Methylenetetrahydrofolate reductase C677T polymorphism is not associated with the risk of nonsyndromic cleft lip/palate: An updated meta-analysis

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Both genetic and environmental factors affect the risk of orofacial clefts. The present meta-analysis aimed to evaluate the association between methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism and risk of nonsyndromic cleft lip/palate (NSCL/P) in cases-control studies. The PubMed/Medline, Scopus, Web of Science, and Cochrane Library databases were searched up to April 2019 with no restrictions. The odds ratios (ORs) and 95% confidence intervals (CIs) in all analyses were calculated by Review Manager 5.3 software. The funnel plot analysis was carried out by the Comprehensive Meta-Analysis version 2.0 software. Subgroup analysis, meta-regression, and sensitivity analysis were performed for the pooled analyses. Thirty-one studies reviewed in this meta-analysis included 4710 NSCL/P patients and 7271 controls. There was no significant association between MTHFR C677T polymorphism and NSCL/P susceptibility related to allelic model (OR = 1.04; P = 0.49), homozygote model (OR = 1.11; P = 0.35), heterozygote model (OR = 0.99; P = 0.91), dominant model (OR = 1.00; P = 0.96), or recessive model (OR = 1.08; P = 0.23). There was no significant association between MTHFR C677T polymorphism and NSCL/P susceptibility based on the ethnicity or the source of cases. There was a significant linear relationship between the year of publication and log ORs for the allele model. The results of the present meta-analysis failed to show an association between MTHFR C677T polymorphism and NSCL/P susceptibility. The subgroup analyses based on the ethnicity and the source of cases further confirmed this result.

Non-syndromic cleft lip/palate (NSCL/P) is a common birth defect worldwide1. In low- and middle-income countries, around 1/730 children is born with cleft lip/palate2. A multifactorial model of genetic inheritance has been recommended for NSCL/P based on the interaction of genetic and environmental factors1. Several lines of evidence have proven a significant association between polymorphism of genes connected to folate metabolism and increased risk of orofacial clefts3. Among genes related to folate metabolism, 5,10-methylenetetrahydrofolate reductase (MTHFR) reportedly has the highest association with NSCL/P. This gene is located on chromosome

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1 at 1p36.3 and translates to MTHFR enzyme that catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a cosubstrate for homocysteine remethylation to methionine. MTHFR is a fundamental enzyme in folate metabolism and DNA synthesis, and MTHFR rs1801133 (C677T) is one of the most common polymorphisms which diminishes the enzyme activity. Regulation of MTHFR activity is pivotal to maintain optimal cellular levels of methionine and S-adenosylmethionine. Folate supplementation or its adequate dietary intake during pregnancy has been shown to prevent or decrease NSCL/P susceptibility. Nutritional factors, such as the adequacy of folic acid in the mother’s diet, are clearly important, but other potential disturbances in ovulation or development of fetus may be due to the activity of key factors such as the MTHFR enzyme in folate metabolism. In addition, the role of other polymorphisms of folate metabolism has been proven in recent meta-analyses. There are six published meta-analyses related to our topic in the literature. However, several other original articles have been recently published. Thus, we aimed to evaluate the association between MTHFR C677T and risk of NSCL/P in an updated meta-analysis of cases-control studies.

### Materials and Methods

#### Protocol.

The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guideline was applied for designing this meta-analysis.

#### Search strategy.

One author (N.G) accomplished the initial search and another author (M.S) re-checked the retrieved articles; disagreements between the two authors were resolved by a third author (M.M.I). A comprehensive search was conducted on the association between MTHFR rs1801133 (C677T) polymorphism and NSCL/P susceptibility in PubMed/Medline, Scopus, Web of Science, and Cochrane Library databases up to April 2019. The search keywords were: (“cleft lip” or “cleft palate” or “orofacial cleft” or “oral cleft”) and (“methylenetetrahydrofolate reductase” or “MTHFR”). The databases were searched without any restrictions. Manual search of all references quoted in published meta-analyses/reviews related to the topic was done by another author (M.S).

#### Eligibility criteria.

Inclusion criteria: (a) original studies; (b) studies reporting the relationship between MTHFR C677T polymorphism and the NSCL/P susceptibility; (c) studies designed as case-control studies; (d)
studies providing sufficient data about the alleles and genotypes of MTTHFR C677T polymorphism in case and control groups. Exclusion criteria: (a) studies not related to the relationship between MTTHFR C677T polymorphism and the NSCL/P susceptibility; (b) duplicate studies/erratum; (c) review/meta-analysis, letter to editors, comments, and conference papers; (d) family-based studies; (e) case-parent triads studies; (f) studies inconsistent with the Hardy-Weinberg equilibrium (HWE) about the control group; (g) studies containing overlapping data.

Data extraction. Two authors (N.G and M.M.I) independently retrieved the data from each study included in this systematic review based on the eligibility criteria. Disagreements between the two authors were resolved through further discussion. The extracted data are presented in Tables 1 and 2.

Quality assessment. To evaluate the study quality, the control group of each study was tested for the HWE. One author (M.S) calculated the HWE for each study.

Statistical analysis. One author (M.S) analyzed the data and other authors independently re-checked them; disagreements were resolved by discussion. The odds ratios (ORs) with the corresponding 95% confidence intervals (CIs) in all analyses were calculated by Review Manager 5.3 to evaluate the strength of the association between MTTHFR C677T polymorphism and the risk of NSCL/P. To examine this association, we used five genetic models namely the allele (T vs. C), homozygote (TT vs. CC), heterozygote (CT vs. CC), dominant (TT + CT vs. CC), and recessive (TT vs. CC + CT) models. The Z test was used for evaluation of the significance of the pooled OR using both fixed effects (FE) (Mantel–Haenszel) and random effects (DerSimonian and Laird) models. Heterogeneity across the studies was evaluated using both the Cochrane Q test and I² metric ranging from 0 to 100%. There was statistically significant heterogeneity if P-value < 0.1 and I² > 50%; in that case, the random-effect model was used to estimate the pooled ORs and CI values. Otherwise, we used the fixed-effect model. The Chi-square test was used for calculation of the HWE for the control group of each study.

| First author, publication year | Case CC | Control TT | Case CC | Control TT | P-value for HWE in controls |
|-------------------------------|---------|-----------|---------|------------|----------------------------|
| Shaw, 1998 | 143     | 127       | 40      | 156        | 178 49 413 207 790 276 0.87 |
| Gaspar, 1999  | 30      | 39        | 8       | 49         | 49 15 99 55 147 79 0.09 |
| Martinelli, 2001 | 64      | 22        | 30      | 46         | 43 17 150 82 135 77 0.20 |
| Grunert, 2002  | 34      | 26        | 6       | 90         | 69 25 94 38 249 119 0.06 |
| Shotelersuk, 2003  | 84      | 25        | 0       | 154        | 46 2 193 25 354 50 0.47 |
| van Rooij, 2003  | 54      | 45        | 6       | 70         | 54 4 153 57 194 62 0.09 |
| Pezzetti, 2004  | 28      | 58        | 24      | 95         | 151 43 114 106 341 237 0.17 |
| Wan, 2006  | 13      | 49        | 14      | 31         | 20 9 75 77 82 38 0.08 |
| Brandalize, 2007  | 49    | 46        | 19      | 45         | 41 14 144 84 131 69 0.35 |
| Chevrier, 2007  | 66      | 60        | 22      | 54         | 81 33 192 104 189 147 0.17 |
| Little, 2008  | 39      | 47        | 10      | 94         | 101 29 125 67 289 159 0.82 |
| Mills, 2008  | 217     | 221       | 54      | 715        | 721 163 655 329 2151 1047 0.34 |
| Ali, 2009  | 225     | 87        | 11      | 176        | 36 2 537 109 388 40 0.91 |
| Guo, 2009  | 19      | 53        | 24      | 22         | 57 24 91 101 101 105 0.27 |
| Sozen, 2009  | 81      | 80        | 18      | 66         | 65 7 242 116 197 79 0.07 |
| Mostowska, 2010  | 81     | 65        | 17      | 78         | 77 16 227 99 233 109 0.67 |
| Chorna, 2011  | 12      | 17        | 4       | 22         | 26 2 41 25 70 30 0.09 |
| Han, 2011  | 46      | 106       | 35      | 74         | 110 29 198 176 258 168 0.24 |
| Semic-Josufagic, 2012  | 25    | 28        | 3       | 44         | 24 8 78 34 112 40 0.10 |
| Kumari, 2013  | 327     | 126       | 15      | 364        | 100 5 780 156 828 110 0.52 |
| Estandia-Ortega, 2014  | 38    | 55        | 39      | 55         | 172 143 131 133 282 458 0.78 |
| Jahabin, 2014  | 20      | 16        | 7       | 46         | 41 14 56 30 133 69 0.32 |
| Murthy, 2014  | 104     | 19        | 24      | 107        | 31 3 227 19 245 37 0.67 |
| Abdollahi-Fakhim, 2015  | 38 | 25        | 2       | 27         | 22 1 101 29 76 24 0.14 |
| Bezerra, 2015  | 74      | 54        | 12      | 85         | 70 20 202 78 240 110 0.34 |
| de Aguiar, 2015  | 137    | 145       | 36      | 319        | 231 48 419 217 869 327 0.50 |
| Jiang, 2015  | 59      | 107       | 38      | 62         | 108 56 225 183 232 220 0.51 |
| Ramírez-Chau, 2016  | 44    | 79        | 42      | 90         | 151 50 167 163 331 251 0.32 |
| Xu, 2016  | 35      | 57        | 28      | 22         | 50 28 127 113 94 106 0.97 |
| Taslim, 2017  | 19      | 5         | 0       | 26         | 19 2 43 5 71 23 0.52 |
| Rafik, 2019  | 44      | 8         | 0       | 97         | 74 11 96 8 268 96 0.53 |

Table 2. Distribution of MTTHFR C677T polymorphism genotype and allele in NSCL/P patients and controls. Abbreviation: HWE, Hardy-Weinberg equilibrium.
Subgroup analysis was performed according to the ethnicity and the source of cases to explore potential heterogeneity. The meta-regression analysis is a technique used to assess heterogeneity between the studies. This statistical approach determines whether there is a significant association between the study period and number of individuals with the pooled OR. The Begg's funnel plot was carried out by the Comprehensive Meta-Analysis version 2.0 software identifying the standard error of log (OR), and the precision of each study was plotted against its log (OR)\(^{25}\). In addition, the results of Egger's linear regression were retrieved using this software\(^{26}\). To estimate the consistency or stability of the results, we used sensitivity analysis namely cumulative analysis and one study was removed. P-value (2-tailed) \(< 0.05\) was statistically significant.

Results
A total of 353 records were retrieved from the databases and after removing the duplicates, 187 records were screened (Fig. 1). Next, 117 records were excluded considering the eligibility criteria. Then, the full-texts of 70 articles were evaluated and 36 articles were excluded with reasons (five studies were systematic reviews/meta-analyses; one study was Erratum; two studies were letter to the editor/research letter; one study was editorial comment; three studies were family-based studies; five studies had irrelevant data; ten studies were case-control parents; three studies had no control group; five studies were case-parent triads; one study reported complex birth defects). On the other hand, by searching the references of meta-analyses, two other articles\(^{27,28}\) were found. Totally, 36 articles were included in this systematic review\(^{6,27-61}\) out of which, 5 studies\(^{59-61}\) had a deviation from the HWE for the control group and were excluded from the meta-analysis. Therefore, 31 articles were included and analyzed in this meta-analysis. In addition, one study\(^{62}\) was excluded for reducing bias compared with other previous meta-analyses, because it was a conference paper and therefore didn’t involve the eligibility criteria.

Table 1 shows the characteristics of each study included in this meta-analysis. The articles had been published from 1998 to 2019. Overall, the studies included 4,710 NSCL/P patients and 7,271 controls. Out of 31 studies, 10 studies were reported in mixed ethnicities, 10 studies had been conducted on Asians, and 11 studies had been
Figure 2. Random-effect forest plot of allele model (T vs. C) for the association between the NSCL/P risk and MTHFR C677T polymorphism.

Figure 3. Random-effect forest plot of homozygote model (TT vs. CC) for the association between the NSCL/P risk and MTHFR C677T polymorphism.
Figure 4. Random-effect forest plot of heterozygote model (CT vs. CC) for the association between the NSCL/P risk and MTHFR C677T polymorphism.

Figure 5. Random-effect forest plot of dominant model (TT + CT vs. CC) for the association between the NSCL/P risk and MTHFR C677T polymorphism.
conducted on Caucasians. In addition, the source of cases (patients) was population-based in 13 studies and hospital-based in 18 studies.

Table 2 shows the distribution of MTHFR C677T polymorphism genotype and allele in NSCL/P patients and controls. All studies followed the HWE for the control group.

**Meta-analysis.** The results of the pooled OR of the association between MTHFR C677T polymorphism and NSCL/P susceptibility are shown in Fig. 2 (T vs. C), Fig. 3 (TT vs. CC), Fig. 4 (CT vs. CC), Fig. 5 (TT + CT vs. CC), and Fig. 6 (T vs. CC + CT). Based on the results, there was no significant association between MTHFR C677T polymorphism and NSCL/P risk. However, the forest plot for the recessive model (TT vs. CC + CT) showed a significant association (OR = 1.13, 95% CI = 1.02, 1.25, P = 0.006).

Table 3. Analysis of non-syndromic cleft lip/palate risk related to MTHFR C677T polymorphism according to ethnicity. Abbreviations: PB, population-based; HB, hospital-based. *P-values were insignificant (P > 0.05) in all analyses. **P means P heterogeneity.
C677T polymorphism and NSCL/P susceptibility related to allelic model [OR = 1.04; 95% CI: 0.93, 1.17; P = 0.49; \( I^2 = 70\% \) (\( P_{\text{heterogeneity}} \) or \( P_{\text{h}} \) < 0.0001)], homozygote model [OR = 1.11; 95% CI: 0.89, 1.38; \( P = 0.35; I^2 = 52\% \) (\( P_{h} = 0.0005 \)], heterozygote model [OR = 0.99; 95% CI: 0.85, 1.16; \( P = 0.91; I^2 = 67\% \) (\( P_{h} < 0.0001 \)], heterozygote model [OR = 0.99; 95% CI: 0.85, 1.16; \( P = 0.91; I^2 = 67\% \) (\( P_{h} < 0.0001 \)], dominant model [OR = 1.00; 95% CI: 0.86, 1.18; \( P = 0.96; I^2 = 70\% \) (\( P_{h} < 0.0001 \)], and recessive model [OR = 1.08; 95% CI: 0.96, 1.21; \( P = 0.23; I^2 = 29\% \) (\( P_{h} = 0.06 \)].

**Subgroup analysis.** The subgroup analysis was performed based on the ethnicity and the source of cases for the association between MTHFR C677T polymorphism and NSCL/P risk (Table 3). There was no significant association between MTHFR C677T polymorphism and NSCL/P susceptibility with regard to the ethnicity (Asian, Caucasian, and mixed ethnicities) or the source of cases (population-based and hospital-based).

**Meta-regression.** Considering the year of publication and the number of individuals as independent variables and the log (OR) as the dependent variable, the fixed-effect meta-regression results are presented in Table 4, Figs. 7 and 8. To estimate the functional relationship of the log OR with the year of publication and the number of individuals, the analysis showed only a significant relationship for the allele model (T vs. C) for the year of publication with a regression coefficient of \(-0.01346\). Therefore, there was a significant linear relationship between the year of publication and log ORs for the allele model (T vs. C), but not for the genetic models.

**Publication bias.** Figure 9 shows the funnel plots of all genetic models to evaluate the association between the NSCL/P risk and MTHFR C677T polymorphism in a fixed-effect model. There was no publication bias between the NSCL/P risk and MTHFR C677T polymorphism in the genetic models. The P-values of Begg's/Egger's tests were 0.21470/0.12123, 0.35933/0.97936, 0.22753/0.29895, 0.25480/0.28137, and 0.93228/0.91342 for T vs. C, TT vs. CC, CT vs. CC, TT + CT vs. CC, and TT vs. CC + CT, respectively.

**Sensitivity analysis.** Two analyses (one study excluded and cumulative analysis) were performed and the pooled ORs did not change qualitatively. Therefore, the analyses showed that the pooled ORs under all genetic models were stable and trustworthy.

**Discussion**
NSCL/P is one of the most common congenital anomalies with high rate of mortality. Its pathogenesis is difficult to be attributed to either environmental or genetic factors. The pathway of folate metabolism plays a significant role in the synthesis, repair, and methylation of DNA involved in NSCL/P pathogenesis. MTHFR enzyme plays an important role in folate intake, and mutations of MTHFR gene significantly impact the stability and thus the function of the enzyme; MTHFR C677T is the most common mutation of this gene. MTHFR C677T polymorphism is related to a reduction in MTHFR activity, raised plasma homocysteine concentration, and lower plasma level of folic acid, which consequently contribute to NSCL/P. The present meta-analysis was performed...
Figure 7. Fixed-effect meta-regression of log odds ratio versus publication year for (A) allele model, (B) homozygote model, (C) heterozygote model, (D) dominant model, and (E) recessive model.

to more precisely assess the relationship between MTHFR C677T polymorphism and NSCL/P susceptibility. In pooled analysis, the meta-analysis showed no significant association between MTHFR C677T polymorphism and NSCL/P risk.

Out of 31 studies included in the present meta-analysis, six studies reported significantly increased risk of T allele, TT genotype, and CT genotype in NSCL/P patients compared with controls, respectively. Also, five studies reported a significantly decreased risk of T allele, TT genotype and CT genotype in NSCL/P patients compared with controls, respectively. In addition, TT + CT genotype was reported to have a significantly increased risk in five studies and significantly decreased risk in four studies in NSCL/P patients compared with controls. Based on the recessive model, three studies reported significantly increased risk of TT genotype and one study reported its significantly decreased risk in NSCL/P patients compared with controls.

A recent meta-analysis with 24 case-control studies investigating the relationship between NSCL/P and MTHFR C677T polymorphism showed that the TT genotype was a risk factor for NSCL/P in Asians in homozygote (OR = 1.96, P < 0.001) and recessive (OR = 1.45, P = 0.028) models. Also, based on mothers with NSCL/P progeny versus control mothers with healthy progeny in 10 studies, the TT genotype of Caucasian mothers may increase progeny NSCL/P morbidity. Another recent meta-analysis of 22 case-control studies showed that MTHFR C677T polymorphism was associated with a higher risk of NSCL/P. Both meta-analyses also included studies with a deviation of HWE. However, in the present meta-analysis, we excluded such studies from the pooled analysis and therefore, reviewed 31 case-control studies and had lower heterogeneity compared with the meta-analysis with 22 studies. Almost similar to the findings of a meta-analysis with 24 studies, our results
showed no association between MTHFR C677T and susceptibility to NSCL/P. In one meta-analysis, definition of ethnicity was different from that in another study and our meta-analysis. The meta-regression showed a linear relationship with a negative slope between the year of publication and log ORs for the allele model and therefore by increasing years of publication, the risk of T allele decreased in NSCL/P patients compared with controls. There were two other meta-analyses with eight and nine case-control studies related to our topic. One of them reported no association and another one on Asian ethnicity showed a significant association between MTHFR C677T and susceptibility to NSCL/P. Luo et al. on nine studies in a meta-analysis didn't show any evidence for significant association between infant or maternal MTHFR C677T polymorphism and NSCL/P risk, but suggested that maternal MTHFR 677TT polymorphism could increase the risk of having a NSCL/P offspring in the white population. Pan et al. on seventeen studies showed that this polymorphism was a risk factor involved in the development of NSCL/P in Asians that definition of ethnicities in this meta-analysis was different from our meta-analysis. The results showed that the effect of each factor alone on the association was low, but such a high heterogeneity among the studies could be due to simultaneous effect of several factors such as differences in the ethnicity of the study populations, source of cases, and number of individuals.

This study had several important limitations including high heterogeneity across studies, unadjusted ORs used in the studies, and intake of folic acid and other supplements that were not considered. Nevertheless, the present study included more studies with meta-regression without any deviation of HWE for controls in all studies compared with other meta-analyses. It did not have publication bias, and the results were stable.

Figure 8. Fixed-effect meta-regression of log odds ratio versus number of individuals for (A) allele model, (B) homozygote model, (C) heterozygote model, (D) dominant model, and (E) recessive model.
In conclusion, the result of the present meta-analysis revealed that MTHFR C677T polymorphism is not associated with susceptibility to NSCL/P, and the subgroup analyses based on the ethnicity and the source of cases further confirmed this result. However, well-designed studies with larger sample size are required taking into account the role of micronutrients such as folic acid in NSCL/P risk.

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Competing interests
The authors declare no competing interests.

Additional information
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