SCIENTIFIC REPORTS natureresearch

OPEN

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

Mohammad Moslem Imani¹, Negin Golchin², Mohsen Safaei ³, Farzad Rezaei⁴, Hooshyar Abbasi⁴, Masoud Sadeghi ^{5*}, Pia Lopez-Jornet⁶, Hamid Reza Mozaffari⁷ & Roohollah Sharifi⁸

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

¹Department of Orthodontics, School of Dentistry, Kermanshah University of Medical Sciences, Kermanshah, 6713954658, Iran. ²Students Research Committee, Kermanshah University of Medical Sciences, Kermanshah, 6715847141, Iran. ³Advanced Dental Sciences Research Laboratory, School of Dentistry, Kermanshah University of Medical Sciences, Kermanshah, 6713954658, Iran. ⁴Department of Oral and Maxillofacial Surgery, Kermanshah University of Medical Sciences, Kermanshah, 6713954658, Iran. ⁵Medical Biology Research Center, Kermanshah University of Medical Sciences, Kermanshah, 6714415185, Iran. ⁶Facultad de Medicina y Odontologia Universidad de Murcia, Hospital Morales Meseguer, Clinica Odontologic Adv Marques Velez s/n, 30008, Murcia, Spain. ⁷Department of Oral and Maxillofacial Medicine, School of Dentistry, Kermanshah University of Medical Sciences, Kermanshah, 6713954658, Iran. ⁸Department of Endodontics, School of Dentistry, Kermanshah University of Medical Sciences, Kermanshah, 6713954658, Iran. ⁸Department of Endodontics, School of Dentistry, Kermanshah University of Medical Sciences, Kermanshah, 6713954658, Iran. *email: sadeghi_mbrc@yahoo.com

First author, publication year	Country	Ethnicity	No. of cases/controls	Source of case	Genotype method
Shaw, 1998 ²⁷	USA	Mixed	310/383	РВ	RFLP-PCR
Gaspar, 1999 ²⁹	Brazil	Mixed	77/113	НВ	PCR
Martinelli, 2001 ²⁸	Italy	Caucasian	116/106	РВ	RFLP-PCR
Grunert, 2002 ³⁰	Germany	Caucasian	66/184	НВ	PCR
Shotelersuk, 2003 ³¹	Thailand	Asian	109/202	РВ	RFLP-PCR
van Rooij, 2003 ³²	Netherlands	Caucasian	105/128	НВ	RFLP-PCR
Pezzetti, 2004 ³³	Italy	Caucasian	110/289	НВ	RFLP-PCR
Wan, 2006 ³⁴	China	Asian	76/60	НВ	RFLP-PCR
Brandalize, 2007 ³⁵	Brazil	Mixed	114/100	НВ	RFLP-PCR
Chevrier, 2007 ³⁶	France	Caucasian	168/148	НВ	RFLP-PCR
Little, 2008 ³⁷	Canada	Mixed	96/224	РВ	MS-PCR
Mills, 2008 ³⁸	Ireland	Caucasian	492/1599	НВ	RFLP-PCR
Ali, 2009 ³⁹	India	Asian	323/214	РВ	RFLP-PCR
Guo, 2009 ⁴⁰	China	Asian	96/103	НВ	PCR
Sozen, 2009 ⁴¹	USA	Mixed	179/138	РВ	PCR
Mostowska, 2010 ⁴²	Poland	Caucasian	174/176	РВ	RFLP-PCR
Chorna, 2011 ⁴³	Ukraine	Caucasian	33/50	НВ	RFLP-PCR
Han, 2011 ⁴⁴	China	Asian	200/213	НВ	RFLP-PCR
Semic-Jusufagic, 2012 ⁴⁵	Turkey	Caucasian	56/76	РВ	PCR
Kumari, 2013 ⁴⁶	India	Asian	467/469	РВ	RFLP-PCR
Estandia-Ortega, 2014 ⁴⁷	Mexico	Mixed	132/370	НВ	KASPar assay system
Jahanbin, 2014 ⁴⁸	Iran	Caucasian	45/101	РВ	RFLP-PCR
Murthy, 2014 ⁴⁹	India	Asian	123/141	НВ	RFLP-PCR
Abdollahi-Fakhim, 2015 ⁵⁰	Iran	Caucasian	65/50	НВ	RFLP-PCR
Bezerra, 2015 ⁵¹	Brazil	Mixed	140/175	РВ	RFLP-PCR
de Aguiar, 2015 ⁵²	Brazil	Mixed	318/598	НВ	Real time-PCR
Jiang, 2015 ⁵³	China	Asian	204/226	РВ	SEQUENOM MassARRAY
Ramírez-Chau, 2016 ⁵⁴	Chile	Mixed	165/291	HB	Real time-PCR
Xu, 2016 ⁵⁵	China	Asian	120/100	PB	PCR
Taslim, 2017 ⁵⁶	Indonesia	Asian	24/47	HB	RFLP-PCR
Rafik, 2019 ⁵⁷	Morocco	Mixed	52/182	НВ	RFLP-PCR

Table 1. Characteristics of the studies included in this meta-analysis (n = 31).

.....

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^{4,5}. 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^{6–8}. Regulation of MTHFR activity is pivotal to maintain optimal cellular levels of methionine and S-adenosylmethionine⁵. Folate supplementation or its adequate dietary intake during pregnancy has been shown to prevent or decrease NSCL/P susceptibility⁹. 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^{10,11}. In addition, the role of other polymorphisms of folate metabolism has been proven in recent meta-analyses^{7,12,13}. There are six published meta-analyses related to our topic in the literature^{7,8,14–17}. 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-analysis¹⁸.

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 C > T (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)

	Case			Contro	l		Case		Contro	1	P-value for HWE	
First author, publication year	CC	СТ	TT	СС	СТ	TT	С	Т	С	Т	in controls	
Shaw, 1998 ²⁷	143	127	40	156	178	49	413	207	790	276	0.87	
Gaspar, 1999 ²⁹	30	39	8	49	49	15	99	55	147	79	0.09	
Martinelli, 2001 ²⁸	64	22	30	46	43	17	150	82	135	77	0.20	
Grunert, 2002 ³⁰	34	26	6	90	69	25	94	38	249	119	0.06	
Shotelersuk, 2003 ³¹	84	25	0	154	46	2	193	25	354	50	0.47	
van Rooij, 2003 ³²	54	45	6	70	54	4	153	57	194	62	0.09	
Pezzetti, 2004 ³³	28	58	24	95	151	43	114	106	341	237	0.17	
Wan, 2006 ³⁴	13	49	14	31	20	9	75	77	82	38	0.08	
Brandalize, 2007 ³⁵	49	46	19	45	41	14	144	84	131	69	0.35	
Chevrier, 2007 ³⁶	66	60	22	54	81	33	192	104	189	147	0.17	
Little, 2008 ³⁷	39	47	10	94	101	29	125	67	289	159	0.82	
Mills, 2008 ³⁸	217	221	54	715	721	163	655	329	2151	1047	0.34	
Ali, 2009 ³⁹	225	87	11	176	36	2	537	109	388	40	0.91	
Guo, 2009 ⁴⁰	19	53	24	22	57	24	91	101	101	105	0.27	
Sozen, 2009 ⁴¹	81	80	18	66	65	7	242	116	197	79	0.07	
Mostowska, 2010 ⁴²	81	65	17	78	77	16	227	99	233	109	0.67	
Chorna, 2011 ⁴³	12	17	4	22	26	2	41	25	70	30	0.09	
Han, 2011 ⁴⁴	46	106	35	74	110	29	198	176	258	168	0.24	
Semic-Jusufagic, 2012 ⁴⁵	25	28	3	44	24	8	78	34	112	40	0.10	
Kumari, 2013 ⁴⁶	327	126	15	364	100	5	780	156	828	110	0.52	
Estandia-Ortega, 2014 ⁴⁷	38	55	39	55	172	143	131	133	282	458	0.78	
Jahanbin, 2014 ⁴⁸	20	16	7	46	41	14	56	30	133	69	0.32	
Murthy, 2014 ⁴⁹	104	19	0	107	31	3	227	19	245	37	0.67	
Abdollahi-Fakhim, 2015 ⁵⁰	38	25	2	27	22	1	101	29	76	24	0.14	
Bezerra, 2015 ⁵¹	74	54	12	85	70	20	202	78	240	110	0.34	
de Aguiar, 2015 ⁵²	137	145	36	319	231	48	419	217	869	327	0.50	
Jiang, 2015 ⁵³	59	107	38	62	108	56	225	183	232	220	0.51	
Ramírez-Chau, 2016 ⁵⁴	44	79	42	90	151	50	167	163	331	251	0.32	
Xu, 2016 ⁵⁵	35	57	28	22	50	28	127	113	94	106	0.97	
Taslim, 2017 ⁵⁶	19	5	0	26	19	2	43	5	71	23	0.52	
Rafik, 2019 ⁵⁷	44	8	0	97	74	11	96	8	268	96	0.53	

Table 2. Distribution of *MTHFR* C677T polymorphism genotype and allele in NSCL/P patients and controls.

 Abbreviation: HWE, Hardy-Weinberg equilibrium.

studies providing sufficient data about the alleles and genotypes of *MTHFR* C677T polymorphism in case and control groups. Exclusion criteria: (a) studies not related to the relationship between *MTHFR* C677T polymorphism and the NSCL/P susceptibility; (b) duplicate studies/erratum; (c) review/meta-analysis, letter to editors, commentaries, 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 *MTHFR* 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^{19,20}. Heterogeneity across the studies was evaluated using both the Cochrane Q test^{21,22} and I² metric^{23,24} ranging from 0 to 100%²⁵. 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.

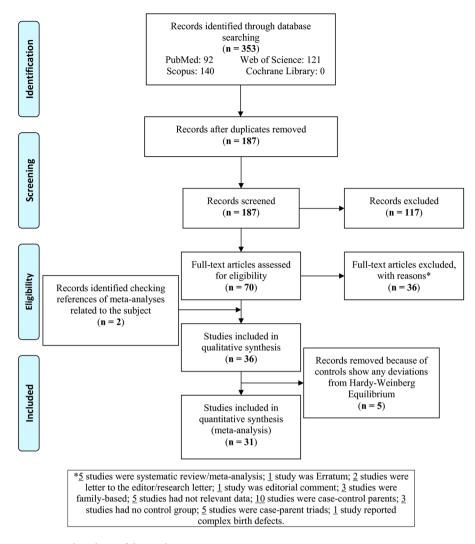


Figure 1. Flowchart of the study.

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 software²⁶. 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; <u>one</u> study was erratum; <u>two</u> studies were letter to editors/research letters; <u>one</u> study was editorial comment; <u>three</u> studies were family-based studies; <u>five</u> studies had irrelevant data; <u>ten</u> studies were case-control parents; <u>three</u> studies had no control group; <u>five</u> studies were case-parent triads; <u>one</u> 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^{6,58-61} 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 study⁶² 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

	NSCL	./P	Cont	rol		Odds Ratio	Odds Ratio
Study or Subgroup	Events	Total	Events	Total	Weight	M-H, Random, 95% CI Year	
Shaw, 1998	207	620	276	1066	4.4%	1.43 [1.16, 1.78] 1998	· · · ·
Gaspar, 1999	55	154	79	226	3.0%	1.03 [0.67, 1.59] 1999	
Martinelli, 2001	82	232	77	212	3.3%	0.96 [0.65, 1.41] 2001	
Grunert, 2002	38	132	119	368	3.0%	0.85 [0.55, 1.31] 2002	· · · · ·
van Rooij, 2003	57	210	62	256	3.1%	1.17 [0.77, 1.77] 2003	, .
Shotelersuk, 2003	25	218	50	404	2.6%	0.92 [0.55, 1.53] 2003	, <u> </u>
Pezzetti, 2004	106	220	237	578	3.8%	1.34 [0.98, 1.83] 2004	. –
Wan, 2006	77	152	38	120	2.6%	2.22 [1.35, 3.65] 2006	; – –
Chevrier, 2007	104	296	147	336	3.7%	0.70 [0.50, 0.96] 2007	·
Brandalize, 2007	84	228	69	200	3.2%	1.11 [0.74, 1.65] 2007	·
Little, 2008	67	192	159	448	3.5%	0.97 [0.68, 1.39] 2008	
Mills, 2008	329	984	1047	3198	4.7%	1.03 [0.89, 1.20] 2008	• +
Sozen, 2009	116	358	79	276	3.6%	1.20 [0.85, 1.68] 2009) .
Guo, 2009	101	192	105	206	3.2%	1.07 [0.72, 1.58] 2009)
Ali, 2009	109	646	40	428	3.3%	1.97 [1.34, 2.89] 2009)
Mostowska, 2010	99	326	109	342	3.7%	0.93 [0.67, 1.29] 2010	
Chorna, 2011	25	66	30	100	2.0%	1.42 [0.74, 2.74] 2011	
Han, 2011	176	374	168	426	4.0%	1.37 [1.03, 1.81] 2011	_ -
Semic-Jusufagic, 2012	34	112	40	152	2.4%	1.22 [0.71, 2.10] 2012	· · · · ·
Kumari, 2013	156	936	110	938	4.1%	1.51 [1.16, 1.96] 2013	,
Murthy, 2014	19	246	37	282	2.3%	0.55 [0.31, 0.99] 2014	·
Estandia-Ortega, 2014	133	264	458	740	4.0%	0.63 [0.47, 0.83] 2014	·
Jahanbin, 2014	30	86	69	202	2.5%	1.03 [0.61, 1.75] 2014	·
de Aguiar, 2015	217	636	327	1196	4.4%	1.38 [1.12, 1.69] 2015	; –
Jiang, 2015	183	408	220	452	4.0%	0.86 [0.66, 1.12] 2015	;
Abdollahi-Fakhim, 2015	29	130	24	100	2.1%	0.91 [0.49, 1.69] 2015	;
Bezerra, 2015	78	280	110	350	3.5%	0.84 [0.60, 1.19] 2015	;
Xu, 2016	113	240	106	200	3.4%	0.79 [0.54, 1.15] 2016	·
Ramírez-Chau, 2016	163	330	251	582	4.0%	1.29 [0.98, 1.69] 2016	
Taslim, 2017	5	48	23	94	1.0%	0.36 [0.13, 1.01] 2017	
Rafik, 2019	8	104	96	364	1.6%	0.23 [0.11, 0.50] 2019	,
Total (95% CI)		9420		14842	100.0%	1.04 [0.93, 1.17]	•
Total events	3025		4762				
Heterogeneity: Tau ² = 0.07	7; Chi² = 1	100.64,	df = 30 (F	o < 0.00	001); I ² = 70	0%	0.1 0.2 0.5 1 2 5 10
Test for overall effect: Z =	0.70 (P =	0.49)					0.1 0.2 0.5 1 2 5 10 Favours [NSCL/P] Favours [control]

Figure 2. Random-effect forest plot of allele model (T vs. C) for the association between the NSCL/P risk and *MTHFR* C677T polymorphism.

	NSCL	/P	Contr	ol		Odds Ratio		Odds Ratio
Study or Subgroup	Events	Total	Events	Total	Weight	M-H, Random, 95% Cl	Year	M-H, Random, 95% CI
Shaw, 1998	40	183	49	205	5.5%	0.89 [0.55, 1.43]	1998	
Gaspar, 1999	8	38	15	64	3.0%	0.87 [0.33, 2.30]	1999	
Martinelli, 2001	30	94	17	63	4.2%	1.27 [0.63, 2.57]	2001	
Grunert, 2002	6	40	25	115	3.0%	0.64 [0.24, 1.68]	2002	
/an Rooij, 2003	6	60	4	74	2.0%	1.94 [0.52, 7.24]	2003	
Shotelersuk, 2003	0	84	2	156	0.5%	0.37 [0.02, 7.71]	2003	· · · ·
Pezzetti, 2004	24	52	43	138	4.5%	1.89 [0.99, 3.64]	2004	
Wan, 2006	14	27	9	40	2.7%	3.71 [1.29, 10.69]	2006	
Brandalize, 2007	19	68	14	59	3.7%	1.25 [0.56, 2.77]	2007	
Chevrier, 2007	22	88	33	87	4.5%	0.55 [0.29, 1.04]	2007	
Little, 2008	10	49	29	123	3.7%	0.83 [0.37, 1.87]	2008	
Mills, 2008	54	271	163	878	6.3%	1.09 [0.77, 1.54]	2008	
Sozen, 2009	18	99	7	73	3.2%	2.10 [0.83, 5.32]	2009	
Guo, 2009	24	43	24	46	3.6%	1.16 [0.50, 2.67]	2009	
Ali, 2009	11	236	2	178	1.6%	4.30 [0.94, 19.66]	2009	· · · · ·
Mostowska, 2010	17	98	16	94	4.0%	1.02 [0.48, 2.17]	2010	
Han, 2011	35	81	29	103	4.7%	1.94 [1.05, 3.59]	2011	
Chorna, 2011	4	16	2	24	1.2%	3.67 [0.58, 23.03]	2011	
Semic-Jusufagic, 2012	3	28	8	52	1.8%	0.66 [0.16, 2.72]	2012	
Kumari, 2013	15	342	5	369	2.8%	3.34 [1.20, 9.29]	2013	
Murthy, 2014	0	104	3	110	0.5%	0.15 [0.01, 2.88]	2014	←
Jahanbin, 2014	7	27	14	60	2.7%	1.15 [0.40, 3.28]	2014	
Estandia-Ortega, 2014	39	77	143	198	5.1%	0.39 [0.23, 0.68]	2014	
Jiang, 2015	38	97	56	118	5.1%	0.71 [0.41, 1.23]	2015	
le Aguiar, 2015	36	173	48	367	5.5%	1.75 [1.08, 2.81]	2015	
Abdollahi-Fakhim, 2015	2	40	1	28	0.7%	1.42 [0.12, 16.48]	2015	
Bezerra, 2015	12	86	20	105	3.8%	0.69 [0.32, 1.50]	2015	
Xu, 2016	28	63	28	50	4.0%	0.63 [0.30, 1.33]	2016	
Ramírez-Chau, 2016	42	86	50	140	5.1%	1.72 [1.00, 2.97]	2016	
Taslim, 2017	0	19	2	28	0.5%	0.27 [0.01, 5.99]	2017	· · · · · · · · · · · · · · · · · · ·
Rafik, 2019	0	44	11	108	0.5%	0.10 [0.01, 1.65]	2019	·
Total (95% CI)		2813		4253	100.0%	1.11 [0.89, 1.38]		•
Total events	564		872					
Heterogeneity: Tau² = 0.1 Test for overall effect: Z =			lf = 30 (P	= 0.000	05); I² = 52%	6		U.05 0.2 1 5 Favours [NSCL/P] Favours [control]

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

_

	NSCL	/P	Contr	ol		Odds Ratio	Odds Ratio
Study or Subgroup	Events	Total	Events	Total	Weight	M-H, Random, 95% CI Yea	M-H, Random, 95% CI
Shaw, 1998	127	270	178	334	4.4%	0.78 [0.56, 1.07] 199	3
Gaspar, 1999	39	69	49	98	2.9%	1.30 [0.70, 2.41] 199)
Martinelli, 2001	22	86	43	89	2.9%	0.37 [0.19, 0.70] 200	·
Grunert, 2002	26	60	69	159	3.0%	1.00 [0.55, 1.82] 200	2
van Rooij, 2003	45	99	54	124	3.3%	1.08 [0.63, 1.84] 200	3
Shotelersuk, 2003	25	109	46	200	3.2%	1.00 [0.57, 1.74] 200	3
Pezzetti, 2004	58	86	151	246	3.4%	1.30 [0.78, 2.19] 200	4 -
Wan, 2006	49	62	20	51	2.2%	5.84 [2.55, 13.41] 200	;
Chevrier, 2007	60	126	81	135	3.5%	0.61 [0.37, 0.99] 200	· · ·
Brandalize, 2007	46	95	41	86	3.1%	1.03 [0.57, 1.85] 200	7 <u> </u>
Mills, 2008	221	438	721	1436	4.9%	1.01 [0.82, 1.25] 200	3
Little, 2008	47	86	101	195	3.5%	1.12 [0.67, 1.87] 200	3
Guo, 2009	53	72	57	79	2.5%	1.08 [0.52, 2.21] 200	
Ali, 2009	87	312	36	212	3.8%	1.89 [1.22, 2.92] 200	
Sozen, 2009	80	161	65	131	3.7%	1.00 [0.63, 1.59] 200	
Mostowska, 2010	65	146	77	155	3.7%	0.81 [0.52, 1.28] 201)
Han, 2011	106	152	110	184	3.7%	1.55 [0.98, 2.44] 201	ı •
Chorna, 2011	17	29	26	48	1.9%	1.20 [0.47, 3.04] 201	I
Semic-Jusufagic, 2012	28	53	24	68	2.5%	2.05 [0.99, 4.28] 201	2
Kumari, 2013	126	453	100	464	4.5%	1.40 [1.04, 1.90] 201	3
Jahanbin, 2014	16	36	41	87	2.3%	0.90 [0.41, 1.96] 201	4
Estandia-Ortega, 2014	55	93	172	227	3.4%	0.46 [0.28, 0.77] 201	4 <u> </u>
Murthy, 2014	19	123	31	138	2.9%	0.63 [0.34, 1.19] 201	4
Jiang, 2015	107	166	108	170	3.8%	1.04 [0.67, 1.63] 201	5
de Aguiar, 2015	145	282	231	550	4.6%	1.46 [1.10, 1.95] 201	5
Bezerra, 2015	54	128	70	155	3.6%	0.89 [0.55, 1.42] 201	5
Abdollahi-Fakhim, 2015	25	63	22	49	2.4%	0.81 [0.38, 1.72] 201	5
Xu, 2016	57	92	50	72	2.8%	0.72 [0.37, 1.38] 201	· · · ·
Ramírez-Chau, 2016	79	123	151	241	3.7%	1.07 [0.68, 1.68] 201	· · ·
Taslim, 2017	5	24	19	45	1.4%	0.36 [0.11, 1.14] 201	
Rafik, 2019	8	52	74	171	2.2%	0.24 [0.11, 0.54] 201	
Total (95% CI)		4146		6399	100.0%	0.99 [0.85, 1.16]	
Total events	1897		3018				
Heterogeneity: Tau ² = 0.12 Test for overall effect: Z =		,	if = 30 (P	< 0.000	001); I² = 67	%	0.1 0.2 0.5 1 2 5 10 Favours [NSCL/P] Favours [control]

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

	NSCL	/P	Contr	ol		Odds Ratio		Odds Ratio
Study or Subgroup	Events	Total	Events	Total	Weight	M-H, Random, 95% Cl	Year	M-H, Random, 95% Cl
Shaw, 1998	167	310	227	383	4.3%	0.80 [0.59, 1.09]	1998	
Gaspar, 1999	47	77	64	113	3.0%	1.20 [0.66, 2.16]	1999	
Martinelli, 2001	52	116	60	106	3.2%	0.62 [0.37, 1.06]	2001	
Grunert, 2002	32	66	94	184	3.1%	0.90 [0.51, 1.58]	2002	
Shotelersuk, 2003	25	109	48	202	3.1%	0.95 [0.55, 1.66]	2003	
van Rooij, 2003	51	105	58	128	3.3%	1.14 [0.68, 1.91]	2003	
Pezzetti, 2004	82	110	194	289	3.4%	1.43 [0.87, 2.35]	2004	
Wan, 2006	63	76	29	60	2.3%	5.18 [2.37, 11.33]	2006	
Brandalize, 2007	65	114	55	100	3.2%	1.09 [0.63, 1.86]	2007	
Chevrier, 2007	82	148	114	168	3.6%	0.59 [0.37, 0.93]	2007	
Little, 2008	57	96	130	224	3.4%	1.06 [0.65, 1.72]	2008	
Mills, 2008	275	492	884	1599	4.7%	1.03 [0.84, 1.26]	2008	
Guo, 2009	77	96	81	103	2.6%	1.10 [0.55, 2.19]	2009	
Ali, 2009	98	323	38	214	3.7%	2.02 [1.32, 3.08]	2009	———
Sozen, 2009	98	179	72	138	3.6%	1.11 [0.71, 1.73]	2009	
Mostowska, 2010	82	163	93	171	3.7%	0.85 [0.55, 1.31]		
Han, 2011	141	187	139	213	3.7%	1.63 [1.05, 2.52]	2011	
Chorna, 2011	21	33	28	50	1.9%	1.38 [0.56, 3.39]	2011	
Semic-Jusufagic, 2012	31	56	32	76	2.6%	1.71 [0.85, 3.42]		
Kumari, 2013	141	468	105	469	4.3%		2013	
Murthy, 2014	19	123	34	141	2.8%	0.57 [0.31, 1.07]	2014	
Jahanbin, 2014	23	43	55	101	2.5%	0.96 [0.47, 1.97]	2014	
Estandia-Ortega, 2014	94	132	315	370	3.5%	0.43 [0.27, 0.69]		
Jiang, 2015	145	204	164	226	3.7%	0.93 [0.61, 1.42]		
de Aguiar, 2015	181	318	279	598	4.4%	1.51 [1.15, 1.99]		
Bezerra, 2015	66	140	90	175	3.6%	0.84 [0.54, 1.31]		
Abdollahi-Fakhim, 2015	27	65	23	50	2.4%	0.83 [0.40, 1.75]		
Ramírez-Chau, 2016	121	165	201	291	3.7%	1.23 [0.80, 1.88]		
Xu, 2016	85	120	78	100	2.9%	0.68 [0.37, 1.27]		
Taslim, 2017	5	24	21	47	1.4%	0.33 [0.10, 1.02]		
Rafik, 2019	8	52	85	182	2.2%	0.21 [0.09, 0.47]		←
Total (95% CI)		4710		7271	100.0%	1.00 [0.86, 1.18]		
Total events	2461		3890					
Heterogeneity: Tau² = 0.1 Test for overall effect: Z =			df = 30 (F	P < 0.00	0001); I ² = 7	0%		0.1 0.2 0.5 1 2 5 Favours [NSCL/P] Favours [control]

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.

. . .

	NSCL	/P	Contr	ol		Odds Ratio		Odds Ratio
Study or Subgroup	Events	Total	Events	Total	Weight	M-H, Fixed, 95% CI Ye	ar	M-H, Fixed, 95% Cl
Shaw, 1998	40	310	49	383	7.4%	1.01 [0.65, 1.58] 19	98	
Gaspar, 1999	8	77	15	113	2.1%	0.76 [0.30, 1.88] 19	99	
Martinelli, 2001	30	116	17	106	2.6%	1.83 [0.94, 3.55] 20	01	
Grunert, 2002	6	66	25	184	2.3%	0.64 [0.25, 1.63] 20	02	
van Rooij, 2003	6	105	4	128	0.7%	1.88 [0.52, 6.84] 20	03	
Shotelersuk, 2003	0	109	2	202	0.3%	0.37 [0.02, 7.70] 20	03 ←	
Pezzetti, 2004	24	110	43	289	3.6%	1.60 [0.92, 2.79] 20	04	+
Wan, 2006	14	76	9	60	1.6%	1.28 [0.51, 3.20] 20	06	
Chevrier, 2007	22	148	33	168	5.1%	0.71 [0.40, 1.29] 20	07	
Brandalize, 2007	19	114	14	100	2.4%	1.23 [0.58, 2.60] 20	07	
Mills, 2008	54	492	163	1599	13.3%	1.09 [0.78, 1.50] 20	08	- -
Little, 2008	10	96	29	224	3.0%	0.78 [0.36, 1.68] 20		
Sozen, 2009	18	179	7	138	1.4%	2.09 [0.85, 5.16] 20	09	+
Ali, 2009	11	323	2	214	0.5%	3.74 [0.82, 17.03] 20		
Guo, 2009	24	96	24	103	3.4%	1.10 [0.57, 2.10] 20	09	
Mostowska, 2010	17	163	16	171	2.7%	1.13 [0.55, 2.32] 20	10	
Chorna, 2011	4	33	2	50	0.3%	3.31 [0.57, 19.22] 20	11	
Han, 2011	35	187	29	213	4.3%	1.46 [0.85, 2.50] 20	11	+
Semic-Jusufagic, 2012	3	56	8	76	1.2%	0.48 [0.12, 1.90] 20	12	
Kumari, 2013	15	468	5	469	0.9%	3.07 [1.11, 8.52] 20	13	· · · · · · · · · · · · · · · · · · ·
Murthy, 2014	0	123	3	141	0.6%	0.16 [0.01, 3.13] 20	14 🔶	
Estandia-Ortega, 2014	39	132	143	370	10.3%	0.67 [0.43, 1.02] 20	14	
Jahanbin, 2014	7	43	14	101	1.4%	1.21 [0.45, 3.24] 20	14	
de Aguiar, 2015	36	318	48	598	5.7%	1.46 [0.93, 2.31] 20	15	—
Abdollahi-Fakhim, 2015	2	65	1	50	0.2%	1.56 [0.14, 17.66] 20	15	
Bezerra, 2015	12	140	20	175	3.2%	0.73 [0.34, 1.54] 20	15	
Jiang, 2015	38	204	56	226	8.4%	0.69 [0.44, 1.11] 20	15	
Ramírez-Chau, 2016	42	165	50	291	5.2%	1.65 [1.03, 2.62] 20	16	
Xu, 2016	28	120	28	100	4.5%	0.78 [0.43, 1.44] 20	16	
Taslim, 2017	0	24	2	47	0.3%	0.37 [0.02, 8.05] 20	17 🔶	· · · ·
Rafik, 2019	0	52	11	182	1.0%	0.14 [0.01, 2.45] 20	19 🔶	
Total (95% CI)		4710		7271	100.0%	1.08 [0.96, 1.21]		•
Total events	564		872					
Heterogeneity: Chi ² = 42.4	9, df = 30	(P = 0	.06); I ² = 3	29%			10.05	
Test for overall effect: Z =	1.21 (P =	0.23)					0.05	Favours [NSCL/P] Favours [control]

Figure 6. Random-effect forest plot of recessive model (TT vs. CC + CT) for the association between the NSCL/P risk and *MTHFR* C677T polymorphism.

.....

	T vs. C	TT vs. CC	CT vs. CC	TT+CT vs. CC	TT vs. CC + CT
Study (n)	OR (95% CI), I ²				
	(%), P _h				
Overall (31)	1.04 (0.93, 1.17),	1.11 (0.89, 1.38),	0.99 (0.85, 1.16),	1.00 (0.86, 1.18),	1.08 (0.96, 1.21),
	70, <0.00001	52, 0.0005	67, <0.00001	70, <0.00001	29, 0.06
Ethnicity					
Asian (10)	1.10 (0.85, 1.43),	1.34 (0.78, 2.29),	1.21 (0.87, 1.67),	1.18 (0.84, 1.67),	1.06 (0.83, 1.35),
	77, <0.00001	61, 0.006	71, 0.0003	76, <0.0001	40, 0.09
Caucasian (11)	1.01 (0.92, 1.13),	1.08 (0.86, 1.34),	0.94 (0.82, 1.08),	0.97 (0.85, 1.11),	1.13 (0.92, 1.39),
	12, 0.33	13, 0.32	47, 0.04	29, 0.17	8, 0.37
Mixed (10)	0.99 (0.79, 1.24),	0.99 (0.67, 1.46),	0.89 (0.68, 1.17),	0.89 (0.66, 1.19),	1.04 (0.87, 1.26),
	81, <0.00001	67, 0.001	71, 0.0003	77, <0.00001	45, 0.06
Source of cases					
PB (13)	1.10 (0.94, 1.28),	1.01 (0.81, 1.25),	1.00 (0.81, 1.24),	1.03 (0.85, 1.24),	1.04 (0.86, 1.27),
	62, 0.002	29, 0.16	61, 0.002	57, 0.006	37, 0.09
HB (18)	1.00 (0.84, 1.18),	1.11 (0.81, 1.53),	0.98 (0.78, 1.24),	0.98 (0.77, 1.25),	1.10 (0.94, 1.28),
	75, <0.00001	60, 0.0005	71, <0.00001	77, <0.00001	26, 0.15

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_h means $P_{heterogeneity}$.

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*

Models for year of publication		Point Estimate	Standard Error	Lower Limit	Upper Limit	Z-value	Р
T vs. C	Slope	-0.01346	0.00548	-0.02420	-0.00271	-2.45454	0.01411
1 VS. C	Intercept	27.12064	11.01569	5.53028	48.71099	2.46200	0.01382
TT vs. CC	Slope	-0.00466	0.01212	-0.02842	0.01910	-0.38437	0.70071
1 1 VS. CC	Intercept	9.44414	27.35460	-38.29000	57.17828	0.38778	0.69818
CT vs. CC	Slope	0.00449	0.00801	-0.01122	0.02019	0.55990	0.57555
CI VS. CC	Intercept	-8.98145	16.09724	-40.53146	22.56857	-0.55795	0.57688
TT+CT vs. CC	Slope	0.00449	0.00801	-0.01122	0.02019	0.55990	0.57555
11+C1 vs. CC	Intercept	-8.98145	16.09724	-40.53146	22.56857	-0.55795	0.57688
TT CC CT	Slope	-0.00788	0.01100	-0.02945	0.01369	-0.71614	0.47391
TT vs. CC + CT	Intercept	15.91868	22.11551	-27.42.692	59.26427	0.71980	0.47165
Models for number of individuals		Point Estimate	Standard Error	Lower Limit	Upper Limit	Z-value	Р
Terro	Slope	0.00004	0.00005	-0.00005	0.00014	0.89668	0.36989
T vs. C	Intercept	0.05212	0.04523	-0.03652	0.14076	1.15249	0.24912
TT vs. CC	Slope	0.00006	0.00011	-0.00015	0.00027	0.56260	0.57371
11 vs. CC	Intercept	0.04092	0.10171	-0.15842	0.24026	0.40233	0.68744
CT vs. CC	Slope	0.00006	0.00007	-0.00008	0.00019	0.83343	0.40460
CI vs. CC	Intercept	-0.00968	0.06556	-0.13818	0.11882	-0.14765	0.88262
TT+CT vs. CC	Slope	0.00006	0.00006	-0.00006	0.00019	0.97564	0.32924
11 + C1 vs. CC	Intercept	0.00136	0.06221	-0.12057	0.12329	0.02180	0.98261
TT vs. CC + CT	Slope	0.00005	0.00010	-0.00015	0.00025	0.48796	0.62558
11 vs. CC+C1	Intercept	0.04821	0.09159	-0.13129	0.22771	0.52639	0.59862

Table 4. Fixed-effect meta-regression of log odds ratio for the publication year and number of individuals.

.....

 $\begin{array}{l} C677T \ polymorphism \ and \ NSCL/P \ susceptibility \ related \ to \ allelic \ model \ [OR = 1.04; 95\% \ CI: 0.93, 1.17; \ P = 0.49; \\ I^2 = 70\% \ (P_{heterogeneity} \ or \ P_h < 0.00001)], \ homozygote \ model \ [OR = 1.11; 95\% \ CI: 0.89, 1.38; \ P = 0.35; \ I^2 = 52\% \ (P_h = 0.0005)], \ heterozygote \ model \ [OR = 0.99; 95\% \ CI: 0.85, 1.16; \ P = 0.91; \ I^2 = 67\% \ (P_h < 0.00001)], \ heterozygote \ model \ [OR = 0.99; 95\% \ CI: 0.85, 1.16; \ P = 0.91; \ I^2 = 67\% \ (P_h < 0.00001)], \ heterozygote \ model \ [OR = 1.00; 95\% \ CI: 0.86, 1.18; \ P = 0.96; \ I^2 = 70\% \ (P_h < 0.00001)], \ and \ recessive \ model \ [OR = 1.08; 95\% \ CI: 0.96, 1.21; \ P = 0.23; \ I^2 = 29\% \ (P_h = 0.06)]. \end{array}$

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, 0.95933/0.97596, 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 pathogenesis⁶³. 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 gene⁶⁴. *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/P⁶⁵. The present meta-analysis was performed

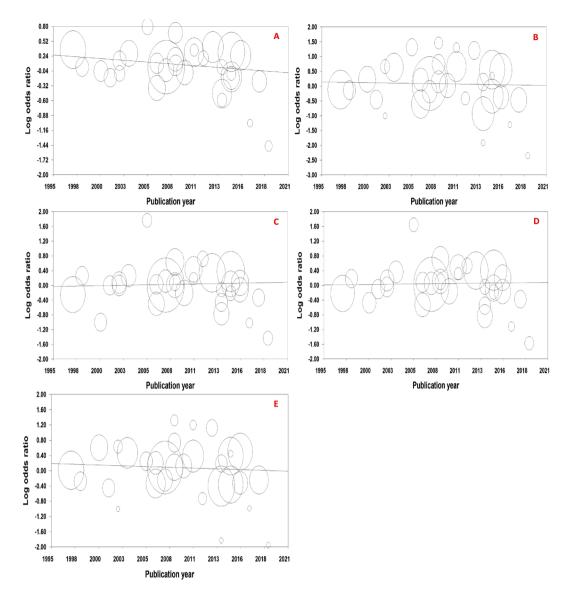


Figure 7. Fixed-effect meta-regression of log odds ratio versus publication year for (A) allele model, (B) homozygote model, (C) heterozygote model, (D) dominant model, and (E) recessive model.

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^{27,34,39,44,45,52}, five studies^{34,39,44,45,52}, and four studies^{34,39,46,52} reported significantly increased risk of T allele, TT genotype, and CT genotype in NSCL/P patients compared with controls, respectively. Also, five studies^{36,47,49,55,57}, two studies^{47,57}, and four studies^{28,36,47,57} 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^{34,39,44,46,52} and significantly decreased risk in four studies^{36,47,55,57} in NSCL/P patients compared with controls. Based on the recessive model, three studies^{39,46,54} reported significantly increased risk of TT genotype and one study⁵⁷ reported its significantly decreased risk in NSCL/P patients compared with controls.

A recent meta-analysis with 24 case-control studies¹⁴ 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 studies¹⁵ 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 studies¹⁵. Almost similar to the findings of a meta-analysis with 24 studies¹⁴, our results

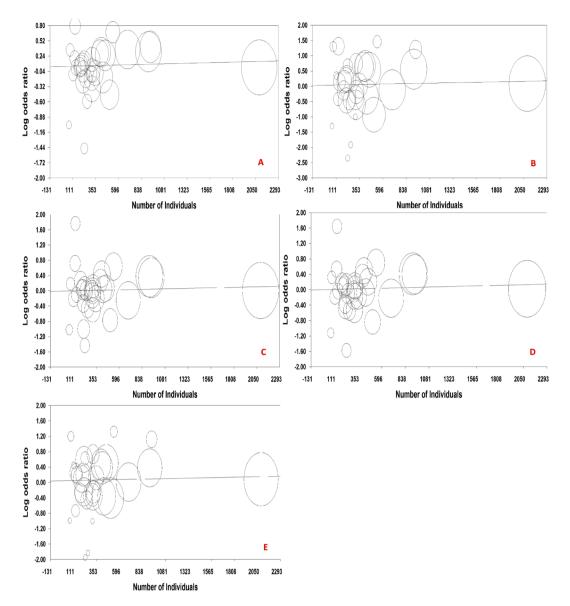


Figure 8. Fixed-effect meta-regression of log odds ratio versus number of individuals for (**A**) allele model, (**B**) homozygote model, (**C**) heterozygote model, (**D**) dominant model, and (**E**) recessive model.

showed no association between *MTHFR* C677T and susceptibility to NSCL/P. In one meta-analysis¹⁵, definition of ethnicity was different from that in another study¹⁴ 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 eight¹⁶ and nine⁷ case-control studies related to our topic. One of them¹⁶ reported no association and another one on Asian ethnicity showed a significant association between *MTHFR* C677T and susceptibility to NSCL/P. Luo *et al.*¹⁷ 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.*⁸ 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.

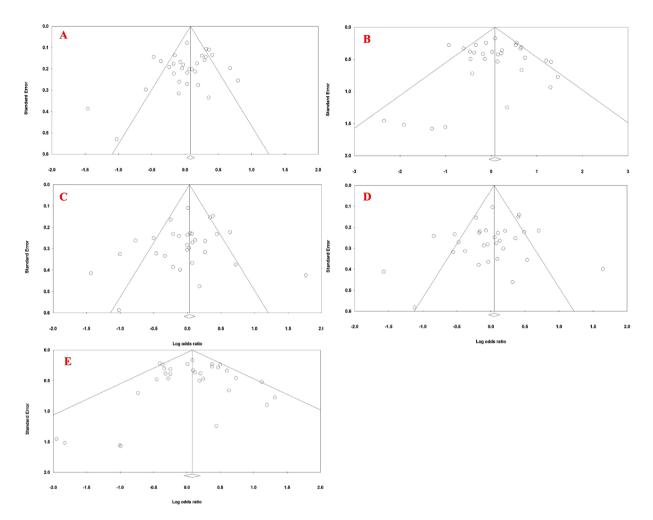


Figure 9. Funnel plot of (**A**) allele model, (**B**) homozygote model, (**C**) heterozygote model, (**D**) dominant model, and (**E**) recessive model for the association between the NSCL/P risk and *MTHFR* C677T polymorphism (fixed-effects model).

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.

Received: 10 July 2019; Accepted: 18 December 2019; Published online: 30 January 2020

References

- 1. Dixon, M. J., Marazita, M. L., Beaty, T. H. & Murray, J. C. Cleft lip and palate: understanding genetic and environmental influences. *Nat. Rev. Genet.* **12**, 167–178 (2011).
- Kadir, A. et al. Systematic Review and Meta-Analysis of the Birth Prevalence of Orofacial Clefts in Low- and Middle-Income Countries. Cleft Palate Craniofac J. 54, 571–581 (2017).
- 3. Rafik, A. & Nadifi, S. Updating genetics polymorphisms of non-syndromic clefts lip-palates. Am. J. Mol. Biol. 8, 178-185 (2018).
- 4. Goyette, P. *et al.* Gene structure of human and mouse methylenetetrahydrofolate reductase (MTHFR). *Mamm. Genome* **9**, 652–656 (1998).
- Yamada., K., Strahler, J. R., Andrews, P. C. & Matthews, R. G. Regulation of human methylenetetrahydrofolate reductase by phosphorylation. *Proc. Natl Acad. Sci. USA* 102, 10454–10459 (2005).
- Ebadifar, A. et al. Maternal Supplementary Folate Intake, Methylenetetrahydrofolate Reductase (MTHFR) C677T and A1298C Polymorphisms and the Risk of Orofacial Cleft in Iranian Children. Avicenna J. Med. Biotechnol. 7, 80–84 (2015).
- 7. Zhao, M., Ren, Y., Shen, L., Zhang, Y. & Zhou, B. Association between MTHFR C677T and A1298C polymorphisms and NSCL/P risk in Asians: a meta- analysis. *PLoS One* **9**, e88242 (2014).
- Pan, Y. et al. Infants' MTHFR polymorphisms and nonsyndromic orofacial clefts susceptibility: a meta-analysis based on 17 casecontrol studies. Am. J. Med. Genet. A 158, 2162–2169 (2012).
- 9. Li, S. S., Li, J., Xiao, Z., Ren, A. G. & Jin, L. Prospective study of MTHFR genetic polymorphisms as a possible etiology of male infertility. *Genet. Mol. Res.* 13, 6367–6374 (2014).
- 10. Li, S. et al. Folic Acid Use and Nonsyndromic Orofacial Clefts in China. Epidemiol. 23, 423-432 (2012).
- 11. Wehby, G. L. & Murray, J. C. Folic Acid and Orofacial Clefts: A Review of the Evidence. Oral. Dis. 16, 11-19 (2010).

- Imani, M. M., Mozaffari, H. R., Sharifi, R. & Sadeghi, M. Polymorphism of reduced folate carrier 1 (A80G) and non-syndromic cleft lip/palate: A systematic review and meta-analysis. Arch. Oral. Biol. 98, 273–279 (2019).
- 13. Zhao, H. *et al.* Is MTHFD1 polymorphism rs 2236225 (c.1958G > A) associated with the susceptibility of NSCL/P? A systematic review and meta-analysis. Version 2. *F1000Res* 4,142 (2015).
- 14. Wang, Y. *et al.* Methylenetetrahydrofolate reductase rs1801133 C > T polymorphism is association with nonsyndromic cleft lip with or without cleft palate susceptibility: a meta-analysis. *Int. J. Clin. Exp. Med.* **10**, 1734–1749 (2017).
- Rai, V. Strong Association of C677T Polymorphism of Methylenetetrahydrofolate Reductase Gene With Nosyndromic Cleft Lip/ Palate (nsCL/P). Indian. J. Clin. Biochem. 33, 5–15 (2018).
- Verkleij-Hagoort, A., Bliek, J., Sayed-Tabatabaei, F., Ursem, N. & Steegers, E. Steegers-& Theunissen, R. Hyperhomocysteinemia and MTHFR polymorphisms in association with orofacial clefts and congenital heart defects: a meta-analysis. Am. J. Med. Gene Part. A 143A, 952–960 (2007).
- 17. Luo, Y. L. et al. Association between MTHFR polymorphisms and orofacial clefts risk: a meta-analysis. Birth Defects Res. A Clin. Mol. Teratol. 94, 237–244 (2012).
- Moher, D., Liberati, A., Tetzlaff, J. & Altman, D. G. & PRISMA Group. Preferred reporting items for systematic reviews and metaanalyses: the PRISMA statement. PLoS Med. 6, e1000097 (2009).
- 19. Mantel, N. & Haenszel, W. Statistical aspects of the analysis of data from retrospective studies of disease. J. Natl Cancer Inst. 22, 719–748 (1959).
- 20. DerSimonian, R. & Laird, N. Meta-analysis in clinical trials. Control Clin. Trials 7, 177-188 (1986).
- 21. Zintzaras, E. & Ioannidis, J. P. Heterogeneity testing in meta-analysis of genome searches. Genet. Epidemiol. 24, 1–15 (2004).
- 22. Zintzaras, E. & Hadjigeorgiou, G. M. The role of G196A polymorphism in the brain-derived neurotrophic factor gene in the cause of Parkinson's disease: a meta-analysis. *J. Hum. Genet.* **50**, 560–566 (2005).
- 23. Higgins, J. P. & Thompson, S. E. Quantifying heterogeneity in a metaanalysis. Stat. Med. 21, 1539-1558 (2002).
- 24. Higgins, J. P., Thompson, S. G., Deeks, J. J. & Altman, D. G. Measuring inconsistency in meta analyses. Br. Med. J. 327, 557-560 (2003).
- 25. Begg, C. B. & Mazumdar, M. Operating characteristics of a rank correlation test for publication bias. *Biometrics* **50**, 1088–1101 (1994).
- 26. Egger, M., Davey Smith, G., Schneider, M. & Minder, C. Bias in metaanalysis detected by a simple, graphical test. *BMJ* **315**, 629–634 (1997).
- Shaw, G. M., Rozen, R., Finnell, R. H., Todoroff, K. & Lammer, E. J. Infant C677T mutation in MTHFR, maternal periconceptional vitamin use, and cleft lip. Am. J. Med. Genet. 80, 196–198 (1998).
- Martinelli, M. et al. C677T variant form at theMTHFR gene and CL/P: a risk factor for mothers? Am. J. Med. Genet. 98, 357–360 (2001).
- Gaspar, D. A. 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. 87, 197–199 (1999).
- Grunert, R. R., Braune, A., Schnackenberg, E., Schloot, W. & Krause, H. R. Genetic differences in enzymes of folic acid metabolism in patients with lip-jaw-palate clefts and their relatives. *Mund. Kiefer Gesichtschir* 6, 31–33 (2002).
- Shotelersuk, V., Ittiwut, C., Siriwan, P. & Angspatt, A. Maternal 677CT/1298AC genotype of the MTHFR gene as a risk factor for cleft lip. J. Med. Genet. 40, e64 (2003).
- Van Rooij, L. A., Swinkels, D. W., Blom, H. J., Markus, H. M. & Stegers-Theunissen, R. P. Vitamin and homocysteine status of mothers and infants and the risk of nonsyndromic orofacial clefts. Am. J. Obstet. Gynecol. 189, 1155–1160 (2003).
- Pezzetti, F. et al. Maternal MTHFR variant forms increase the risk in offspring of isolated nonsyndromic cleft lip with or without cleft palate. Hum. Mutat. 24, 104–105 (2004).
- Wan, W. D. et al. Relationship between nonsyndromic cleft lip with or without cleft palate (NSCL/P) and genetic polymorphisms of MTHFR C677T and A1298C. Zhonghua Zheng Xing Wai Ke Za Zhi 22, 8–11 (2006).
- Brandalize, A. P. et al. Polymorphisms in genes MTHFR, MTR and MTRR are not risk factors for cleft lip/palate in South Brazil. Braz. J. Med. Biol. Res. 40, 787–791 (2007).
- Chevrier, C. et al. Fetal and maternal MTHFR C677T genotype, maternal folate intake and the risk of nonsyndromic oral clefts. Am. J. Med. Genet. A 143, 248–257 (2007).
- Little, J. et al. Folate and clefts of the lip and palate-a U.K.-based case-control study: part II: biochemical and genetic analysis. Cleft Palate Craniofac J. 45, 428–438 (2008).
- 38. Mills, J. L. *et al.* Folate-related gene polymorphisms as risk factors for cleft lip and cleft palate. *Birth Defects Res. A* 82, 636–643 (2008).
- Ali, A., Singh, S. K. & Raman, R. MTHFR 677TT alone and IRF6 820GG together with MTHFR 677CT, but not MTHFR A1298C, are risks for non-syndromic cleft lip with or without cleft palate in an Indian population. *Genet. Test. Mol. Biomark* 13, 355–360 (2009).
- Guo, J. Z. et al. Relationship between genetic polymorphisms of MTHFR C677T and nonsyndromic cleft lip with or without palate. Beijing Da Xue Xue Bao 41, 432–436 (2009).
- Sozen, M. A., Tolarova, M. M. & Spritz, R. A. The common MTHFR C677T and A1298C variants are not associated with the risk of non-syndromic cleft lip/palate in northern Venezuela. J. Genet. Genomics 36, 283–288 (2009).
- 42. Mostowska, A., Hozyasz, K. K., Wojcicki, P., Dziegelewska, M. & Jagodzinski, P. P. Associations of folate and choline metabolism gene polymorphisms with orofacial clefts. *J. Med. Genet.* 47, 809–815 (2010).
- Chorna, L. B., Akopyan, H. R., Makukh, H. V. & Fedoryk, I. M. Allelic polymorphisms in the MTHFR, MTR and MTRR genes in patients with cleft lip and/or palate and their mothers. *Cytolo Genet.* 45, 177–181 (2011).
- Han, Y. et al. Methylenetetrahydrofolate reductase C677T and A1298C polymorphisms and nonsyndromic orofacial clefts susceptibility in a southern Chinese population. DNA Cell Biol. 30, 1063–1068 (2011).
- Semiç-Jusufagiç, A. et al. Association between C677T and A1298C MTHFR gene polymorphism and nonsyndromic orofacial clefts in the Turkish population: a case-parent study. Turkish J. Pediatr. 54, 617–625 (2012).
- 46. Kumari, P., Ali, A., Sukla, K. K., Singh, S. K. & Raman, R. Lower incidence of nonsyndromic cleft lip with or without cleft palate in females: is homocysteine a factor? J. Biosci. 38, 21–26 (2013).
- Estandia-Ortega, B. et al. 5,10-Methylenetetrahydrofolate reductase single nucleotide polymorphisms and gene–environment interaction analysis in analysis in non-syndromic cleft lip/palate. Eur. J. Oral. Sci. 122, 109–113 (2014).
- Jahanbin, A. et al. Analysis of MTHFR Gene C.677 C > T and C.1298 A > C Polymorphisms in Iranian Patients with Non-Syndromic Cleft Lip and Palate. Iran. J. Public. Health 43, 821–827 (2014).
- Murthy, J., Gurramkonda, V. B., Karthik, N. & Lakkakula, B. V. MTHFR C677T and A1298C polymorphisms and risk of nonsyndromic orofacial clefts in a south Indian population. *Int. J. Pediatr. Otorhinolaryngol.* 78, 339–342 (2014).
- Abdollahi-Fakhim, S. *et al.* Common Mutations of the Methylenetetrahydrofolate Reductase (MTHFR) Gene in Non-Syndromic Cleft Lips and Palates Children in North-West of Iran. *Iran. J. Otorhinolaryngol.* 27, 7–14 (2015).
- Bezerra, J. F. et al. Genetic and non-genetic factors that increase the risk of non-syndromic cleft lip and/or palate development. Oral. Dis. 21, 393–399 (2015).
- 52. de Aguiar, P. K. *et al.* rs1801133C > T polymorphism in MTHFR is a risk factor for nonsyndromic cleft lip with or without cleft palate in the Brazilian population. *Birth Defects Res. A Clin. Mol. Teratol.* **103**, 292–298 (2015).

- Jiang, C. et al. Lack of Association Between MTHFR, MTR, MTRR, and TCN2 Genes and Nonsyndromic CL ± P in a Chinese Population: Case-Control Study and Meta-Analysis. Cleft Palate Craniofac J. 52, 579–587 (2015).
- Ramírez-Chau, C., Blanco, R., Colombo, A., Pardo, R. & Suazo, J. MTHFR c.677 C > T is a risk factor for non-syndromic cleft lip with or without cleft palate in Chile. Oral. Dis. 22, 703–708 (2016).
- Taslim, T., Joenoes, H., Sulistyani, L. D., Latief, B. S. S. & Ibrahim, E. MTHFR C677T polymorphism in indonesian patients with oral cleft. JIDMR 10, 723–728 (2017).
- Xu, X., Pan, H., Yu, L. & Hong, Y. Association of MTHFR polymorphisms with nsCL/P in Chinese Uyghur population. Egypt. J. Med. Hum. Genet. 17, 311–316 (2016).
- Rafik, A., Rachad, L., Kone, A. S. & Nadifi, S. MTHFR C677T polymorphism and risk of nonsyndromic cleft lip with or without cleft palate in the Moroccan population. Appl. Clin. Genet. 12, 51–54 (2019).
- Aşlar, D., Özdiler, E., Altuğ, A. T. & Taştan, H. Determination of Methylenetetrahydrofolate Reductase (MTHFR) gene polymorphism in Turkish patients with nonsyndromic cleft lip and palate. *Int. J. Pediatr. Otorhinolaryngol.* 77, 1143–1146 (2013).
- Gaspar, D. A. *et al.* Maternal MTHFR interacts with the offspring's BCL3 genotypes, but not with TGFA, in increasing risk to nonsyndromic cleft lip with or without cleft palate. *Eur. J. Hum. Genet.* 12, 521–526 (2004).
- Wang, W., Jiao, X. H., Wang, X. P., Sun, X. Y. & Dong, C. MTR, MTRR, and MTHFR Gene Polymorphisms and Susceptibility to Nonsyndromic Cleft Lip With or Without Cleft Palate. *Genet. Test. Mol. Biomarkers* 20, 297–303 (2016).
- Nan, X., Liu, M. & Yuan, G. Relationship between genetic polymorphism of MTHFR C677T and nonsyndromic cleft lip with or without cleft palate in Shanxi Province of China. *Zhonghua Zheng Xing Wai Ke Za Zhi* 30, 265–269 (2014).
- 62. Tolarova, M. M. *et al.* A common mutation in the MTHFR gene is a risk factor for non-syndromic cleft lip and palate anomalies. *Am. J. Hum. Genet.* **63**, A27 (1998).
- 63. Hopper, R. A. Cleft lip and Palate: Embryology, Principles and Treatment, In: C. H. Thorne (Ed.), Grabb Smith's Plast. Surg., seventh, Lippincott Williams & Wilkins, Philadelphia. pp. 173–199 (2013).
- 64. Thaler, C. J. Folate Metabolism and Human Reproduction. Geburtshilfe Frauenheilkd. 74, 845-851 (2014).
- 65. Frosst, P. et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat. Genet. 10, 111–113 (1995).

Acknowledgements

This work was performed in partial fulfillment of the requirements for the degree of general dentistry by "Negin Golchin" at Faculty of Dentistry, Kermanshah University of Medical Sciences, Kermanshah, Iran. The authors gratefully acknowledge the Research Council of Kermanshah University of Medical Sciences (Grant number: 980686) for the financial support.

Author contributions

Conceptualization, M.M.I. and Ma.S.; Data curation, M.M.I., N.G. and Ma.S.; Formal analysis, Ma.S.; Funding acquisition, M.M.I.; Investigation, N.G. and Ma.S.; Methodology, M.M.I.; Project administration, M.M.I, Mo.S. and F.R.; Resources, H.A., P.L.-J. and H.R.M.; Software, M.S.; Supervision, M.M.I.; Validation, P.L.J. and M.S.; Visualization, M.M.I., Ma.S. and R.S.; Writing – original draft, Ma.S; Writing – review & editing, M.M.I., N.G., Mo.S., F.R., H.A., Ma.S., P.L.-J., H.R.M. and R.S.

Competing interests

The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to M.S.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2020