or both, present a greater *MTHFR 677T* allele frequency

and a greater frequency of homozygosity for the *677C > T* mutation (van der Put *et al.*, 1995; Shields *et al.*, 1999; Kirke *et al.*, 2004) whereas others do not find this association (Barber *et al.*, 1999; Lucock *et al.*, 2000, Felix *et al.*, 2004). Studies which have evaluated the association between the *1298C* allele and NTDs are less common and the results reported to date have been inconsistent (van der Put *et al.*, 1998; Felix *et al.*, 2004; De Marco *et al.*, 2002).

Most of these studies have included various NTDs analyzed as if they were a homogeneous group. This reduces the power of finding associations between the genetic marker and the disease (Relton *et al.*, 2003) since different NTDs could have different etiologies (Holmes *et al.*, 1976; Khoury *et al.*, 1982), owing to the fact that the process which causes the closing of the neural tube is different at the anterior pole, where the principal mechanism for closure is neurulation, whereas the closure at the posterior pole is caused by canalization (Elwood *et al.*, 1992). Thus, the risk factors may act in different ways at one level or another; and this is why it is necessary to evaluate the effect of the polymorphisms on specific NTDs.

The Mexican population has one of the highest *MTHFR 677T* allele frequencies in the world (50%) (Mutchinick *et al.*, 1999) and one of the highest frequencies of NTDs, with a birth prevalence of anencephaly, spina bifida and encephalocele, consisting of 8, 9 and 2 per 10 000 live births, respectively (International Clearinghouse for Birth Defects Monitoring System, 2002).

The aim of this study was to evaluate the association between *MTHFR 677C > T* and *1298A > C* polymorphisms in mothers and the risk of anencephaly in their offspring, in three states of the Mexican Republic presenting high frequencies of NTDs: Puebla, Estado de Mexico and Guerrero.

Materials and Methods

A case–control study was carried out, based on the Record of the Epidemiological Surveillance System of Neural Tube Defects (SVEDTN, from the Spanish: Registro del Sistema de Vigilancia Epidemiológica de los Defectos del Tubo Neural), in three states of the Mexican Republic: Puebla, State of Mexico and Guerrero. The SVEDTN forms part of the National Epidemiological Surveillance System which compiles information emanating from all the institutions within the National Health System, such as the Fetal Death Certificates and Death Certificates (in Mexico two kinds of death certificate exist: a Fetal Death Certificate, which is used in the case of offspring who are born dead, and a Death Certificate, which is used when new born babies die after birth).

The study was approved by the Institutional Review Board of National Institute of Public Health of Mexico. All participating mothers were given a letter of informed consent, which was signed prior to participation.

Selection of cases and controls

Cases of 20 or more weeks of gestational age (live birth or stillbirths) that were ascertained by the local SVEDTN between the 1st of March 2000 and the 28th of February 2001 and whose cause of death was anencephaly (International Classification of Diseases, Tenth Revision, code Q00.0) were potentially eligible.

For each of the cases, a control was selected at the same childbirth healthcare center where the cases were born. The control was the next child born alive, without anencephaly or another congenital malformation apparent at the time of birth. For cases as well as controls, the inclusion criteria were that mothers should have resided during the year prior to the birth in the corresponding state and that it should be possible to locate them during the first 3 months post-partum.

In the studied period, 189 cases were identified, which fulfilled the inclusion criteria. Of these, 157 (83.1%) case mothers agreed to participate in the study, whereas 32 refused.

Once a case mother agreed to participate in the study, we contacted the control mother. If the case mother accepted participation but the first eligible control mother did not agree to participate in the study, the following potentially eligible control was considered. A total of 160 control mothers were contacted, of which 151 (94.4 %) agreed to participate in the study. For six cases, it was not possible to find a control that fulfilled the inclusion criteria.

Data collection

A structured questionnaire was administered to mothers of cases and controls who agreed to participate in the study. The questionnaire included questions on reproductive history, sociodemographic characteristics (age, marital/cohabitant status, maternal education and occupation and family income), lifetime tobacco and alcohol consumption, as well as factors referring to the periconceptional period (3 months prior to conception to 1 month after conception), presence of chronic diseases, acute illnesses and fever during the periconceptional period, consumption of multivitamin supplements and medicines during this period and characteristics of prenatal care (month of initiation and number of prenatal consultations).

A standard 85-item food frequency questionnaire was also used to assess nutrient intake from diet. This instrument has been validated for use in epidemiologic studies in Mexico (Hernández-Ávila *et al.*, 1998).

Mothers who refused to participate in the study answered a brief questionnaire which included summarized information on the socioeconomic characteristics (education, income and occupation) and reproductive history.

The questionnaires were administered at home by previously trained nursing personnel. Interviewers had no knowledge of the main hypotheses of the study.

A blood sample could be obtained for 118 case mothers and 112 control mothers in order to determine the maternal genotype. Blood was obtained from the antecubital vain using the vacutainer system and collected in glass test tubes which contained EDTA as an anticoagulant. The samples were stored at 4°C and then transported to the Instituto Nacional de Perinatología (National Institute of Perinatology), where they were centrifuged in order to separate the buffy-coat which was maintained frozen at −70°C until extraction of DNA and genotyping. The laboratory personnel who carried out the genotypification were blinded to the case or control status of the participating mothers.

Genotyping

The DNA was extracted from leukocytes using a Genomic DNA extraction Kit Wizard® (Promega™). *MTHFR* genotype was analyzed by polymerase chain reaction (PCR) in a thermal cycler Eppendorf™, using 5 μl of 10x PCR Buffer (Promega™), 2 mM $MgCl_2$, 5% DMSO, 50 μM of dNTP's mix, 10 pmol of each primer forward: 5′-GCA GGG AGC TTT GAG GCT GAC-3′ and reverse: 5′-AGG ACG GTG CGG TGA GAT G-3′, and 0.5 U of Taq polymerase (Promega™) in a total reaction volume of 50 μl. PCR conditions were as follows: denaturation at 92ºC for 1 min, annealing at 60ºC for 30 s, and extension at 72ºC for 30 s by 35 cycles, followed by a final extension to 72ºC (7 min) (Fig. 1). A 15 μl aliquot of PCR product was incubated at 37ºC for 3 h with 1 unit of *Hinf*I restriction enzyme (New England Biolabs™) and restriction fragments were electrophoresed in a 4% agarose gel stained with ethidium bromide and visualized under UV light in a Fotodyne TM transilluminator. If the *677T MTHFR* allele is present, a restriction fragment is generated for *Hinf*I restriction enzyme and the PCR 228 bp fragment is cut into two fragments of 172 and 56 bp (Fig. 2). For *1298A > C* polymorphism, oligonucleotides forward, 5′-ATG TGG GGG GAG GAG CTG AC-3′ and reverse, 5′-GTC TCC CAA CTT ACC CTT CTC CC-3′ were used with slight PCR reaction modifications. A 241 bp PCR product was amplified and a 15 μl aliquot was digested with 1 unit of *Mbo*II restriction enzyme (New England Biolabs™) at 37ºC for 2 h. Digestion product was electrophoresed in a 4% agarose gel (van der Put and Blom, 2000).

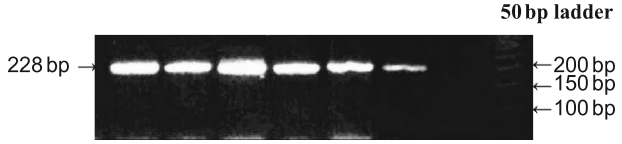

Figure 1: PCR reaction showing the 228 bp fragment, identified through 2% agarose gel electrophoresis, stained with ethidium bromide.

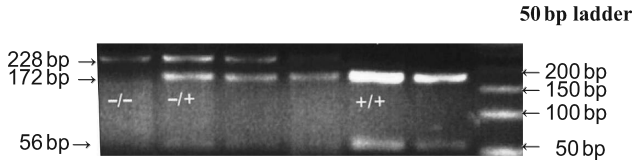

Figure 2: The 677 C → T genotype generates a RFLP through *Hinf*I restriction enzyme digestion, allowing the appearance of two bands of 172 and 56 bpPattern (−/−) 228 bp uncut band represents a wild-type subject, heterozygote state (+/−) is represented by 228, 172 and 56 bp bands and two bands of 172 and 56 bp are a double mutated subject.

Statistical analysis

Cases and controls were compared on their general characteristics by means and proportions. The allelic distribution and maternal genotype for the *MTHFR 677C > T* and *1298A > C* polymorphism among cases and controls was initially compared by proportions and then crude and adjusted odds ratios were estimated using logistic regression.

Confounding was assessed for each one of the variables for which information was available. Those variables that were associated with maternal genotype as well as with anencephaly and modified the odds ratios corresponding to the association between maternal genotype and anencephaly by 10% or more were considered to be confounders, as well as those which were considered conceptually fundamental for explaining the phenomenon under study. The final multivariate model included the following co-variables: maternal age (as a continuous variable), maternal education (divided into two categories: <completed junior high school and completed junior high school or more) and levels of folate consumption in the daily maternal diet categorized into ≤750 μg and more than 750 μg; this limit was selected in agree with the current recommendations for folate consumption among Mexican pregnant women (Bourges-Rodríguez *et al.*, 2004).

We calculated retrospectively the power of the study. We took into account the prevalence of polymorphisms, a significance level of 0.05, the observed odds ratios and a prevalence of anencephaly of 0.0008.

Analyses were carried out using the statistical program Stata 7. (Stata Corporation, College Station, TX, USA) and the Power program version 3.0.0 (National Cancer Institute, USA)

Results

The evaluation of the association of socioeconomic factors and the risk of anencephaly in this population has been reported elsewhere (Blanco Muñoz *et al.*, 2005). However, 78 of the interviewed women did not provide a blood sample. The basic characteristics (education, income and reproductive history) of participants and non-participants mothers were similar, except that those who did not give a blood sample are an average of 1 year older than those who did, 25 versus 24, respectively, $P < 0.05$ (data not shown).

The principal characteristics for case and control mothers are listed in Table 1. As can be observed, the case and control mothers differed significantly in terms of education level and family income, smoking during pregnancy, number of pregnancies and the frequency of adverse reproductive antecedents. Only three women, two case mothers and one control mother, reported the use of multivitamins containing folic acid during periconceptional period.

The distribution of the genotype for the *677C > T* polymorphism in the control population was found to agree with that of Hardy–Weinberg ($P = 0.55$). Among the case mothers the frequency of the

Table 1: Sociodemographic, reproductive and life style characteristics of case and control mothers

Variables	Cases		Controls	
	n[a]	%	*n*[a]	%
Age				
< 20	23	21.50	31	30.10
20–34	73	68.22	68	66.02
≥ 35	11	10.28	4	3.88
Education*				
≥ Junior high complete	23	21.50	39	38.24
< Junior high complete	84	78.50	63	61.76
Monthly family income (in US dollars)*				
> 100	50	46.73	68	67.33
≤ 100	57	53.27	33	32.67
Folate consumption in diet (in μg)				
> 750	12	11.76	18	17.82
≤ 750	90	88.24	83	82.18
Tobacco during periconceptional period**				
No	106	99.07	96	93.20
Yes	1	0.93	7	6.80
Alcohol during periconceptional period				
No	101	94.39	93	91.18
Yes	6	5.61	9	8.82
Number of pregnancies*				
1	40	37.38	49	47.57
2–3	35	32.71	39	37.86
> 3	32	29.91	15	14.56
Adverse reproductive antecedents*				
First time pregnancies	40	37.74	49	49.00
Multi-pregnancies without antecedents	37	34.91	37	37.00
Multi-pregnancies with antecedents	29	27.36	14	14.00
Sex of offspring				
Feminine	55	53.92	47	46.53
Masculine	47	46.08	54	53.47

[a]Due to missing values numbers for some variables do not equal the total of participants.
*$P < 0.05$ by the χ^2 test.
**$P < 0.05$ by the Fisher's exact test.

Table 2: Genotype distribution and allelic frequency of the 677C > T polymorphism among mothers of cases and control mothers

Variable	Cases $n = 118$	%	Controls $n = 112$	%	*P*-value*
Genotype					
CC	14	11.86	25	22.32	
CT	54	45.76	57	50.89	0.018
TT	50	42.37	30	26.79	
Allele C	82	34.75	107	47.77	
Allele T	154	65.25	117	52.23	0.006

*By the χ^2 test

677T allele and the *677TT* genotype was significantly higher than among control mothers (Table 2).

In terms of the *1298A > C* polymorphism, there were no significant differences between case and controls mothers, either concerning the allelic frequency or the genotype distribution (Table 3).

Table 4 presents the odds ratios for *677C > T* polymorphism. After adjusting for maternal age, education and folate consumption in the daily diet, the risk from the mutated homozygous mothers (*677TT* genotype) was significantly higher than that from mothers with *677CC* genotype (OR 3.16, 95% CI 1.29–7.73). In the case of the heterozygous mothers, an increased risk of anencephaly was also observed, even though this was not statistically significant (OR 1.81 95% CI 0.78–4.25).

The power of the study concerning *677TT* genotype was 93%.

No association was found between the maternal *1298C* allele or any of the corresponding genotypes, and anencephaly (data not presented). Nor did we find a significant association with the maternal *677CT/1298AC* genotype (OR: 1.46, 95% CI 0.49–4.38).

Discussion

The results of this study indicate that maternal homozygosity for the *677T* allele increases the risk of anencephaly in their offspring. Even though the heterozygous mothers showed higher risk levels than the 'wild-type' homozygote mothers, this association was not statistically significant. We did not find a positive association between homozygosity or maternal heterozygosity for the *1298C* allele and the risk of anencephaly or when considering the *677CT/1298AC* genotype.

Compared with other studies carried out in Mexico, this one includes the largest number of cases with a single type of NTD. It is also the first study in Mexico to evaluate the association between the *1298A > C* polymorphism and any kind of NTD. In terms of the *677T* mutation among mothers, our results are consistent with those reported by Martínez de Villarreal *et al.* (2001), but differ from those reported by Dávalos *et al.* (2000) and by González-Herrera *et al.* (2002), who did not find an association between this mutation and the risk of anencephaly. Nor did Barber *et al.* (2000), who studied an Hispanic population residing in the USA, find an increased risk of NTDs among women with the mutated allele.

The controversial results regarding the *677T* allele and NTDs might be partially explained by the use of different methods. Many of the studies have treated NTDs as a homogeneous group, not evaluating a specific defect; which might be relevant, if the mutation under consideration is associated with more serious malformations, as in the case of anencephaly, where up to 50% of outcomes are still births and the rest die in the first 48 h of life (Hunter, 1993). It has also been found that maternal homozygosity for the *677T* allele is associated with a greater risk of recurring spontaneous abortion (Nelen *et al.*, 1997; Zetterberg, 2004), which would also indicate that the viability of the pregnancy is reduced. This could explain the discrepancies found between our results and those reported by González-Herrera *et al.* (2002) whose cases were born alive with spina bifida, and who did not find a significant association with the maternal genotype. Dávalos, who included among their cases, the mothers and fathers of children affected by anencephaly, encephalocele and spina bifida, also found no differences between the cases and the control groups concerning the maternal genotype or allelic frequency, although they grouped all the NTDs, without specifying the frequency of each one of the defects included (Dávalos *et al.*, 2000). Martínez de Villarreal *et al.* (2001), whose study included 38 cases of NTDs, of which 34 (89.5%) were anencephalic, had results consistent with ours.

The *677T* allele may only be a risk factor in populations with a poor folate diet, which could explain the lack of consistency among studies (Shields *et al.*, 1999). Supporting this is the fact that the phenotype expression at a metabolic level (increasing levels of plasmatic homocysteine) can be modified through folate supplementation (Nelen *et al.*, 1998) and that the polymorphism influences DNA methylation through an interaction with folate status (Friso *et al.*, 2002). *In vitro* studies found that an impaired folate and homocysteine metabolism affect neural crest cell formation and migration (Boot *et al.*, 2003).

The controversy concerning the association between the *677C > T* variant and NTDs reveals the complexity of the etiology of these kind of malformations and suggests that there are interactions either; gene–gene, gene–environment and gene–nutrition, which contribute to determine the final malformation.

Table 3: Genotype distribution and allelic frequency for the 1298A > C polymorphism among cases and control mothers

Variable	Cases $n = 92$	%	Controls n = 80	%	*P*-value*
Genotype					
AA	66	71.74	51	63.75	
AC	21	22.83	20	25.00	0.32
CC	5	5.43	9	11.25	
Allele A	153	83.15	122	76.25	
Allele C	31	16.85	38	23.75	0.14

*By the χ^2 test.

Table 4: Crude and adjusted odds ratios for anencephaly according to 677C > T genotype distribution in case and controls mothers

Variable	Cases $n = 118$	Controls $n = 112$	Crude odds Ratio [CI 95%]	Adjusted odds ratio[a] [CI 95%]
Genotype				
CC	14	25	1	1
CT	54	57	1.69 [0.79, 3.59]	1.81 [0.78, 4.25]
TT	50	30	2.98 [1.34, 6.59]	3.16 [1.29, 7.73]

[a]Adjusted for maternal age, maternal education and maternal folate consumption in daily diet ($\leq$750 μg; >750 μg).

Our results do not support Barber's hypothesis, which upholds that the association between the mutated genotype and NTDs would be evident in populations where the prevalence of the *677T* allele is low and scarce in populations where the mutation is common (Barber *et al.*, 2000). They are, however, consistent with the observation that at an ecological level a gradient of association exists between the frequency of the mutated allele and the frequency of NTDs (Botto *et al.*, 1999; Botto and Yang, 2000).

Concerning the absence of an association between the *1298A > C* polymorphism and anencephaly our results are consistent with those reported by other authors of the region (Barber *et al.*, 2000; Felix *et al.*, 2004) and do not support the results of van der Put who found that the heterozygotic combination *677T/1298C* was more common among patients affected by NTDs than among their controls (van der Put *et al.*, 1998). De Marco *et al.* (2002) found that among the Italian population, both heterozygosity and maternal homozygosity for the mutated *1298C* allele, increased the risk of DTN, this being to date the only study which found such this association.

The *1298C* allele frequency in our control population (24%) is relatively high and similar to that found in Canada by Weisberg *et al.* (1998), among Ashkenazi Jews (Rady *et al.*, 1999) and among the Italian population (De Marco *et al.*, 2002).

Although efforts were made to avoid potential bias, the nature of this observational study calls for caution in the interpretation of the results. Selection bias was evaluated by comparing the characteristics of the basic characteristics of the women who gave a blood sample and those who did not give one; and all characteristics were similar, except that those who did not give a sample are an average of 1 year older than those who did (25 versus 24, $P < 0.05$). However, it is not likely that older women have a different genotype distribution in a way to affect the direction of the association. However, it reduced the sample size, which is reflected in wide confidence intervals.

Another source of potential selection bias could be differential survival of the pregnancies up to 20 or more weeks of gestational age, since spontaneous abortions were not included in the study. If the women with *677C > T* allele had a greater probability of spontaneously aborting a fetus with anencephaly, the associations between *677CT* or *677TT* genotypes and anencephaly might be underestimated.

Differential recall bias by maternal genotype is not likely since participating women did not know their genotype and the laboratory personnel who carried out the genotypification did not know whether each mother was case or control.

The complex mixture of ethnic groups in Mexico makes it very difficult to stratify by ethnicity, which is 'mestizo' for most of the Mexicans; thus, we cannot rule out the presence of ethnic stratification, nor control for this factor in the analysis. However, given that each control was selected from the same state and in the same childbirth health-care center where the case was identified, we think that both come from the same population and that, if stratification exists, the distribution of the strata would be similar between the case and the control mothers and the association would not be biased.

Additional research also needs to be done regarding the effect of the 677C > T mutation on other specific NTDs that are frequent among this population, such as spina bifida. Also, future studies should be carried out to search the molecular basis that explain the mechanism with which the mutation induces anencephaly.

In terms of public health, we think that the use of genetic tests in order to identify women at high risk entails ethical considerations, especially in terms of confidentiality of the information. Also, in terms of cost–benefit, for the moment it is more efficient to provide supplements for all women rather than carrying out this type of test.

Considering the high prevalence of maternal *MTHFR 677T* allele among Mexican women of fertile age, increased folate intake either through periconceptional folic acid supplementation or food folate fortification, which has proved to modify this genetic risk in other populations, could alleviate the effect of reduced MTHFR activity.

Acknowledgments

To all the case and control mothers, because without them it would not have been possible to carry out this study. To the Health Services in the states of Puebla, Guerrero and Estado de Mexico for their logistic support. This project was supported by the National Council of Science and Technology (CONACYT) no. 28203-M.

References

Bagley PJ, Selhub J. A common mutation in the methylenetetrahydrofolate reductase gene is associated with an accumulation of formylated tetrahydrofolates in red blood cells. *Proc Natl Acad Sci USA* 1998;**95**:13217–20.

Barber RC, Lammer EJ, Shaw GM *et al.* The role of folate transport and metabolism in neural tube defect risk. *Mol Genet Metab* 1999;**66**:1–9.

Barber R, Shalat S, Hendricks K *et al.* Investigation of folate pathway gene polymorphisms and the incidence of neural tube defects in a Texas hispanic population. *Mol Genet Metab* 2000;**70**:45–52.

Blanco Muñoz J, Lacasaña Navarro M, Torres Sánchez LE *et al.* Socioeconomic factors and the risk of anencephaly in a mexican population: a case-control study. *Public Health Rep* 2005;**120**:39–45.

Boot MJ, Steegers-Theunissen RP, Poelmann RE *et al.* Folic acid and homocysteine affect neural crest and neuroepithelial cell outgrowth and differentiation in vitro. *Dev Dyn* 2003;**227**:301–308.

Botto LD, Moore CA, Khoury MJ *et al.* Neural-tube defects. *N Engl J Med* 1999;**341**:1509–1519.

Botto LD, Yang Q. 5,10-Methylenetetrahydrofolate reductase gene variants and congenital anomalies: a HuGE review. *Am J Epidemiol* 2000;**151**:862–877.

Bourges-Rodríguez H, Casanueva E, Rosado JL (eds). Recomendaciones de ingestión de nutrimentos para la población mexicana. Bases fisiológicas, tomo 1. Vitaminas y nutrimentos inorgánicos. Editorial Panamericana, México, 2004.

Czeizel AE, Dudas I. Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation. *N Engl J Med* 1992;**327**:1832–1835.

Dávalos IP, Olivares N, Castillo MT *et al.* The C677T polymorphism of the methylenetetrahydrofolate reductase gene in Mexican mestizo neural-tube defect parents, control mestizo and native populations. *Ann Genet* 2000;**43**:89–92.

De Marco P, Calevo MG, Moroni A *et al.* Study of MTHFR and MS polymorphisms as risk factors for NTD in the Italian population. *J Hum Genet* 2002;**47**:319–324.

Elwood JM, Little J, Elwood JH. *Epidemiology and Control of Neural Tube Defects*. Oxford, England, Oxford University Press: 1992.

Felix TM, Leistner S, Giugliani R. Metabolic effects and the methylenetetrahydrofolate reductase (MTHFR) polymorphism associated with neural tube defects in southern Brazil. *Birth Defects Res A Clin Mol Teratol* 2004;**70**:459–463.

Friso S, Choi SW, Girelli D *et al.* A common mutation in the 5,10-methylentetra-hydrofolate reductase gen affects genomic DNA methylation thorugh an interaction with folate status. *Proc Natl Acad Sci USA* 2002;**99**:5607–5611.

Frosst P, Blom MJ, Lios R *et al.* A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet* 1995;**10**:111–113.

González-Herrera L, García-Escalante G, Castillo-Zapata I *et al.* Frequency of the thermolabile variant C677T in the MTHFR gene and lack of association with neural tube defects in the State of Yucatan, Mexico. *Clin Genet* 2002;**62**:394–398.

Hernández-Ávila M, Romieu I, Parra S *et al.* Validity and reproducibility of a food frequency questionnaire to assess dietary intake of women living in Mexico City. *Salud Pública Mex* 1998;**40**:133–140.

Holmes LB, Driscoll SG, Atkins L. Etiologic heterogenecity of neural-tube defects. *N Engl J Med* 1976;**294**:365–369.

Hunter AGW. Brain and spinal cord. In Stevenson RE, Hall JG, Goodman RM (eds) *Human Malformations and Related Anomalies*. New York, Oxford Univ. Press: USA, 1993,109–137.

International Clearinghouse for Birth Defects Monitoring System. *Annual Report*. With data for 2000. The International Center for Birth Defects, 2002.

Khoury MJ, Erickson JD, James LM. Etiologic heterogenecity of neural tube deffects: clues from epidemiology. *Am J Epidemiol* 1982;**115**:358–548.

Kirke PN, Mills JL, Molloy AM *et al.* Impact of the MTHFR C677T polymorphism on risk of neural tube defects: case-control study. *BMJ* 2004;**328**:1535–1536.

Lucock M, Daskalakis I, Briggs D *et al.* Altered folate metabolism and disposition in mothers affected by a spina bifida pregnancy: influence of 677c → t methylenetetrahydrofolate reductase and 2756a → g methionine synthase genotypes *Mol Genet Metab* 2000;**70**:27–44.

Martínez de Villarreal LE, Delgado-Enciso I, Valdez-Leal R *et al.* Folate levels and N(5),N(10)-methylenetetrahydrofolate reductase genotype (MTHFR) in mothers of offspring with neural tube defects: a case-control study. *Arch Med Res* 2001;**32**:277–282.

Mutchinick OM, Lopez MA, Luna L *et al.* High prevalence of the thermolabile methylenetetrahydrofolate reductase variant in Mexico: a country with a very high prevalence of neural tube defects. *Mol Genet Metab* 1999;**68**:461–467.

Nelen WL, van der Molen EF, Blom HJ *et al.* Recurrent early pregnancy loss and genetic-related disturbances in folate and homocysteine metabolism. *Br J Hosp Med* 1997:**58**:511–513.

Nelen WLDM, Blom HJ, Thomas CMG *et al.* Methylentetrahidrofolate reductase polymorphism affects tehe change in homocysteine and folate concentrations resulting from low dose folic acid supplementation in women with unexplained recurrent abortion. *J Nutr* 1998;**128**: 1336–1341.

Rady PL, Tyring SK, Hudnall SD *et al.* Methylenetetrahydrofolate reductase (MTHFR): the incidence of mutations C677T and A1298C in the Ashkenazi Jewish population. *Am J Med Genet* 1999;**86**:380–384.

Relton CL, Wilding CS, Jonas PA *et al.* Genetic susceptibility to neural tube defect pregnancy varies with offspring phenotype. *Clin Genet* 2003;**64**:424–428

Shields DC, Kirke PN, Mills JL *et al.* The 'thermolabile' variant of methylenetetrahydrofolate reductase and neural tube defects: an evaluation of genetic risk and the relative importance of the genotypes of the embryo and the mother. *Am J Hum Genet* 1999;**64**:1045–1055.

van der Put NM, Blom HJ. Reply to Donelly, Letters to de Editor. *Am J Hum Genet* 2000;**66**:744–745.

van der Put NM, Gabreels F, Stevens EM *et al.* A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects?. *Am J Hum Genet* 1998;**62**:1044–1051.

van der Put NM, Steegers-Theunissen RP, Frosst P *et al.* Mutated methylenetetrahydrofolate reductase as a risk factor for spina bifida. *Lancet* 1995;**346**:1070–1071.

Weisberg I, Tran P, Christensen B *et al.* A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity. *Mol Genet Metab* 1998;**64**:169–172.

Zetterberg H. Methylenetetrahydrofolate reductase and transcobalamin genetic polymorphisms in human spontaneous abortion: biological and clinical implications. *Reprod Biol Endocrinol* 2004;**17**:2–7.

Submitted on December 20, 2006; resubmitted on February 27, 2007; accepted on February 28, 2007