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; homogygosity 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; Lucquet 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 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/cohabitation status, maternal education and occupation and family income), lifetime tobacco and alcohol consumption, as well as factors referring to the periconception 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-Avila 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 vein 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 genotyping 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 MgCl2, 5% DMSO, 50 μM of dNTP’s mix, 10 pmol of each primer forward: 5'-GCA GGG AGC TTT GAG GCT-3' and reverse: 5’-AGG ACG GTG CCG 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 92°C for 1 min, annealing at 60°C for 30 s, and extension at 72°C for 30 s by 35 cycles, followed by a final extension to 72°C (7 min) (Fig. 1). A 15 μl aliquot of PCR product was incubated at 37°C for 3 h with 1 unit of Hinfl 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 Hinfl 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 GAT GC-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 MboII restriction enzyme (New England Biolabs™) at 37°C 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.
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: 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 agreement 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

<table>
<thead>
<tr>
<th>Variables</th>
<th>Cases</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Age&lt;br&gt;&lt; 20</td>
<td>23</td>
<td>21.50</td>
</tr>
<tr>
<td>20–34</td>
<td>73</td>
<td>68.22</td>
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<tr>
<td>≥ 35</td>
<td>11</td>
<td>10.28</td>
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<tr>
<td>Education*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ Junior high complete</td>
<td>23</td>
<td>21.50</td>
</tr>
<tr>
<td>&lt; Junior high complete</td>
<td>84</td>
<td>78.50</td>
</tr>
<tr>
<td>Monthly family income (in US dollars)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; 100</td>
<td>50</td>
<td>46.73</td>
</tr>
<tr>
<td>≤ 100</td>
<td>57</td>
<td>53.27</td>
</tr>
<tr>
<td>Folate consumption in diet (in µg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; 750</td>
<td>12</td>
<td>11.76</td>
</tr>
<tr>
<td>≤ 750</td>
<td>90</td>
<td>88.24</td>
</tr>
<tr>
<td>Tobacco during periconceptional period**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>106</td>
<td>99.07</td>
</tr>
<tr>
<td>Yes</td>
<td>1</td>
<td>0.93</td>
</tr>
<tr>
<td>Alcohol during periconceptional period</td>
<td></td>
<td></td>
</tr>
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<td>No</td>
<td>101</td>
<td>94.39</td>
</tr>
<tr>
<td>Yes</td>
<td>6</td>
<td>5.61</td>
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<tr>
<td>Number of pregnancies*</td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>40</td>
<td>37.38</td>
</tr>
<tr>
<td>2–3</td>
<td>35</td>
<td>32.71</td>
</tr>
<tr>
<td>&gt; 3</td>
<td>32</td>
<td>29.91</td>
</tr>
<tr>
<td>Adverse reproductive antecedents*</td>
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<td>First time pregnancies</td>
<td>40</td>
<td>37.74</td>
</tr>
<tr>
<td>Multi-pregnancies without antecedents</td>
<td>37</td>
<td>34.91</td>
</tr>
<tr>
<td>Multi-pregnancies with antecedents</td>
<td>29</td>
<td>27.36</td>
</tr>
<tr>
<td>Sex of offspring</td>
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<tr>
<td>Feminine</td>
<td>55</td>
<td>53.92</td>
</tr>
<tr>
<td>Masculine</td>
<td>47</td>
<td>46.08</td>
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</tbody>
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*Due to missing values numbers for some variables do not equal the total of participants.
*P < 0.05 by the χ² test.
**P < 0.05 by the Fisher’s exact test.
677T allele and the 677TT genotype was significantly higher than among control mothers (Table 2).

In terms of the I298A > C polymorphism, there were no significant differences between case and controls mothers, either concerning the allelic frequency or the genotype distribution (Table 3).

Table 3 presents the odds ratios for 1298A > 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 I298C 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 I298A > 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.
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 I298A > 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 I298C 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.

Additionally 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.

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### References


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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Cases n = 118</th>
<th>Controls n = 112</th>
<th>Crude odds ratio [CI 95%]</th>
<th>Adjusted odds ratio* [CI 95%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>14</td>
<td>25</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CT</td>
<td>54</td>
<td>57</td>
<td>1.69 [0.79, 3.59]</td>
<td>1.81 [0.78, 4.25]</td>
</tr>
<tr>
<td>TT</td>
<td>50</td>
<td>30</td>
<td>2.98 [1.34, 6.59]</td>
<td>3.16 [1.29, 7.73]</td>
</tr>
</tbody>
</table>

*Adjusted for maternal age, maternal education and maternal folate consumption in daily diet (≤750 μg; >750 μg).

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