Exploring the Effects of Methylenetetrahydrofolate Reductase Gene Variants C677T and A1298C on the Risk of Orofacial Clefts in 261 Norwegian Case-Parent Triads

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Folic acid and the methylenetetrahydrofolate reductase (MTHFR) gene have both been implicated in the etiology of orofacial clefts. The authors selected 261 case-parent triads (173 cases with cleft lip with or without cleft palate (CL/P) and 88 cases with cleft palate only (CPO)) from a Norwegian population-based study of orofacial clefts (May 1996–1998). A case-parent triad design was used to examine whether MTHFR variants C677T and A1298C, and their haplotypes, are risk factors for orofacial clefts. Among CL/P cases, the child’s genotype at C677T or A1298C did not influence the risk. However, children of mothers carrying the C677T variant allele had a lower risk of CL/P. For CPO, children carrying the C677T variant allele had about a twofold increased risk, whereas the mother’s genotypes did not contribute to the risk. The haplotype-based transmission/disequilibrium test showed that except for 677T/1298A (p = 0.06), none of the other haplotypes showed evidence of excess transmission to the offspring. The authors also explored interaction of C677T with maternal use of folic acid among children with CPO. Surprisingly, the risk associated with the child’s carrying either CT or TT was higher (fourfold) when the mother used folic acid. These findings suggest a possible role of MTHFR and folic acid in the causation of orofacial clefts, but the strength and direction of these effects remain to be clarified.

abnormalities; cleft lip; cleft palate; folic acid; genes; genetic predisposition to disease; haplotypes; vitamins

Abbreviations: CI, confidence interval; CL/P, cleft lip with or without cleft palate; CPO, cleft palate only; MTHFR, methylenetetrahydrofolate reductase; PCR, polymerase chain reaction.

With a birth prevalence of approximately two per 1,000 livebirths, orofacial clefts rank among the most common birth defects in humans. On the basis of patterns of familial recurrence and embryological characteristics, orofacial clefts are commonly categorized as cleft lip with or without cleft palate (CL/P) and cleft palate only (CPO) (1, 2). Both genetic and environmental factors are likely to contribute to the risk of clefts. The risk of recurrence in first-degree relatives of affected persons is about 40-fold greater than in the general population, which suggests a strong genetic component (3–5). However, even among first-degree relatives, the absolute risk is no more than 5 percent. Furthermore, the great majority of babies born with orofacial clefts have no close relatives who are affected. Therefore, the role of nongenetic factors must also be taken into account. The considerable heterogeneity in orofacial clefts complicates both case ascertainment and measurements of prevalence. Certain populations consistently show higher clefting rates (6, 7). Norway, for example, has one of the highest rates of CL/P in the world (8). This fact, coupled with the
highly organized systems of registry, makes Norway a promising setting in which to conduct population-based genetic and environmental studies of orofacial clefts.

There is no conclusive evidence that intake of vitamins is related to the risk, but maternal periconceptional use of folic acid has been linked to a reduced risk of orofacial clefts (9, 10). An increased risk associated with maternal use of the epilepsy drugs phenytoin and phenobarbital has also been related to the effects of these drugs may have on folic acid metabolism (11). If intake of folic acid affects the risk of orofacial clefts in some pregnancies, one might further hypothesize that variants of genes involved in metabolism of folic acid could also be associated with risk. Such hypotheses have initiated studies of the methylenetetrahydrofolate reductase (MTHFR) gene, which encodes a key enzyme in folic acid metabolism.

MTHFR is localized to chromosomal region 1p36.3 (12). C677T (alanine to valine) was the first common MTHFR variant identified, and it was shown to correlate with reduced enzyme activity and increased thermolability in both the heterozygous and homozygous state (13, 14). A second common MTHFR variant, A1298C (glutamate to alanine), has also been associated with decreased enzyme activity, although to a lesser extent than C677T (15, 16). Furthermore, certain genotype combinations of these two MTHFR variants have been reported to occur at very low frequencies (16, 17). The more frequent occurrence of the 677T/1298C haplotype among spontaneously aborted embryos has led to speculation as to whether this haplotype might compromise fetal viability (18, 19).

We used a case-parent triad design to examine whether MTHFR variants C677T and A1298C, and their haplotypes, are risk factors for CL/P and CPO. This paper reports on that study.

MATERIALS AND METHODS

Subjects

This study was approved by the Regional Committee on Research Ethics for Western Norway and by the Institutional Review Board of the National Institute of Environmental Health Sciences. A total of 261 complete case-parent triads were selected from within a larger population-based case-control study of orofacial clefting in Norway. Cases were recruited during the period May 1996–1998 from the two surgical treatment centers that together treat all babies with cleft lip and cleft palate in Norway. Most cases were recruited within 2 months after birth, and the overall participation rate was 93 percent. Of the 261 families, 173 included a child with CL/P and 88 included a child with CPO (table 1); 156 CL/P and 63 CPO cases had no accompanying defects. We chose to perform initial analyses on the two main categories of clefts (including cases with defects other than clefts) because 1) there was limited statistical power to pursue the individual effects for each case group subdivided by type of anomaly, and 2) no definitive prior knowledge exists about which subgroup of cases would be more susceptible to the effects of the MTHFR variants studied here. When there was evidence of an effect, we also performed subanalyses in subcategories of cases, especially for those without other defects or syndromes.

After informed written consent was obtained, blood was drawn from cases during surgery, and parents also donated blood. Information on maternal use of folic acid and multivitamins, as well as the mother’s age and number of previous pregnancies and the baby’s sex, was obtained from a self-administered questionnaire (“Pregnancy, Heredity and Environment,” available online at the following Internet Web site: http://dir.niehs.nih.gov/direb/norwayclefts/home_norwayclefts.htm). Mothers of affected children completed this questionnaire about 3 months following the birth of their baby. Mothers who reported any form of folic acid supplementation, including multivitamins that contained folic acid, during the last month before they became pregnant or during the first 2 months of pregnancy were categorized as folic acid users (mothers were also asked to provide vitamin bottles so that the ingredients could be verified). All other mothers were categorized as nonusers. In Norway, there is no general fortification of common food items.

Genetic analysis

Genomic DNA was extracted manually from whole blood by using standard phenol/chloroform extraction methods. All 261 case-parent triads were checked for discrepancies in Mendelian inheritance by genotyping for a tetranculotide repeat marker D5S2842 from the Cooperative Human Linkage Center (http://gai.nic.ni.nih.gov/html-chlc/Chlc-Markers.html) and by analyzing band patterns on denaturing polyacrylamide gels for nonmatching genotypes. All genotyping was performed blinded to the subject’s cleft status (CL/P or CPO) and to maternal use of folic acid supplementation. We used the 5′-exonuclease (or TaqMan (Applied Biosystems, Foster City, California)) assay (20) for genotyping MTHFR variants C677T and A1298C. Briefly, this method relies on the use of two allele-specific TaqMan probes that anneal to their corresponding target sequences. Each probe is connected to a fluorescent reporter dye at the 5′-end and a quencher dye at the 3′-end. The quencher prevents the reporter from emitting fluorescence; hence, no signal is detected. The 5′-exonuclease activity of the Taq DNA polymerase excises the reporter more efficiently if the probe hybridizes to a perfectly matched complementary target sequence, causing release of the reporter and emission of a quantifiable fluorescence. We performed the TaqMan assays for the two MTHFR variants essentially as described by Ulvik and Ueland (21), and we analyzed the allele-specific fluorescence signals after polymerase chain reaction (PCR) (i.e., in endpoint reactions and not in real time).

The PCR-based restriction fragment length polymorphism assay previously reported by Frostell et al. (14) for genotyping C677T was modified slightly to result in better fragment separation and none of the fragments running off the gel. This test was used to verify results obtained by using the TaqMan assay, especially for samples whose signal traces were difficult to interpret. The test consisted of amplifying a 176 base-pair product by using standard PCR conditions, forward primer 5′-cca ccc cca agc agg gag ctt tg, and reverse primer 5′-tgg gaa aga tcc cgg gga cga tg. (To view the...
MTHFR nucleotide sequence, enter accession number U09806 in the GenBank database (National Institutes of Health, Bethesda, Maryland) at the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/Genbank/index.html). Thermal cycling consisted of an initial denaturing step at 94°C for 3 minutes followed by 35 cycles at 94°C for 30 seconds, annealing at 60°C for 60 seconds, and primer extension at 72°C for 60 seconds. A total of 10 µl of PCR product was added to 20 U of Hinf I, 2 µl 10 x RE buffer (New England Biolabs Inc., Beverly, Massachusetts), and double-distilled water was added to a final volume of 20 µl. After 3–4 hours of incubation at 37°C, the reaction was stopped by heat inactivation at 80°C for 20 minutes. The digests were then electrophoresed on a 3 percent NuSieve agarose gel (BioWhittaker Molecular Applications, Rockland, Maine) at 80 volts for approximately 1 hour. Hinf I-treated PCR products of known C677T genotypes and a sample of uncut PCR product served as controls and were run alongside samples being tested. The wild-type CC genotype yields an intact 176 base-pair product, whereas the CT genotype results in three fragments of 176, 113, and 63 base pairs. Results based on the PCR-restriction fragment length polymorphism method corroborated those obtained from the TaqMan assay.

<table>
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<td>Mother's age (years)†</td>
<td>No. %</td>
<td>No. %</td>
</tr>
<tr>
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</tr>
<tr>
<td>20–24</td>
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<tr>
<td>During first trimester</td>
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<tr>
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<td>34 39</td>
</tr>
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<td>31 18</td>
<td>13 15</td>
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<td>12 14</td>
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</table>

* CL/P, cleft lip with or without cleft palate; CPO, cleft palate only.
† For both categories, mean age = 29.2 years.
‡ Defined as a self-report of drinking at least two or more units of alcohol per drinking occasion and drinking at least twice per month during the first 2 months of pregnancy.
§ Yes, used folic acid in some form; no, did not use any supplements identified as containing folic acid; unknown, used supplements but did not know the folic acid content or used supplements but could not tell whether use occurred during the months of interest.
TABLE 2. Distribution of triad types for variant alleles of the methylenetetrahydrofolate reductase gene in 173 cleft lip with or without cleft palate triads selected from a Norwegian population-based study of newborns, May 1996–1998

<table>
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<tr>
<th>MFC†</th>
<th>Mating type</th>
<th>MTHFR&lt;sup&gt;*&lt;/sup&gt;-C677T</th>
<th>MTHFR&lt;sup&gt;*&lt;/sup&gt;-A1298C</th>
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<tr>
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<td>4</td>
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</tr>
<tr>
<td>1 2 2</td>
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<td>2</td>
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</tr>
<tr>
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<td>3</td>
<td>6</td>
</tr>
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</tr>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
<td>0 0 0</td>
<td>6</td>
<td>46</td>
<td>47</td>
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</table>

* MFC, number of copies of the variant allele in the mother, father, and child; MTHFR, methylenetetrahydrofolate reductase gene.
† Mating type is defined by the number of copies of the variant allele carried by each of the two parents.

Statistical analysis

Gene effects. A log-linear-based method was used to analyze the asymmetric distribution of a particular variant allele among affected offspring and their biologic parents (22–24). This method was based on genotypes of cases and their parents stratified into the 15 possible types of triads (tables 2 and 3), which enabled estimation of the relative risks associated with either the mother’s or the offspring’s genotype. The log-linear model was also fitted to prevent mutual confounding of the effects of the mother’s and the child’s genotypes. The models can be fitted with or without the assumption of Hardy-Weinberg equilibrium. We assessed Hardy-Weinberg equilibrium both by the goodness of fit of these models and by testing for deviation from expected counts in the genotype distributions among the 522 families by type of facial defect. In both cleft categories, the average maternal age was 29.2 years. There was the expected predominance of males with CL/P (64 percent) and the less-skewed sex distribution among CPO cases (49 percent male). Ten percent of the CL/P babies had other reported defects, whereas the proportion was 28 percent.

Haplotype analysis. We used the TRANSMIT software program, version 2.5.4 (25), to analyze haplotypes defined by the C677T and A1298C variants. This test is based on a generalization of the transmission/disequilibrium test and can be used to test for excess transmission of multilocus haplotypes in a case-parent triad design. Using this method, we estimated the unknown haplotype frequencies among parents and performed a hypothesis test of excess transmission of particular haplotypes to the offspring.

Gene-environment interactions. We assessed interaction between folic acid intake and the effect of MTHFR variants by comparing the strength of the genetic associations after stratifying by whether the mother was or was not a folic acid user (26). As a variation of the case-only study, the case-parent triad design cannot be used to estimate the main effects of exposures. However, a different effect of genotype by level of exposure would indicate that an exposure might have an effect within certain genotypes. We dichotomized case-parent triads in the CL/P and CPO categories by maternal use or nonuse of folic acid. If possible, we assumed that the variant allele had either a dominant or a recessive effect to reduce the number of parameters in the Poisson regression model and to increase statistical power. If justified, these analyses were also performed under the assumption of Hardy-Weinberg equilibrium. For descriptive statistics, SPSS statistical software, version 10 for Windows (SPSS, Inc., Chicago, Illinois), was used, and all other statistical analyses were performed by using STATA 7 software (Stata Corporation, College Station, Texas).

RESULTS

Table 1 provides descriptive characteristics of the case families by type of facial defect. In both cleft categories, average maternal age was 29.2 years. There was the expected predominance of males with CL/P (64 percent) and the less-skewed sex distribution among CPO cases (49 percent male). Ten percent of the CL/P babies had other reported defects, whereas the proportion was 28 percent.
among CPO cases. More than 90 percent of mothers were Norwegian by birth. Two thirds of mothers reported using folic acid supplements during pregnancy. Tables 2 and 3 provide the specific frequency distributions of the 15 possible types of triads for the two MTHFR markers, C677T and A1298C, for each cleft subtype. Using parental data alone, we checked whether the observed genotype counts for the two markers differed significantly from the expected counts under the assumption of Hardy-Weinberg equilibrium. They did not: based on a chi-square test of equilibrium, $p$ values were 0.77 for C677T and 0.13 for A1298C.

**CL/P**

Figure 1 shows the relative risks for CL/P associated with each of the two MTHFR variants. There was little evidence that carrying either variant of MTHFR affected the child’s risk of CL/P. For mothers, however, the C677T variant allele appeared to lower the risk of CL/P in the offspring. When analyzed under a dominant model, the relative risks were 0.71 (95 percent confidence interval (CI): 0.49, 1.04) for offspring of mothers carrying one copy of the variant allele and 0.38 (95 percent CI: 0.17, 0.86; $p = 0.02$) when mothers were homozygous. When the analyses were repeated under the assumption of a recessive model, the relative risk was 0.53 (95 percent CI: 0.26, 1.10). A1298C showed no associations with CL/P whether carried by the mother or her child.

**CPO**

For CPO, there was a modest increased risk among children carrying one or two copies of the C677T variant allele (figure 2). The relative risks were 2.1 (95 percent CI: 1.2, 3.6) with one copy and 1.7 (95 percent CI: 0.6, 5.4) with two copies (a dominant pattern). When we restricted the analysis to the 63 isolated CPO triads, the relative risk estimates were 2.4 (95 percent CI: 1.2, 4.6) and 1.4 (95 percent CI: 0.3, 6.1) with one and two copies of the variant allele, respectively.

In contrast, a recessive pattern of reduced risk was found among children carrying the A1298C genotypes. When the child was homozygous for the A1298C variant allele, the reduction in risk was estimated at 0.30 (95 percent CI: 0.09, 1.04). The same alleles carried by the mother showed no particular patterns of risk in the offspring.

**Haplotype-based transmission/disequilibrium tests for C677T and A1298C**

As expected, the 677T and 1298A alleles were in strong linkage disequilibrium (table 4). Only a single parent had the 677T/1298C haplotype, which was not transmitted to the child. Tests for association did not indicate excess transmission of any of the haplotypes to the CL/P offspring. In the CPO category, however, 677T/1298A appeared to be transmitted to the offspring more often than would have been predicted from its distribution among the parents ($p = 0.06$). Restricting the analyses to the isolated CPO triads ($n = 63$) did not materially change the results ($p = 0.07$).

**Interaction between C677T and folic acid**

The C677T variant has been implicated in the metabolism of folic acid. Because we found the suggestion of an association of C677T with the risk of cleft palate (when the variant allele was carried by the child), we decided to explore a possible interaction of this variant with the mother’s periconceptional intake of folic acid. We assumed Hardy-Weinberg equilibrium and a dominant effect of the variant allele (figure 2). The effect of the allele was estimated separately for folic acid users and nonusers. Surprisingly, there was a higher relative risk of 4.3 (95 percent CI: 1.6, 12) for children whose mothers used folic acid supplements compared with children whose mothers did not use them (relative risk = 1.4, 95 percent CI: 0.7, 2.8) (figure 3). There was statistically significant heterogeneity among the relative risks ($p = 0.03$).

As mentioned earlier, there was little evidence that carrying the C677T genotypes affected the child’s risk of CL/P (figure 1). Nonetheless, we looked for possible interaction between maternal intake of folic acid and infant’s C677T genotypes. No evidence of an interaction was observed; the $p$ values for interaction were 0.66 under a recessive model and 0.14 under a dominant model.
DISCUSSION

Maternal genes may shape the early environment of the fetus, and genes that metabolize essential nutrients or harmful chemicals are particularly relevant. Thus, it is important to study the effects of both maternal and infant genotypes when evaluating susceptibility loci for orofacial clefts. This analysis was part of a larger project to explore genetic risk factors for orofacial clefts using the case-parent triad design. In an earlier study, we considered genes that are involved in embryonic development and that prior studies have suggested as candidate genes (27). In this study, we focused on folic-acid-metabolizing gene \( \text{MTHFR} \), which has also been implicated in the causation of orofacial clefts.

The benefits of folic acid supplementation in reducing the risk of neural tube defects in the newborn are well established (28–32). The biologic basis is thought to involve overcoming a substrate deficiency in the pathway for folic acid metabolism (33, 34). Several studies have shown that folic acid may also have a similar beneficial effect on reducing the risk of orofacial clefts (35–40), but others could not substantiate these findings (29, 41). Some indirect evidence of the protective effects of folic acid has also been gained through studies of the effects of folic acid antagonists on the risk of orofacial clefts (42). Furthermore, studies in animal models and humans have also suggested a possible link between folic acid and clefts (9, 43–47).

To explore the contribution of \( \text{MTHFR} \) variants to the risk of orofacial clefts, we used a case-parent triad approach. Statistical analysis based on a log-linear method enables the effects of genetic variants harbored by the child and the mother to be assessed separately. We report the actual distribution of genotypes among the 261 case-parents triads (tables 2 and 3), which enables pooling of these data with those from other studies for future meta-analyses. Our aim was to examine whether two common variants of \( \text{MTHFR} \), \( C677T \) and \( A1298C \), and haplotypes of these two variants, are risk factors for CL/P and CPO. Although our study lacked statistical power for an extensive study of interactions, we did select the gene effect that was statistically the strongest to evaluate possible interaction with maternal folic acid intake.

CL/P

For variant alleles carried by the child, our data did not indicate an increased risk of CL/P with any of the \( \text{MTHFR} \) variants examined. However, there was the suggestion of a maternal effect through the \( C677T \) genotypes. Surprisingly, mothers carrying either one or two copies of the \( C677T \) variant seem to reduce the risk of CL/P in the child in a manner that appears to be dose specific (figure 1). Evidence from functional studies on the MTHFR enzyme has shown that the \( C677T \) variant produces a thermolabile enzyme with reduced activity (14, 33). Mothers with the \( CT \) and \( TT \) genotype would thus be expected to have a poorly functioning MTHFR enzyme, which does not seem to correspond with
the reduced risk observed among offspring of mothers with the variant allele.

CPO

We observed a dominant pattern of increased risk of CPO with the child’s C677T genotypes. Most previous studies focused on the risk of CL/P with C677T, and there are only a few reports on CPO. In a case-control study in Ireland, Mills et al. (48) estimated a higher risk with the TT genotype based on a comparison of 27 CPO cases with 848 controls (odds ratio = 3.2, 95 percent CI: 1.3, 7.4). Two other studies have been negative. A case-control study in California compared 117 CPO cases with 383 controls (49) and found no increase in risk of CPO among infants homozygous for the C677T variant allele (odds ratio = 0.6, 95 percent CI: 0.3, 1.3). In a recent study, Beaty et al. (50) applied different statistical tests to evaluate (in addition to other susceptibility loci) the risk of having certain genotype combinations of C677T and A1298C. Among 83 CPO cases tested, no evidence of an association was found with the MTHFR markers (p = 0.14).

C677T and A1298C haplotypes

When haplotypes of C677T and A1298C were examined among the 522 parents, almost complete linkage disequilibrium was observed between the 677T and 1298A alleles (table 4). Such tight linkage between 677T and 1298A suggests that these two alleles may form part of an ancestral haplotype in which recombination has not had sufficient time to shuffle the alleles between the two homologues. The proximity of these two polymorphisms (approximately 1.9 kilobases apart) also contributes to upholding this tight linkage. This skewed distribution of C677T and A1298C haplotypes has also been observed in other studies (16, 18, 19), and the rare occurrence of the 677TT/1298C haplotype has been hypothesized to result from a selective disadvantage. Since the variant allele of one of the two markers almost always follows the normal allele of the other, an effect of one of the MTHFR variants could hypothetically produce an apparent reverse effect of the other variant. This possibility indeed appears to be the case, as seen in figures 1 and 2, when the relative risk associated with the 677-1298T genotype is negatively correlated with the corresponding risk estimates for 1298-CC. Thus, our data are consistent with a scenario in which the C677T variant accounts for the observed increase in risk, whereas the risk associated with A1298C reflects the tight linkage.

Results from the haplotype-based transmission/disequilibrium tests for association (table 4) were consistent with those obtained when each MTHFR variant was analyzed separately for each cleft category (figures 1 and 2). Again, the most noteworthy findings were in the CPO category. In particular, there was the suggestion of excess transmission of the 677TT/1298A haplotype to the offspring (p = 0.06). This result is consistent with the observations that the C677T variant on its own is a risk factor for CPO (figure 2) and that the 677T allele invariably cosegregates with 1298A (table 4).

Interaction between C677T and maternal folic acid intake

Our sample size limited exploration of interactions between the variant alleles and vitamin intake. The allele most strongly associated with orofacial clefts was the C677T variant of MTHFR (a risk factor for CPO when carried by the child). We found a significant increase in the risk of CPO for children with one or two copies of the C677T variant allele, which is consistent with an effect of poor folic acid metabolism among these children. When we stratified these cases by whether the mother did or did not use folic acid vitamins during the crucial early stages of pregnancy, we found that the effect was enhanced (rather than reduced) when the mother used folic acid supplements (figure 3). The triad design does not enable us to compare absolute levels of risk; only the relative effects of the genetic variant can be determined for each stratum of exposure. Therefore, we were not able to distinguish a scenario in which the risk was reduced by folic acid among children without the MTHFR variant from a scenario in which folic acid increased the risk for children with the MTHFR variant (51). Both scenarios are consistent with our results.

These results are not unlike the findings of Shaw et al. (52), who evaluated the risk of another congenital anomaly (spina bifida) by infant C677T genotypes and maternal periconceptional use of vitamin supplements containing folic acid. Among infants heterozygous for C677T, the risk of spina bifida was highest among those whose mothers were early vitamin users (odds ratio = 1.9, 95 percent CI: 0.9, 3.8). However, before our observation on interaction can be
regarded as biologically based, it needs to be replicated in an independent study.

At this stage of analysis, we chose to perform all analyses on the two main categories of clefts, CL/P and CPO, which also included cases with other birth defects. There may be heterogeneity between subgroups of cases with or without other defects. However, we know of no prior evidence to suggest that the presence of other genes or genetic aberrations associated with syndromes affects fetal susceptibility to the effects of the MTHFR variants. Despite the large number of triads used in this study, our sample size was still too limited to further pursue effects among subgroups of cases. Until a sufficiently large sample size becomes available for a more definitive analysis, we will not be able to test whether there was a difference in the effects of the MTHFR variants in the subgroup of cases with other birth defects.

Nevertheless, we did perform subanalyses for the larger category of isolated cases whenever there were signs of an overall effect. In all analyses restricted to cleft cases without other defects, the results did not change substantially.

In conclusion, our study suggests that children who carry the C677T variant of the MTHFR gene may have an increased risk of cleft palate. This finding supports the hypothesis that folic acid may play a role in the etiology of cleft palate. However, the observation that the risk associated with this allele was apparently higher when the mothers used folic acid points to the possible complexity of this etiologic association. Studies across a range of designs with even larger sample sizes and more complete measures of vitamin consumption are needed to clarify the role of MTHFR in orofacial clefts.

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act either directly or through maternal effects and that may be subject to parental imprinting. Am J Hum Genet 1998;62:969–78.