The C677T mutation of the 5,10-methylenetetrahydrofolate reductase gene is a moderate risk factor for spina bifida in Italy

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Abstract

Objective—To estimate the risk for spina bifida associated with the common mutation C677T of the MTHFR gene in a country with a relatively low prevalence of NTDs.

Design—Case-control study.

Subjects—Cases: 203 living patients affected with spina bifida (173 myelomeningocele and 30 lipomeningocele); controls: 583 subjects (306 young adults and 277 unselected newborns) from northern and central-southern Italy.

Setting—Cases: three spina bifida centres; young adult controls: DNA banks; newborn controls: regional neonatal screening centres.

Main outcome measures—Prevalence of the C677T genotypes in cases and controls by place of birth; odds ratios for spina bifida and estimated attributable fraction.

Results—The prevalence of T/T, T/C, and C/C genotype was 16.6%, 53.7%, and 29.7% in controls and 25.6%, 43.8%, and 30.6% in cases, respectively. We found no differences between type of defect or place of birth. The odds ratio for spina bifida associated with the T/T genotype v C/C plus T/C was 1.73 (95% CI 1.15, 2.59) and the corresponding attributable fraction was 10.8%. No increased risk was found for heterozygous patients (OR=0.79, 95% CI 0.53-1.18).

Conclusion—This study, as well as the meta-analysis we updated, shows that homozygosity for the MTHFR C677T mutation is a moderate risk factor in Europe, and even in Italy where there is a relatively low prevalence of spina bifida. The estimated attributable fraction associated with this risk factor explains only a small proportion of cases preventable by periconceptional folic acid supplementation. Thus, other genes involved in folate-homocysteine metabolism, their interaction, and the interaction between genetic and environmental factors should be investigated further.

Keywords: spina bifida; MTHFR; folic acid; meta-analysis

Based on several observational studies and two randomised clinical trials, it is widely accepted that periconceptional folate supplementation prevents about 50-70% of all neural tube defects (NTD)1,2. The biochemical and genetic determinants of such a striking effect, however, are largely unknown. A growing body of evidence suggests that alterations in the metabolism of folate and homocysteine may play a role, since spina bifida patients as well as mothers of NTD offspring have moderately increased levels of plasma homocysteine.3,4 However, this is probably not because of a simple deficiency of folic acid in the mothers.

Recent research has focused on genetic factors that could potentially influence the metabolism of these nutrients, perhaps interacting with suboptimal folate supply. The metabolic pathways are complex and include many genes.5 Among these, much interest has been focused on the gene for 5,10-methylenetetrahydrofolate reductase (MTHFR) which has a key role in the metabolism of folates and homocysteine. It has been reported that homozgyosity for the common C677T mutation of the MTHFR gene causes thermolability and reduction of the enzymatic activity.6 These findings have stimulated a number of studies in various countries7-11 to evaluate the role of the C677T mutation in the gene encoding for MTHFR as a possible risk factor for NTD, predisposing subjects with the mutated alleles to so-called functional folate deficiency.12 A further issue that may be of importance in the prevention strategies in various countries is whether the impact of periconceptional folic acid supplementation could be different in populations with various levels of background prevalence of NTDs. It is well known that the occurrence of NTDs shows striking variation by country;13 furthermore, the prevalence seems to be declining in many countries, according to some data, mainly because of improvements in nutrition. Thus, it is an open question whether the effects of folic acid use and the risk associated with the C677T mutation are similar in populations with a different risk of NTD.

We have studied the C677T mutation in Italy, where the prevalence of NTD is lower than that observed elsewhere.14,15

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Table 1  Prevalence of the MTHFR genotypes in controls and spina bifida patients, by type of controls, defect type, and area of birth

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Total (No) %</th>
<th>North (No) %</th>
<th>Central-south (No) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>(583) (235)</td>
<td>(348)</td>
<td></td>
</tr>
<tr>
<td>T/T</td>
<td>16.6</td>
<td>16.6</td>
<td>16.7</td>
</tr>
<tr>
<td>T/C</td>
<td>53.7</td>
<td>49.8</td>
<td>56.3</td>
</tr>
<tr>
<td>C/C</td>
<td>29.7</td>
<td>33.6</td>
<td>27.0</td>
</tr>
<tr>
<td>Newborns</td>
<td>(277) (182)</td>
<td>(95)</td>
<td></td>
</tr>
<tr>
<td>T/T</td>
<td>15.9</td>
<td>14.8</td>
<td>17.9</td>
</tr>
<tr>
<td>T/C</td>
<td>56.3</td>
<td>52.7</td>
<td>63.2</td>
</tr>
<tr>
<td>C/C</td>
<td>27.8</td>
<td>32.5</td>
<td>16.9</td>
</tr>
<tr>
<td>Young adults</td>
<td>(306) (53)</td>
<td>(253)</td>
<td></td>
</tr>
<tr>
<td>T/T</td>
<td>17.3</td>
<td>22.6</td>
<td>16.2</td>
</tr>
<tr>
<td>T/C</td>
<td>51.3</td>
<td>39.6</td>
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</tr>
<tr>
<td>C/C</td>
<td>31.4</td>
<td>37.8</td>
<td>30.0</td>
</tr>
<tr>
<td>Spina bifida</td>
<td>(203) (65)</td>
<td>(138)</td>
<td></td>
</tr>
<tr>
<td>T/T</td>
<td>25.6</td>
<td>26.2</td>
<td>25.4</td>
</tr>
<tr>
<td>T/C</td>
<td>43.8</td>
<td>47.7</td>
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<tr>
<td>C/C</td>
<td>30.6</td>
<td>26.1</td>
<td>32.6</td>
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<tr>
<td>Myelo-MC</td>
<td>(173) (45)</td>
<td>(128)</td>
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<tr>
<td>T/T</td>
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<td>25.0</td>
</tr>
<tr>
<td>T/C</td>
<td>45.1</td>
<td>51.1</td>
<td>43.0</td>
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<td>C/C</td>
<td>28.9</td>
<td>20.0</td>
<td>32.0</td>
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<tr>
<td>Lipo-MC</td>
<td>(30) (20)</td>
<td>(10)</td>
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<td>30.0</td>
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<td>C/C</td>
<td>40.0</td>
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</table>

**Subjects and methods**

**PATIENTS**

We studied 203 patients affected by spina bifida, recruited from three Italian spina bifida centres (Genova, Roma, and Napoli) with the assistance of the Italian Federation of Spina Bifida and Hydrocephalus Associations. All children were affected by spina bifida aperta, either in isolation or associated with a related defect (for example, hydrocephalus). Children with unrelated major defects were excluded. All of them were the first affected children in the nuclear family; 173 of them were affected by myelomeningocele (85.2%) and 30 (14.8%) by lipomeningocele. The level of the defect of the myelomeningocele was not always available and could not be considered in the present study.

**CONTROLS**

We used 583 subjects as a control group. Three hundred and six unrelated young adults (age range 20-49 years) were recruited from an accessible bank of previously collected anonymous DNA samples. They were either volunteers or parents of children with various genetic diseases (mainly cystic fibrosis and FRAXA). None of them reported significant medical problems and had never had any offspring affected by NTD. Two hundred and seventy controls were newborn infants. The blood spots, collected on filter paper, were obtained from three regional newborn mass screening centres (Liguria 87, Piemonte 95, and Marche 95). The regions, and the areas within the regions, were chosen because they had a static population.

**RESULTS**

A recent study showed a high frequency of the C677T mutation in northern Italy.20 For this reason we divided patients and controls into two broad groups: those born in northern (mainly in north-western) regions and those born in the central-southern regions. Since migration is limited in Italy, the birth place of newborns and adult subjects was used as the origin of the subjects studied. All subjects born in Sardinia were excluded from the present report since differences in the prevalence of the C677T mutation were found in a preliminary study.

**BLOOD COLLECTION**

Ten ml of blood were drawn from adult subjects and used for DNA extraction. For newborns, blood spots were collected on filter paper commonly used for the neonatal Guthrie test. The latter method was chosen because of the high number of samples required and to speed up the blood sample collection.

**DNA ANALYSIS**

DNA was extracted from blood according to standard procedures. When blood had been collected on filter paper, it was directly used for PCR amplification. Half of the blood spot was cut and submerged in 130 µl of distilled water, covered with a drop of paraffin oil, and heated at 96°C for at least half an hour. At the end of this time, 25 µl of the PCR amplification mix were added directly to the template (25 µl of lysate). DNA amplification and restriction analysis by HinfI were carried out as previously described.6

**STATISTICAL ANALYSIS**

Heterogeneity of prevalence of the genotypes by defect type and birth place was tested by the chi-square statistic. A p value cut off of 0.05 was used for considering data statistically not heterogeneous. Hardy-Weinberg equilibrium was tested with the chi-square statistic for goodness of fit (1 degree of freedom).9 Maximum likelihood estimate of odds ratios (OR) for the association between genotypes and case status were calculated with their exact mid-p 95% confidence interval (CI) using Epil software.10 OR heterogeneity among studies included in a meta-analysis was tested by the Breslow-Day test.21 Pooled Mantel-Haenszel weighted OR was computed when heterogeneity was not statistically significant (p<0.05). The attributable fraction, representing the proportion of all cases in the target population that is attributable to the presence of the mutation(s), was estimated using the equation:10

\[ \text{AF} = [\text{OR} - (\text{OR}-1)]/\text{OR} \]

where \( \text{OR} \) is the fraction of cases with the genotype under study and \( \text{OR} \) is the odds ratio.

**Results**

Two hundred and three cases with spina bifida and 583 controls were genotyped for the C677T mutation of the MTHFR gene.
The prevalence of T/T, T/C, and C/C genotypes in controls was 16.6%, 53.7%, and 29.7%, respectively (table 1). The frequency of the mutated T allele was 43.5% and that of the wild C allele 56.5%.

The distribution of genotypes was no different in male and female subjects and no statistically significant heterogeneity was found in the distribution of the genotypes or alleles by type of controls or by area.

A slight discordance from Hardy-Weinberg equilibrium was found in the total material ($\chi^2 = 4.96$, df=1 for goodness of fit, $p=0.026$). This was because of the 95 newborns born in the Marche region (central-south area) where an excess of heterozygotes (63.2%, n=60) was found ($\chi^2 = 6.59$, df=1 for goodness of fit, $p=0.010$).

The prevalence of T/T, T/C, and C/C genotypes in patients affected by spina bifida was 25.6%, 43.8%, and 30.6% respectively. The frequency of the mutated T allele was 47.5% and that of the wild C allele was 52.4%. No statistically significant heterogeneity was found in the distribution of the genotypes or alleles by type of defect or by area. The distribution of genotypes was in accordance with Hardy-Weinberg equilibrium ($\chi^2 = 2.97$, df=1 for goodness of fit, $p=0.085$).

The odds ratio for spina bifida to be associated with the T/T genotype was 1.50 (95% CI 0.93, 2.40) when the comparison subjects were only those with the wild type genotype (C/C) (table 2). The odds ratio increased to 1.73 (95% CI 1.15, 2.59) when the comparison group included subjects with the wild genotype and those heterozygous for the mutation (C/C plus T/C).

Excluding the set of 95 control newborns born in the Marche region with apparent Hardy-Weinberg disequilibrium, the odds ratios did not change, although the comparison between T/T and C/C only reached formal statistical significance (OR=1.62; 95% CI 1.03, 2.57). The results did not change when the Mantel-Haenszel adjusted value for area of birth was considered, showing that area of birth was not a confounding factor. When the analysis was conducted for the two different types of spina bifida, no heterogeneity of ORs was found.

Assuming that the association between the homozygosity for the C677T mutation and spina bifida was causal, the proportion of all spina bifida cases that in the whole target population could be attributed to the presence of the homozygous C677T mutation was 8.5% or 10.8% depending on the comparison group used for the risk estimate (T/T v C/C only or T/T v C/C plus C/T).

No increased risk was found for heterozygous patients (OR=0.79, 95% CI 0.53, 1.18).

**META-ANALYSIS WITH OTHER PUBLISHED DATA**

Table 3 summarises the results of seven available studies including the present one. When possible, only data for spina bifida were considered (in the UK study the NTD type was not specified) since four studies, including this one, did not include anencephaly. One found a higher risk for spina bifida than for other NTDs, and one found the 3 T/T genotype only in 31 spina bifida cases and not in 12 anencephaly cases. In other words, studies on the possible association between the MTHFR C677T mutation and NTDs seem to be based more on spina bifida than on other NTDs.

Among the seven studies analysed, a higher risk for spina bifida (or NTDs when not specified) associated with the T/T genotype was found in Atlanta (USA), Ireland, and the Netherlands, as well as Italy, with the OR estimator ranging from 1.7 to 11.9. No increase in risk was found in the UK or France, but the limited sample sizes could not exclude an increased risk in either study. Heterogeneity of the estimated ORs associated with the T/T genotype was present ($p=0.05$), owing to the high OR found in spina bifida patients studied in Atlanta (USA), irrespective of the comparison group (T/C plus C/C or C/C only). Thus, considering only the other six studies, all from Europe, with a homogeneous estimate of the OR ($p=0.29$), the resulting pooled OR was 1.99 (95% CI 1.51, 2.61) when the comparison group included the two T/C plus C/C genotypes and 1.67 (95% CI 1.19, 2.35) when the comparison group included only the C/C genotype (the last figure is based on five studies, since in one data were lacking).

The meta-analysis did not confirm the small increased risk for heterozygous patients suggested by a previous meta-analysis. The pooled OR for heterozygotes was 0.98 (95% CI 0.76, 1.25).

**Discussion**

We have studied the C677T mutation of the MTHFR gene in the Italian population, which has a relatively low prevalence of NTDs, to evaluate its role as a genetic risk factor for spina bifida.

Selection of cases and controls is critical in these kinds of studies. We used as cases children with isolated spina bifida aperta. The majority of them had myelomeningoceles, while a few had lipomeningoceles. All of them were the first occurrence in the nuclear family. They were all alive with an age range between 1 month and 7 years. According to Posey et al., this sample may represent a weakness, because the use of prevalence instead of incidence may
raise the concern that morbidity and mortality associated with the C677T mutation from vascular diseases may affect fetal or neonatal survival and bias the estimates of the possible associated risk.

As controls we used two sets of subjects, young adults and newborns. Young adults were recruited from accessible DNA banks. Some of them were normal volunteers and others were parents of children affected by genetic diseases. Samples were stored anonymously without indication of the status of their children. Among the little information available was the place of birth, age range, and sex. No differences could be found in these three variables. Some heterogeneity could exist in this group since healthy volunteers and parents of children with genetic diseases were present. None of them had a first degree relative affected by NTD. A number of newborns were also used in the present study to validate the frequency of the genotypes first studied in the young adults. We did not find any difference in the frequency of the C677T mutation in these two groups.

In one of the three sets of newborns, an apparent Hardy-Weinberg disequilibrium was found. It was based on 95 subjects and it was because of an excess of heterozygotes. We have no explanation for this finding, except a possible random variation (p=0.01).

Since a high prevalence of the C677T mutation was reported in northern Italy, we analysed the data considering two main areas of Italy, north and central-south. The region of birth of the subjects studied was used as their origin, given the low migration within this country. We found no difference in the genotype frequency among controls or cases by area of birth. Subjects classified as of northern origin were actually almost all born in two north-western regions, Liguria and Piemonte. We excluded from the present study cases and controls from Sardinia since in a continuing study we are observing differences in the prevalence of the MTHFR mutated allele.

In the present study we found a 16.6% prevalence of the T/T genotype among controls, which is the highest prevalence so far published after the 19.0% found in one study in Japan among young adults. Our finding cannot be biased because of the consistency across the different groups of subjects we have investigated, including three sets of non-selected newborns. Moreover, the high prevalence of the T/T genotype in the healthy Italian subjects has also been reported in other independent samples.

In spite of the high prevalence of the 677T/T genotype among healthy subjects we found an increased risk associated with spina bifida. The increased risk was evident, irrespective of the group that was used for comparison, namely subjects with the T/C or C/C genotype (OR=1.73, 95% CI 1.15, 2.59) or only subjects with the wild C/C genotype (OR=1.50, 95% CI 0.93, 2.40). The problem of the composition of the comparison group is not yet defined. Some researchers have used the first alternative, others have used the second one. The underlying rationale is the activity of the enzyme in the heterozygous subjects. Heterozygotes appear to have levels of activity and thermolability that are intermediate between homozygous normal (C/C) and homozygous mutant (T/T). However, a clear effect of heterozygosity for the C677T mutation on total plasma homocysteine levels has not definitely been established yet.

The finding of an increased risk for subjects with the T/T genotype to be affected by spina bifida in Italy is intriguing. It is well documented that in Italy the prevalence of spina bifida and NTDs is lower than in other countries. Data from different areas of Italy (Sicily and Campania in southern Italy, Toscana in central Italy, Emilia Romagna and Veneto in northern Italy) show, during the period 1990-1994, a prevalence rate ranging from 0.28 to 0.38 per 1000 for spina bifida and 0.46 to 0.66 per 1000 for all NTDs. The corresponding figures for the same period in other European countries are 0.47-1.00 for spina bifida and 0.91-1.59 for all NTDs. The low prevalence of spina bifida and all NTDs in Italy is commonly attributed to a high folate intake entirely derived from a diet rich in fresh vegetables and fruit, as periconceptional vitamin supplementation is very rare in Italy. If a significant proportion of NTDs or spina bifida has been already prevented by foltates contained in the normal diet, one might expect that a mutation linked to folate metabolism should not be a significant risk factor for spina bifida or NTDs, or at least should have a very marginal role compared to other countries where the prevalence of spina bifida and NTDs is higher and the diet less rich in folate. However, the present study shows that the T/T genotype is linked to spina bifida, and that the strength of the association, as well as its attributable fraction, is quite similar to that found elsewhere (table 3).

Assuming that the T/T genotype is causally associated with about a two-fold increased risk for spina bifida in Europe, the corresponding attributable fraction should be around 5-15%. This estimate falls well below the 50-70% risk reduction usually estimated by adequate periconceptional folate consumption. Therefore, there is a considerable preventable fraction still unexplained. The possible explanations may be linked to (1) other vitamins (for example, B12, B6) or nutrients (for example, zinc) interacting with foltates, (2) other enzymes involved in folate-homocysteine metabolism (for example, cystathionine β synthase), (3) gene-gene interaction, or (4) gene-environment interaction.

In conclusion, the present study shows that a moderate increased risk for spina bifida is associated with homozygosity for the C677T mutation of the MTHFR gene, even in a country where the prevalence of NTDs is relatively low.
MTHFR mutation and spina bifida

1 US Public Health Service. Recommendation for the use of folic acid to reduce the number of cases of spina bifida and other neural tube defects. MMWR 1992;41:1-7.
22 Miettinen OS. Proportion of disease caused or prevented by a given exposure, trait or intervention. Am J Epidemiol 1974;99:325-32.