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Folate-Related Gene Polymorphisms as Risk Factors for Cleft Lip and Cleft Palate

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Abstract

BACKGROUND—Cleft lip with or without cleft palate (CLP) and cleft palate only (CPO) have an inherited component and, many studies suggest, a relationship with folate. Attempts to find folate-related genes associated with clefts have, however, often been inconclusive. This study examined four SNPs related to folate metabolism (*MTHFR* 677 C→T, *MTHFR* 1298 A→C, *MTHFD1* 1958 G→A, and *TC* II 776 C→G) in a large Irish population to clarify their relationship with clefts.

METHODS—Cases and their parents were recruited from major surgical centers performing cleft repairs in Ireland and a support organization. Data on risk factors, medical history, and DNA were collected. Controls were pregnant women from the greater Dublin area (n = 1,599).

RESULTS—CLP cases numbered 536 and CPO cases 426 after exclusions. CPO mothers were significantly more likely than controls to be *MTHFR* 677 TT, OR 1.50 (95% CI: 1.05–2.16; p = .03). Log-linear analysis showed a borderline association (p = .07). Isolated CPO case mothers were significantly more likely than controls to be homozygous for the *MTHFD1* 1958 G→A variant, OR 1.50 (95%CI: 1.08–2.09; p = .02). When multiple cases were added, both CPO cases and case mothers were significantly more likely to be AA (p = .02 and p = .007, respectively). The CLP case-control and mother-control analyses also showed significant effects, ORs 1.38 (95% CI: 1.05–1.82; p = .03) and 1.39 (95% CI: 1.04–1.85; p = .03), respectively.

CONCLUSIONS—Associations were found for both CPO and CLP and *MTHFD1* 1958 G→A in cases and case mothers. *MTHFR* 677 C→T could be a maternal risk factor for clefts but the association was not strong. Because multiple comparisons were made, these findings require additional investigation. Given the known association between *MTHFD1* 1958 G→A and NTDs, these findings should be explored in more detail.

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Keywords

cleft lip; cleft palate; oral clefts; folate; folate genes; vitamin B12; transcobalamin gene

INTRODUCTION

There is considerable evidence suggesting that folate-related genes play a role in the etiology of oral facial clefts. Clefts are known to have a strong genetic component. Nonsyndromic clefts are complex traits and it is likely that genetic factors interact with environmental factors. Folate has long been considered one such factor. A number of observational studies (Czeizel et al., 1996, 1999; Itikala et al., 2001; Loffredo et al., 2001; Shaw et al., 1995; Tolarova and Harris, 1995; van Rooij et al., 2004; Boyles et al., 2008), although not all (Hayes et al., 1996; Czeizel, 1993), have reported lower rates of clefts in the offspring of mothers who took folic acid during the periconceptional period. Some studies have found a decline in cleft rates since food fortification with folic acid began (Canfield et al., 2005; Yazdy et al., 2007), although others have not found a change (Ray et al., 2003; Simmons et al., 2004). These observations have led investigators to look for associations between clefts and folate enzyme genes, including methylenetetrahydrofolate reductase (*MTHFR*) (Jugessur et al., 2003; Gaspar et al., 1999; Blanton et al., 2002; Martinelli et al., 2001; Prescott et al., 2002; Shotelersuk et al., 2003; Shaw et al., 1998; van Rooij et al., 2003; Vieira et al., 2005; Mills et al., 1999) and methylenetetrahydrofolate dehydrogenase—the trifunctional cytoplasmic enzyme methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase/formyltetrahydrofolate synthetase—*MTHFD1* (Boyles et al., 2008; Mostowska et al., 2006). Because of the interrelationship between vitamin B12 and folate, the plasma vitamin B12 transporter, transcobalamin 2 (*TCII*), has also been investigated for a role in clefts (Boyles et al., 2008). The results of these investigations have been mixed: some demonstrating strong associations and others failing to find any significant associations. This lack of consensus may be the result of genetic differences, nutritional factors, or the lack of sufficient numbers of subjects to find an effect.

We sought to clarify the role of common variants in folate genes that are known to be expressed during development using a large, homogeneous population from an area, Ireland, where food fortification is not mandated, and folic acid supplement use is uncommon.

METHODS

Subjects

Subjects with cleft palate only (CPO) or cleft lip with or without cleft palate (CLP) along with their mothers and fathers were identified by several mechanisms. Samples were collected at the Dublin Cleft Centre in Ireland, which consists of two units with a single database and common protocols between two surgeons and the cleft teams. The majority (80%) of Irish patients with cleft lip and palate are treated through the Centre. Subjects were also recruited from the membership of the Cleft Lip and Palate Association of Ireland as previously described (Mills et al., 1999). Cleft subjects or their parents were either interviewed or completed the interview questionnaire themselves and, when necessary, had an interviewer review their answers with them in order to collect data on socio-demographic variables, obstetrical history, family history, and relevant data on the affected pregnancy including medication use, folic acid exposure, alcohol, and smoking. Recruitment was conducted in two phases with a more detailed questionnaire being used in the second phase (985 families) than the first phase (103 families). Medical information was provided by the attending surgeons for 87% of the cleft cases. Written informed consent was obtained from all participants. Ethical approval was granted by the

Research Ethics Committees of the Health Research Board of Ireland, the participating hospitals, and the Institutional Review Board at NIH.

Control Samples

Between 1986 and 1990, blood samples were obtained from a population of 56,049 pregnant women attending the three main maternity hospitals in the Dublin area. Controls for this study were a random sample (n = 1,000), selected after all mothers who had given birth to a child with a known malformation, including NTD, orofacial cleft, congenital heart defect, Down syndrome, or limb deformity had been removed. Mothers with a previous history of an NTD-affected pregnancy were also excluded. Additional controls (n = 599) selected from the same pool of 56,049 mothers for previous work (Parle-McDermott et al., 2006) were used to augment the 1,000 random controls for this analysis. Subjects gave consent for the samples.

Genotyping Methods

Whole blood or buccal swab samples were used for the extraction of genomic DNA using a QIAamp DNA Blood Mini Kit (Qiagen, UK). For all polymorphisms, genotyping quality was tested by repeat genotyping approximately 10% of the samples using the initial genotyping method. In addition, the assays had been previously verified by replicate genotyping approximately 10% of samples using an independent assay. All genotypes were independently double scored with repeat genotyping reaching 95% agreement with the original genotyping results in order to achieve a high level of quality control. The *MTHFD1* 1958G→A, R653Q, (rs2236225) polymorphism assay was performed by restriction fragment length polymorphism (PCR-RFLP) using *Msp* I as previously described (Brody et al., 2002). The *MTHFR* 677C→T, A222V (rs1801133) polymorphism was performed by PCR-RFLP using *Hinf* I as previously described (Frosst et al., 1995). The *MTHFR*1298A→C A429E (rs1801131) polymorphism was PCR amplified as described in van der Put et al. (1998) and genotyping was carried out via allele specific oligonucleotide analysis as described in Parle-McDermott et al. (2003). The *TCII* 776C→G, P259R, (rs1801198) polymorphism was genotyped using an allele-specific primer extension assay and scored by matrix-assisted laser desorption/ionization-time of flight MALDI-TOF mass spectrometry (Sequenom, San Diego, CA). The success rates and agreement rates for repeat genotyping were, respectively, for *MTHFD1* 1958G→A, R653Q 99.2% and 98.4%, for *MTHFR* 677C→T, A222V 99.9% and 95.1%, for *MTHFR*1298A→C, A429E 99.3% and 95.6% and for *TCII* 776C→G, P259R 93.5% and 94.9%. Not all subjects were genotyped for all SNPs. At the start of the study it was decided that when it was clear after the first group of subjects was genotyped that the results would remain negative even if all the remaining samples were genotyped, no further analysis would be performed for cost and logistic reasons. In some instances sample was not available to perform all SNP analyses.

Statistical Methods

Tests for Hardy-Weinberg equilibrium (HWE) were conducted within each subject class (case, control, mother, and father) for each SNP by chi-squared test for genotype proportions.

Cases were analyzed as CLP or CPO. Initial analysis was restricted to isolated defects only, with additional analysis on the full group of isolated or multiple defects. Each case-control group was tested for association with each SNP using the two genotype groups corresponding to either a dominant or recessive disease model using Fisher's exact test for 2 × 2 tables. The strength of association was characterized by the odds ratio (OR) estimates from logistic regression. Similarly, mother-control associations were tested for each SNP using the same methodology.

Triads (case, mother, and father) were used to test for genotype relative risk using two log-linear models. The first uses a single parameter for the number of risk alleles in the case

genotype, giving a 1-df test of association equivalent to the transmission disequilibrium test (TDT) of Spielman et al. (1993). This model also uses a single parameter for maternal genotype, yielding an analogous test of association. The second log-linear model uses two parameters each for the case and maternal genotypes, allowing a separate effect for one or two copies of the risk allele (Weinberg et al., 1998). This last model was also fit using the EM algorithm to incorporate the incomplete families. Finally, to address the possibility of an interaction effect of gene and exposure, a separate log-linear model was fit for each of the following exposures: alcohol use, tobacco use, family history of clefts, and folic acid supplementation (broken into five time periods; one before and four after conception). Each log-linear model had separate terms for case genotype with and without the exposure. Tests for interaction were based on the 4-df Wald test for case genotype*exposure.

RESULTS

There were 1,088 cases recruited for this study. Five were excluded because the mother had diabetes, 12 because the mother had epilepsy, and 27 because the mother was exposed to potentially teratogenic drugs. In addition, we dropped nine cases with chromosomal abnormalities, 49 with other syndromes, three because the type of cleft could not be classified, and 21 who were not genotyped. Of the remaining cases, 536 were CLP. This group consisted of 494 cases with isolated defects, 23 with one additional defect, 18 with multiple defects, and one with Pierre Robin. There were 426 cases with CPO. This group consisted of 321 cases with isolated defects, 15 with one additional defect, 21 with multiple defects, and 69 with Pierre Robin Sequence. There were 783 complete triads (case, mother, and father), 134 case-mother pairs, eight case-father pairs, 36 cases without parents, and one mother without a case.

Study subjects' characteristics are shown in Table 1. More than a third (38%) of the mothers smoked during the affected pregnancy and 55% drank some alcohol. Most women had previous pregnancies; 3% reported previous stillbirths and over 30% had at least one prior pregnancy loss. There was at least one affected sibling in 5% of the families. Family history was positive in 16% of the fathers and 20% of the mothers.

In the primary analysis only isolated cases of CLP and CPO and their parents were included. In the secondary analysis, nonsyndromic cases with other defects were included to see if the results were changed. ORs are reported for the recessive model except as noted. Genotype distributions in all groups were in HWE except as noted below. The genotyping results are shown in Table 2.

MTHFD1 1958G→A, (R653Q)

It has been noted previously by us that the control subjects were not in HWE for *MTHFD1* 1958G→A, R653Q, and the possible biological reasons have been discussed (Brody et al., 2002).

CPO—There was no significant association between case status and the Q allele (OR 1.31; CI: 0.94–1.82; $p = .13$). The TDT and log linear analyses did not show any association between the Q allele and case status. Mothers were significantly more likely to be QQ compared to controls (OR 1.50; CI: 1.08–2.09; $p = .02$) but not in the log-linear analysis ($p > .05$). Adding the multiple case families changed the results for the case analysis. The case-control OR became significantly positive (OR 1.41; CI: 1.05–1.90; $p = .02$), but the log-linear analysis did not become significantly positive. The statistical findings in the mother-control analysis became more positive (OR 1.51; CI: 1.13–2.03; $p = .007$). The log-linear analysis remained negative.

CLP—Both the case-control and mother-control analyses showed a significant effect, ORs 1.38 (CI: 1.05–1.82; $p = .03$) and 1.39 (CI: 1.04–1.85; $p = .03$), respectively. TDT testing did not show increased transmission of the Q allele ($p = .16$). There was borderline evidence ($p = .09$) for a maternal association in the log-linear analysis but not a case effect. When the multiple case families were added to the analysis, there were minor changes. The case-control effect became slightly more pronounced ($p = .02$) while the mother-control finding became slightly weaker ($p = .05$). The overall maternal effect in the log-linear analysis remained the same, but the log-linear examination of the effect of one maternal copy of the variant allele versus none became significantly positive ($p = .03$). It is worth noting that the term for two copies of the variant allele versus zero was not significantly positive.

MTHFR 677C→T, (A222V)

CPO—CPO cases did not show a significant association with the *MTHFR* 677C→T, A222V variant. The OR was 0.96 (CI: 0.64–1.43; $p = .92$). The TDT and log-linear analyses showed no association. On the other hand, there was evidence that the variant was a maternal risk factor for CPO. Mothers were significantly more likely than controls to be TT (OR 1.50; CI: 1.05–2.16; $p = .03$). Log-linear analysis showed a borderline association in mothers ($p = .07$).

When multiple cases were included in the analysis there was no change in the findings regarding cases. The effect in mothers was less strong; the OR was 1.37 (CI: 0.98–1.90; $p = .07$). No significant effect was found in the log-linear analysis ($p = .15$).

CLP—The 677C→T SNP was not associated with being a CLP case in the isolated case-control analysis (OR 1.08; CI: 0.78–1.50), nor was it preferentially transmitted ($p > .05$). The 677C→T SNP was also not more common in mothers in the mother-control analysis (OR 1.00; CI: 0.71–1.41) or in the log-linear analysis. When the multiple defect families were added to the analysis, the results were not substantially changed.

MTHFR 1298A→C (A429E)

CPO—*MTHFR* 1298A→C (A429E) is well known to be in linkage disequilibrium with *MTHFR* 677 C→T. Mothers in the CPO group were not in HWE.

The OR in the isolated case-control analysis was 0.83 (CI: 0.49–1.40; $p = .53$). The TDT analysis showed no association between CPO and the C allele ($p > .05$). Log-linear analysis showed no case effect. Both the mother-control and log-linear analyses showed no significant association. The combined analysis of isolated and multiple cases also showed no significant associations between the C allele and case or mother status.

CLP—As was the case with CPO, there was no association between the C allele and CLP. In the isolated case families, the case-control analysis, mother-control analysis, TDT, and log-linear analyses revealed no significant associations (all $p > .05$). Adding the multiple group did not change the results substantially.

TCII 776 C→G (P259R)

CPO—There was no association between having the 776C→G (P259R) variant of the *TC II* gene and CPO in isolated cases. The ORs for being either a case (1.05; CI: 0.73–1.52) or a case mother (1.00; CI: 0.68–1.46) were not statistically significant. Neither log-linear analyses for maternal or case associations, nor TDT testing, showed any association between CPO and the G allele (all $p > .05$). The analysis was repeated adding multiple cleft cases; all p values remained nonsignificant.

CLP—In the CLP cases (OR 1.05; CI: 0.77–1.43) and mothers (OR 1.10; CI 0.81–1.50), the findings were similar. There were no significant associations between the 776C→G SNP and CLP in the case-control, mother-control, log-linear, or TDT comparisons (all $p > .05$). Adding multiple CLP cases did not alter the above results. However, the mother-control comparison did show a significant ($p = .04$) effect (OR 1.35; CI: 1.02–1.77) when a dominant gene model was used. The log-linear analysis also produced a significant ($p = .03$) maternal effect for one copy of the gene. It should be noted that the dominant model using only isolated cases ($p > .05$), the log-linear analysis testing the effect of two copies of the gene ($p > .05$), and the recessive model reported above did not show a significant effect.

Additional Analysis

Next, all the SNP analyses were tested for interactions with environmental factors. Alcohol and tobacco use, positive family history, and folic acid use preconception and in each of the first 4 months of pregnancy were considered. There were no significant interactions with any of these factors in any of the SNPs tested. Adding incomplete triads to the analyses did not change any of the results substantially. We examined the CLP and CPO cases that had one or more additional defects to determine whether any patterns suggestive of syndromes were present. None were found. The other defects are shown in Table 3.

DISCUSSION

We examined four SNPs from genes related to folate directly or indirectly via vitamin B12. *MTHFR* 677 C→T and 1298 A→C have been studied extensively, but with inconsistent results. *MTHFD1* 1958 G→A and *TC II* 776C→G have only been examined in a few studies (Boyles et al., 2008), also with conflicting results.

The most interesting results appeared in our investigation of *MTHFD1* 1958 G→A. As has been reported by us previously (Brody et al., 2002), the control population was not in HWE. This finding complicates the interpretation of the case-control and mother-control analyses. We found that mothers of isolated ($p = .02$) and multiple defect CPO cases were significantly ($p = .007$) more likely to be homozygous for the A (Q) variant than controls, although A was not a significant risk factor in the log-linear analysis. In the case-control analysis, adding multiple cases increased the case control OR from 1.31 ($p = .13$) to 1.50 and produced a statistically significant result ($p = .02$). As in the mothers, the log-linear analysis was not significant. In the CLP group, both cases and mothers showed a significant association with the variant allele (both $p = .03$). The log-linear analysis showed at most a borderline association ($p = .09$) in the mothers and no significance in the cases. In contrast to our findings, the other studies that looked at cases (Boyles et al., 2008; Palmieri et al., 2008) found no effect, as did the two studies that looked for an effect in mothers (Boyles et al., 2008; Mostowska et al., 2006).

Interestingly, *MTHFD1* has been shown by our group to be a maternal, but not an embryonic, risk factor for NTDs in two studies of Irish subjects (Brody et al., 2002; Parle-McDermott et al., 2006). *MTHFD1* is a logical candidate gene to explore for possible associations with birth defects. It catalyzes the conversion of tetrahydrofolate to the corresponding 10 formyl, 5,10 methenyl, and 5,10 methylene derivatives. 10-Formyltetrahydrofolate and 5,10 methenyltetrahydrofolate act as carbon donors for the de novo synthesis of purines and pyrimidines and hence are required for DNA synthesis. Insufficient DNA synthesis by the mother could damage the developing embryo.

In contrast to our smaller, earlier study, we found that the *MTHFR* 677C→T variant was not a risk factor in CPO or CLP cases in any of our analyses. The same was true in mothers of CLP cases. In mothers of isolated CPO cases, however, the *MTHFR* 677 C→T variant was

significantly more common in the mother-control analysis and a borderline risk factor ($p = .07$) in the log-linear analysis. Thus, the *MTFHR* 677 C→T variant in mothers may play a role in CPO in the Irish population, although these findings could be due to chance.

Results of previous investigations have been mixed. Some studies of *MTHFR* 677 C→T have shown no association with CLP in either cases or mothers while others have reported associations in mothers, or in mothers who did not use folic acid periconceptionally (Jugessur et al., 2003; Gaspar et al., 1999; Blanton et al., 2002; Martinelli et al., 2001; Prescott et al., 2002; Shotelersuk et al., 2003; Shaw et al., 1998; van Rooij et al., 2003; Mills et al., 1999). One recent study (Chevrier et al., 2007) found that the TT genotype was significantly protective in their case control analysis, although the authors noted that the sample size was small and that the TT rate in controls was higher than expected. CPO has not been studied as extensively. Shaw et al. (1999) found no association between *MTHFR* 677 C→T and CPO. Jugessur et al. (2003) found no association in mothers but an increased risk when the mother was heterozygous CT.

The *MTHFR* 1298 A→C variant was not found to be a risk factor for CPO or CLP in any of our analyses. Neither mothers nor cases showed any associations in the case-control, mother-control, log-linear, or TDT analyses. These results are consistent with the findings of a recent meta-analysis (Verkleij-Hagoort et al., 2007) that showed ORs close to 1 for CLP in both mothers and cases. CPO was not included in the meta-analysis. The only previous study (Jugessur et al., 2003) of 1298 A→C in CPO found, as we did, no association in either cases or mothers.

Our investigation of the role of the B12 transporter gene variant 776 C→G showed no association with either CPO or CLP. Only two other studies have examined this variant. Boyles et al. (2008) found no association except in the parent of origin test, and this was based on only 10 cases in which the allele was inherited from the father and six in which it was inherited from the mother. Martinelli et al. (2006) found a positive association with CLP. There are several possible reasons for these discordant results. Their subjects came from genetically different populations. Martinelli et al. (2006) had a higher proportion of familial cases (87/218) and chance may have played a role; the authors noted that their findings required confirmation. Thus, our data and data from the other large study, Boyles et al. (2008), do not suggest that *TC II* 776C→G is an important risk factor in CLP, but additional investigation would help to clarify the situation.

Our study has numerous strengths. It included a large number of triads and controls. The Irish population is genetically homogeneous, which reduces concerns that stratification could have occurred and increases our power. We were able to document other potential effect modifiers and confounders including smoking, alcohol, and folic acid use. The study has some limitations as well. Folate levels during the pregnancy of interest could not be measured. Our study included many tests of genotype-phenotype association so that our statistically significant findings could have occurred by chance. Syndromic cases were identified by the attending surgeons and excluded, but clinical geneticists did not examine the cases. We did, however, record the other defects seen in cases with multiple defects and there were no patterns of defects suggestive of syndromes present.

In summary, ours is the first study to find that the *MTHFD1* 1958G→A variant is a maternal risk factor for CLP. *MTHFR* 677 C→T was not a risk factor in cases; however, there was modest evidence that it was a maternal risk factor in CPO by both mother-control and log-linear analyses. In light of the fact that multiple comparisons were made, the positive findings require additional investigation. Nonetheless, our findings suggest that the *MTHFD1* 1958

G→A variant may be important in the etiology of CPO and CLP and this variant merits additional attention.

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References

Blanton SH, Patel S, Hecht JT, et al. MTHFR is not a risk factor in the development of isolated nonsyndromic cleft lip and palate. Am J Med Genet 2002;110:404–405. [PubMed: 12116219]

Boyles AL, Wilcox AJ, Taylor JA, et al. Folate and one-carbon metabolism gene polymorphisms and their associations with oral facial clefts. Am J Med Genet A 2008;146:440–449. [PubMed: 18203168]

Brody LC, Conley M, Cox C, et al. A polymorphism, R653Q, in the trifunctional enzyme methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase/ formyltetrahydrofolate synthetase is a maternal genetic risk factor for neural tube defects: report of the Birth Defects Research Group. Am J Hum Genet 2002;71:1207–1215. [PubMed: 12384833]

Canfield MA, Collins JS, Botto LD, et al. Changes in the birth prevalence of selected birth defects after grain fortification with folic acid in the United States: findings from a multi-state population-based study. Birth Defects Res A Clin Mol Teratol 2005;73:679–689. [PubMed: 16240378]

Chevrier C, Perret C, Bahuau M, et al. Fetal and maternal MTHFR C677T genotype, maternal folate intake and the risk of nonsyndromic oral clefts. Am J Med Genet A 2007;143:248–257. [PubMed: 17219389]

Czeizel AE. Prevention of congenital abnormalities by periconceptional multivitamin supplementation. BMJ 1993;306:1645–1648. [PubMed: 8324432]

Czeizel AE, Timar L, Sarkozi A. Dose-dependent effect of folic acid on the prevention of orofacial clefts. Pediatrics 1999;104:e66. [PubMed: 10586000]

Czeizel AE, Toth M, Rockenbauer M. Population-based case control study of folic acid supplementation during pregnancy. Teratology 1996;53:345–351. [PubMed: 8910980]

Frosst P, Blom HJ, Milos R, et al. A candidate genetic risk factor for vascular disease: A common mutation in methylene-tetrahydrofolate reductase. Nature Genet 1995;10:111–113. [PubMed: 7647779]

Gaspar DA, Pavanello RC, Zatz M, et al. Role of the C677T polymorphism at the MTHFR gene on risk to nonsyndromic cleft lip with/without cleft palate: results from a case-control study in Brazil. Am J Med Genet 1999;87:197–199. [PubMed: 10533038]

Hayes C, Werler MM, Willett WC, et al. Case-control study of periconceptional folic acid supplementation and oral clefts. Am J Epidemiol 1996;143:1229–1234. [PubMed: 8651221]

Itikala PR, Watkins ML, Mulinare J, et al. Maternal multivitamin use and orofacial clefts in offspring. Teratology 2001;63:79–86. [PubMed: 11241430]

Jugessur A, Wilcox AJ, Lie RT, et al. Exploring the effects of methylenetetrahydrofolate reductase gene variants C677T and A1298C on the risk of orofacial clefts in 261 Norwegian case-parent triads. Am J Epidemiol 2003;157:1083–1091. [PubMed: 12796044]

Loffredo LC, Souza JM, Freitas JA, et al. Oral clefts and vitamin supplementation. Cleft Palate Craniofac J 2001;38:76–83. [PubMed: 11204686]

Martinelli M, Scapoli L, Palmieri A, et al. Study of four genes belonging to the folate pathway: transcobalamin 2 is involved in the onset of non-syndromic cleft lip with or without cleft palate. Hum Mutat 2006;27:294. [PubMed: 16470748]

Martinelli M, Scapoli L, Pezzetti F, et al. C677T variant form at the MTHFR gene and CL/P: a risk factor for mothers? Am J Med Genet 2001;98:357–360. [PubMed: 11170082]

Mills JL, Kirke PN, Molloy AM, et al. Methylenetetrahydrofolate reductase thermolabile variant and oral clefts. Am J Med Genet 1999;86:71–74. [PubMed: 10440833]

Mostowska A, Hozyasz KK, Jagodzinski PP. Maternal MTR genotype contributes to the risk of non-syndromic cleft lip and palate in the Polish population. Clin Genet 2006;69:512–517. [PubMed: 16712703]

Palmieri A, Masiero E, Martinelli M, et al. The MTHFD1 gene is not involved in cleft lip with or without palate onset among the Italian population. Ann Hum Genet 2008;72:297–299. [PubMed: 18261183]

Parle-McDermott A, Kirke PN, Mills JL, et al. Confirmation of the R653Q polymorphism of the trifunctional C1-synthase enzyme as a maternal risk for neural tube defects in the Irish population. Eur J Hum Genet 2006;14:768–772. [PubMed: 16552426]

Parle-McDermott A, Mills JL, Kirke PN, et al. Analysis of the MTHFR 1298A-→C and 677C-→T polymorphisms as risk factors for neural tube defects. J Hum Genet 2003;48:190–193. [PubMed: 12730722]

Prescott NJ, Winter RM, Malcolm S. Maternal MTHFR genotype contributes to the risk of non-syndromic cleft lip and palate. J Med Genet 2002;39:368–369. [PubMed: 12011160]

Ray JG, Meier C, Vermeulen MJ, et al. Association between folic acid food fortification and congenital orofacial clefts. J Pediatr 2003;143:805–807. [PubMed: 14657833]

Shaw GM, Lammer EJ, Wasserman CR, et al. Risks of orofacial clefts in children born to women using multivitamins containing folic acid periconceptionally. Lancet 1995;346:393–396. [PubMed: 7623568]

Shaw GM, Rozen R, Finnell RH, et al. Infant C677T mutation in MTHFR, maternal periconceptional vitamin use, and cleft lip. Am J Med Genet 1998;80:196–198. [PubMed: 9843036]

Shaw GM, Todoroff K, Finnell RH, et al. Maternal vitamin use, infant C677T mutation in MTHFR, and isolated cleft palate risk. Am J Med Genet 1999;85:84–85. [PubMed: 10377018]

Shotelersuk V, Ittiwut C, Siriwan P, et al. Maternal 677CT/1298AC genotype of the MTHFR gene as a risk factor for cleft lip. J Med Genet 2003;40:e64. [PubMed: 12746418]

Simmons CJ, Mosley BS, Fulton-Bond CA, et al. Birth defects in Arkansas: is folic acid fortification making a difference? Birth Defects Res A Clin Mol Teratol 2004;70:559–564. [PubMed: 15368553]

Spielman RS, McGinnis RE, Ewens WJ. Transmission Test for Linkage Disequilibrium: The Insulin Gene Region and Insulin-dependent Diabetes Mellitus (IDDM). Am J Hum Genet 1993;52:506–516. [PubMed: 8447318]

Tolarova M, Harris J. Reduced recurrence of orofacial clefts after periconceptional supplementation with high-dose folic acid and mul-tivitamins. Teratology 1995;51:71–78. [PubMed: 7660324]

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. [PubMed: 9545395]

van Rooij IA, Ocke MC, Straatman H, et al. Periconceptional folate intake by supplement and food reduces the risk of nonsyndromic cleft lip with or without cleft palate. Prev Med 2004;39:689–694. [PubMed: 15351534]

van Rooij IA, Vermeij-Keers C, Kluijtmans LA, et al. Does the interaction between maternal folate intake and the methylenetetrahydrofolate reductase polymorphisms affect the risk of cleft lip with or without cleft palate? Am J Epidemiol 2003;157:583–591. [PubMed: 12672677]

Verkleij-Hagoort A, Bliek J, Sayed-Tabatabaei F, et al. Hyperhomo-cysteinemia and MTHFR polymorphisms in association with orofacial clefts and congenital heart defects: a meta-analysis. Am J Med Genet A 2007;143:952–960. [PubMed: 17431894]

Vieira AR, Murray JC, Trembath D, et al. Studies of reduced folate carrier 1 (RFC1) A80G and 5,10-methylenetetrahydrofolate reductase (MTHFR) C677T polymorphisms with neural tube and orofacial cleft defects. Am J Med Genet A 2005;135:220–223. [PubMed: 15880745]

Weinberg CR, Wilcox AJ, Lie RT. A Log-Linear Approach to Case-Parent-Triad Data: Assessing Effects of Disease Genes That Act Either Directly or Through Maternal Effects and That May be Subject to Parental Imprinting. Am J Hum Genet 1998;62:969–978. [PubMed: 9529360]

Yazdy MM, Honein MA, Xing J. Reduction in orofacial clefts following folic acid fortification of the U.S. grain supply. Birth Defects Res A Clin Mol Teratol 2007;79:16–23. [PubMed: 17177274]

Table 1

Characteristics of the Study Population

	CLP isolated *n* = 494		Not isolated *n* = 42		CPO isolated *n* = 321		Not isolated *n* = 105	
	n	**%**	***n***	**%**	***n***	**%**	***n***	**%**
Maternal age at birth of case								
<20	15	3.0	0	0.0	10	3.1	4	3.8
20–29	192	38.9	14	33.3	142	44.2	37	35.2
30–39	251	50.8	22	52.4	145	45.2	63	60.0
≥40	18	3.6	5	11.9	13	4.1	1	0.9
Unknown	18	3.6	1	2.4	11	3.4	0	0.0
Year of birth of case*								
1931–1979	54	10.9	2	4.8	8	2.5	1	0.9
1980–1989	139	28.1	12	28.6	73	22.7	9	8.6
1990–1999	217	43.9	13	31.0	146	45.5	57	54.3
2000–2004	83	16.8	15	35.7	94	29.3	38	36.2
Unknown	1	0.2	0	0.0	0	0.0	0	0.0
Maternal smoking								
Yes	173	35.0	16	38.1	133	41.4	45	42.9
No	248	50.2	21	50.0	166	51.7	54	51.4
Unknown	73	14.8	5	11.9	22	6.9	6	5.7
Alcohol use during pregnancy								
Yes	251	50.8	25	59.5	192	59.8	60	57.1
No	168	34.0	12	28.6	107	33.3	39	37.1
Unknown	75	15.2	5	11.9	22	6.9	6	5.7
Live births								
1	73	14.8	7	16.7	59	18.4	24	22.9
2	124	25.1	14	33.3	81	25.2	26	24.8
3	121	24.5	11	26.2	87	27.1	30	28.6
4	87	17.6	8	19.1	51	15.9	13	12.4
5 or more	81	16.4	2	4.8	38	11.8	12	11.4
Unknown	8	1.6	0	0.0	5	1.6	0	0.9
Stillbirths								

	CLP isolated *n* = 494		Not isolated *n* = 42		CPO isolated *n* = 321		Not isolated *n* = 105	
	n	**%**	***n***	**%**	***n***	**%**	***n***	**%**
0	460	93.1	41	97.6	310	96.6	103	98.1
1 or more	20	4.1	1	2.4	4	1.3	2	1.9
Unknown	14	2.8	0	0.0	7	2.2	0	0.0
Miscarriages								
0	328	66.4	26	61.9	225	70.1	69	65.7
1	99	20.0	12	28.6	56	17.4	26	24.8
2 or more	53	10.7	4	9.5	33	10.3	10	9.5
Unknown	14	2.8	0	0.0	7	2.2	0	0.0
Family history of clefts								
Maternal								
Yes	93	18.8	10	23.8	70	21.8	18	17.1
No	361	73.1	31	73.8	228	71.0	85	81.0
Unknown	40	8.1	1	2.4	23	7.2	2	1.9
Paternal								
Yes	94	19.0	3	7.1	45	14.0	12	11.4
No	313	63.4	28	66.7	224	69.8	78	74.3
Unknown	87	17.6	11	25.2	52	16.2	15	14.3
Marital status								
Married	386	78.1	33	78.6	239	74.4	75	71.4
Living with partner	27	5.5	0	0.0	29	9.0	14	13.3
Single/divorced/separated/widowed	68	13.8	9	21.4	49	15.3	16	15.2
Unknown	13	2.6	0	0.0	4	1.3	0	0.0

*Year of birth was significantly different in the isolated versus the not isolated cases in both the CLP and CPO groups.

Table 2

Genotyping Results in Controls and Triads (Cases, Mother and Fathers) for CLP and CPO (Isolated) or with Other Defects (Multiple)

***TCII* 776C→G**	**Isolated defects**						**Multiple defects**					
	CC		**CG**		**GG**		**CC**		**CG**		**GG**	
CLP	***n***	**%**	***n***	**%**	***n***	**%**	***n***	**%**	***n***	**%**	***n***	**%**
Controls	347	31.6	532	48.5	218	19.9						
Case	99	31.3	153	48.4	64	20.2	106	30.6	166	48.0	74	21.4
Mother	88	27.8	162	51.3	66	20.9	91	26.4	180	52.3	73	21.2
Father	86	32.3	128	48.1	52	19.6	94	32.4	140	48.3	56	19.3
	CC		CG		GG		CC		CG		GG	
CPO	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Case	65	31.5	99	48.1	42	20.4	85	30.1	136	48.2	61	21.6
Mother	62	30.2	99	48.3	44	21.5	90	32.4	133	47.8	55	19.8
Father	60	32.4	93	50.3	32	17.3	74	29.5	133	53.0	44	17.5
MTHFR 677C→T	Isolated defects						Multiple defects					
	CC		CT		TT		CC		CT		TT	
CLP	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Controls	715	44.7	721	45.1	163	10.2						
Case	217	44.1	221	44.9	54	11.0	236	44.2	235	44.0	63	11.8
Mother	205	44.1	212	45.6	48	10.3	221	43.8	233	46.1	51	10.1
Father	174	44.2	177	44.9	43	10.9	186	43.8	196	46.1	43	10.1
	CC		CT		TT		CC		CT		TT	
CPO	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Case	148	46.1	142	44.2	31	976	194	45.6	187	44.0	44	10.4
Mother	139	45.1	125	40.6	44	14.3	191	46.4	166	40.3	55	13.3
Father	115	42.3	130	47.8	27	9.9	157	42.9	172	47.0	37	10.1

***TCII* 776C→G**	**Isolated defects**						**Multiple defects**					
	CC		**CG**		**GG**		**CC**		**CG**		**GG**	
CLP	***n***	**%**	***n***	**%**	***n***	**%**	***n***	**%**	***n***	**%**	***n***	**%**

MTHFR 1298A→C	Isolated defects						Multiple defects					
	AA		AC		CC		AA		AC		CC	
CLP	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Controls	519	49.4	439	41.8	92	8.8						
Case	202	49.6	172	42.3	33	8.1	223	50.6	183	41.5	35	7.9
Mother	179	48.9	164	44.8	23	6.3	198	49.7	175	44.0	25	6.3
Father	160	50.2	127	39.8	32	10.0	171	49.7	140	40.7	33	9.6
	AA		AC		CC		AA		AC		CC	
CPO	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Case	126	47.5	120	45.3	19	7.2	168	48.1	155	44.4	26	7.4
Mother	132	53.4	86	34.8	29	11.7	175	53.0	123	37.3	32	9.7
Father	100	46.5	95	44.2	20	9.3	135	46.9	124	43.1	29	10.1

MTHFD1 1958G→A	Isolated defects						Multiple defects					
	GG		AG		AA		GG		AG		AA	
CLP	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Controls	290	27.3	570	53.7	202	19.0						
Case	106	27.2	189	48.6	94	24.2	112	26.5	208	49.2	103	24.3
Mother	112	30.5	163	44.4	92	25.1	122	30.6	180	45.1	97	24.3
Father	84	26.6	165	52.2	67	21.2	88	25.8	178	52.2	75	22.0
	GG		AG		AA		GG		AG		AA	

***TCII* 776C→G**	**Isolated defects**						**Multiple defects**					
	CC		**CG**		**GG**		**CC**		**CG**		**GG**	
CLP	***n***	**%**	***n***	**%**	***n***	**%**	***n***	**%**	***n***	**%**	***n***	**%**
CPO	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Case	55	21.9	138	55.0	58	23.1	70	21.0	182	54.5	82	24.6
Mother	68	27.6	113	45.9	65	26.4	84	25.5	158	48.0	87	26.4
Father	56	26.4	106	50.0	50	23.6	73	25.6	138	48.4	74	26.0

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Table 3

Associated Defects in Subjects with Cleft Lip with or without (+/−) Cleft Palate and Cleft Palate Only Who Did Not have Recognized Syndromes*

	Cleft lip +/− palate *n* = 41	Cleft palate only *n* = 36
Central nervous system	4	4
Neuro-muscular	1	0
Facial	4	4
Eyes	3	4
Ears	0	6
Jaw/mouth	0	6
Gastro-intestinal	4	1
Respiratory	0	2
Cardiac	4	8
Skeletal/limb	13	7
Genito-urinary	8	4
Dysmorphic features	2	2

* Subjects may have more than one entry. Pierre Robin sequence and micrognathia not included.