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Folic Acid Supplementation Use and the *MTHFR* C677T Polymorphism in Orofacial Clefts Etiology: An Individual Participant Data Pooled-Analysis

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Abstract

Background—This study examines gene-environment interaction (GEI) between the *MTHFR* C677T polymorphism and folic acid in the etiology of orofacial clefts (OFC). We used a pooled-analytic approach on four studies that used similar methods.

Methods—We used logistic regression to analyse the pooled sample of 1149 isolated cases and 1161 controls. Fetal and maternal *MTHFR* C677T genotypes, and maternal periconceptional exposure to smoking, alcohol, vitamin containing folic acid and folic acid supplements were contrasted between the cleft types [non-syndromic clefts lip or without cleft palate (CL(P)) and non syndromic cleft palate (CP)] and control groups.

Results—There was a reduced risk of CL(P) with maternal folic acid use ($p=0.008$; OR=0.70, 95% CI: 0.65–0.94) and with supplements containing folic acid ($p=0.028$, OR=0.80, 95% CI: 0.65–0.94). Maternal smoking increased the risk of both CL(P) ($p<10e-3$; OR=1.62, 95% CI: 1.35–1.95) and CP ($p=0.028$; OR=1.38, 95% CI: 1.04–1.83). No significant risk was observed with either maternal or fetal *MTHFR* C677T genotypes.

Conclusion—This individual participant data (IPD) meta-analysis affords greater statistical power and can help alleviate the problems associated with aggregate-level data-sharing. The result of this IPD meta-analysis is consistent with previous reports suggesting that folic acid and smoking influence OFC outcomes.

Keywords

Cleft lip and palate; *MTHFR*; Folic acid; Individual patient data; pooled-analysis

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CONFLICT OF INTEREST

None

INTRODUCTION

Orofacial clefts (OFC) include cleft lip with or without cleft palate (CL(P)) and cleft palate only (CP). Collectively, they are among the most common birth defects and the most frequent congenital malformations of the head and neck area. The worldwide prevalence is around 1 in 700 (WHO, 2002). This rate varies across ethnic groups and geographic regions. The rate of CL(P) is higher in Latin American and Asian countries (China, Japan) compared with Israel, South Africa, and southern Europe. Similarly, the rate of CP is higher in Canada and parts of northern Europe compared with parts of Latin America and South Africa (Mossey *et al.*, 2009). The management of OFC requires a multidisciplinary approach involving surgical, nutritional, dental, speech, medical and behavioral interventions (Wehby and Cassell, 2009).

The development of craniofacial structures is the product of an exquisitely coordinated sequence of event that involves the growth of several independently-derived facial primordia. Genetic and environmental factors, and their interactions, may disrupt these events and cause a cleft of the lip and/or the palate (Jugessur *et al.*, 2009). Motivated by the successes of maternal folic acid use in the prevention of neural tube defects (Smithells *et al.*, 1981), several studies have investigated the role of folic acid in orofacial cleft aetiology. Since cleft lip and cleft palate appear to be genetically (Dixon *et al.*, 2011) and embryologically (Thomson and Dixon, 2009) distinct entities, it is possible that the effect of folic acid varies across the two cleft subtypes. Several studies have reported a reduced risk of CL(P) when mothers used either folic acid supplements or dietary folate during the periconceptional period (Chevrier *et al.*, 2007; Wilcox *et al.*, 2007; Jia *et al.*, 2011; Kelly *et al.*, 2012). However, there are some notable exceptions (Little *et al.*, 2008a; Sayed *et al.*, 2008). A review of the existing literature on the effect of dietary folate and folic acid fortification on CL(P) (Johnson and Little, 2008) highlights varying degrees of heterogeneity between studies and no strong evidence of association between CL(P) and dietary folate alone. This could be the result of confounding by other lifestyle factors, by other B-vitamins present in these multivitamin pills, or by any other vitamin and/or minerals that might have a beneficial effect on OFC risk. Current evidence is still equivocal, as illustrated by a recent Cochrane review on the impact of periconceptional folate supplementation on birth defects prevention (De-Regil *et al.*, 2010), in which the authors found no statistically significant evidence for a protective effect of folic acid on the risk of cleft palate, cleft lip, congenital cardiovascular defects, miscarriages, and other birth defects. However, the individual papers included in the review reported effects that are statistically significant without clear evidence of clinical significance.

Increased risk for CP has been reported with maternal *MTHFR* 677 TT genotypes (Zhu *et al.*, 2006; Mills *et al.*, 2008). A reduced risk for CL(P) has also been reported with maternal *MTHFR* C677T genotypes (Jugessur *et al.*, 2003; Little *et al.*, 2008b). In contrast, Boyles *et al.* (2008) did not find any associated risk with maternal *MTHFR* CT or TT genotypes for either CL(P) or CP. On the other hand, Jugessur *et al.* (2003) observed a dominant pattern of increased risk of cleft palate only with the child's C677T genotypes. More recently, a meta-analysis on the role of *MTHFR* polymorphisms in both mothers and children did not find any significant associations for CL(P) (Verkleij-Hagoort *et al.*, 2007).

Several studies have explored gene-environment interactions between a range of environmental and genetic factors in the aetiology of orofacial clefts (Skare *et al.*, 2012). Studies by van Rooij *et al.* (2003) and Chevrier *et al.* (2007) found reduced risks for CL(P) among mothers carrying the *MTHFR* genotypes CT and TT, who were among those with the highest intake of dietary folate or took folic acid supplements. A higher risk of CP was

reported by Jugessur *et al.* (2003) for children who carried the *MTHFR* 677TT genotype, where the mothers took folic acid supplements.

In order to circumvent some of the problems associated with aggregate-level data analysis, we performed an individual participant data (IPD) pooled-analysis in order to increase statistical power to assess the effects of folic acid, *MTHFR* genotypes and the interaction between folic acid and the *MTHFR* C677T polymorphism on OFC risk.

METHODS

Search strategies and inclusion criteria

A literature search was carried out to identify studies that had examined the interaction between genetic and environmental factors in the etiology of CL(P). Studies up to 2009 were included. The search terms used were: orofacial cleft, cleft lip, cleft palate, gene-environment interactions and etiology. However, we only selected articles that met the inclusion criteria (provided further below) for our analysis and we focused on the interaction between maternal folic acid use and *MTHFR* C677T genotypes for the IPD pooled-analysis because of the uncertainty in the field and the population-specific nature of the *MTHFR*/folic acid results reported in the literature.

The following eligibility criteria were applied: European study; case-control or case-parent triad studies; non-syndromic infants and mothers, controls without birth defects and mothers as participants; studies that provided quantifiable data on folic acid; and studies that provided data on genotypes. Quality assessment was built into the extraction of information protocol and these included selection criteria (studies in humans, cleft lip and palate as an outcome in newborns, and gene-environment interaction investigated) and count data on genetic and environmental factors. Three independent investigators (AB, PAM and ILM) manually checked the identified studies and excluded studies that did not fit the above criteria. Studies having the following information were deemed eligible for analysis: 1) reported the population studied, (2) inclusion and exclusion criteria for index cases and controls, (3) provided empirical data for their results. A consensus on eligible studies was reached by the study analysts (AB, PAM, and ILM).

Protocol for pooled-analysis

The four studies that met our search criteria used similar methods and reported specific information suitable for a pooled analysis. Authors of these studies were informed about the aim of the IPD pooled-analysis, and they were asked to provide the following data on each participant: unique patient study ID, maternal age at delivery, educational level, folic acid use (either folic acid supplements and/or dietary folate intake), vitamin supplement use, maternal smoking, maternal alcohol intake, maternal medication use, medical and reproductive history, dietary habits, and *MTHFR* C677T genotypes.

Statistical analysis

Analyses were based on individual patient data and all authors provided data on case-parent and control-parent triads, thus ensuring uniformity. We used the Statistical Package for the Social Sciences and Epi Info version 7 for the analysis. Frequency data were generated for all the variables listed in the protocol. Data were first pooled together from all studies before performing an unmatched case-control analysis (adjusted for gender). We stratified the analysis by cleft types comparing CL(P) to controls and CP to controls separately. The risk estimates were expressed in odd ratios. Logistic regression models were used to predict the outcomes CL(P) and CP using maternal variables such as smoking, alcohol use and folic acid use. A step-wise logistic regression analysis was done to study maternal variables that

potentially contribute to the outcomes of CL(P) and CP. We adjusted for location, gender and age during the analyses and dummy variables were used to define cases and controls. The threshold for significance was set as $p < 0.05$

RESULTS

The number of cases and controls recruited in each study are displayed in Table 1. Standardized data were collected on the following variables: maternal age at delivery, educational level, dietary folate intake, vitamin supplementation containing folic acid, folic acid alone, maternal medication use, maternal tobacco use, alcohol consumption, medical and obstetrical history, previous reproductive history, dietary habits and *MTHFR C677T* genotypes. Consistent with previous findings, there was a significant difference ($p=0.01$) between males and females with CL(P). There was a non-significant difference ($p=0.48$) between females and males with CP. The average gestational period and average maternal age were similar in both groups. However, there is a significant difference ($p=0.003$) in the gestational period when cases (CL(P) and CP combined) were compared to controls. There was a significant difference between cases and controls ($p=0.014$) when maternal education was compared between the two groups.

Environmental and genetic variables

The case-control comparison in Table 2 shows that there is a statistically significant reduction in risk of CL(P) with maternal folic acid use ($p=0.008$; OR= 0.78, 95% CI: 0.65–0.94) and use of supplements containing folic acid ($p=0.028$; OR=0.80, 95% CI:0.66–0.98). Smoking significantly increased the risk for CL(P) ($p<10e-3$; OR=1.62, 95% CI: 1.35–1.95) and CP ($p=0.028$; OR=1.38, 95% CI: 1.04–1.83). These risks remained unchanged after adjusting for gender.

Table 3a shows that only folic acid and smoking were significant in the model. Table 3b shows the final model; i.e. any of these models can be used interchangeably for further analysis; CL(P) can be predicted by folic acid alone or folic acid and smoking. It further shows that inclusion of other variables in the model has no significant effect on CL(P) outcomes. A reduced risk of CP was found with alcohol use in the model (Table 3c).

DISCUSSION

Maternal folic acid use

The results of the case/control comparison indicate that folic acid reduces the risk of CL(P) in the periconceptional period (OR= 0.78, 95%CI: 0.65–0.94). These findings are consistent with those reported by Wilcox *et al.* (2007), where maternal use of folic acid was also found to significantly reduce the risk of CL(P) (OR=0.61; 95% CI: 0.39–0.96). For the CP analysis, our results suggest that folic acid does not influence the risk of CP (OR=1.2; 95% CI: 0.89–1.57). This is also consistent with findings from the study by Wilcox *et al.*(2007) where folic acid provided no protection against cleft palate alone (OR=1.07; 95% CI: 0.56 to 2.03). Our result for CL(P) conflicts with those reported in the meta-analysis by Johnson and Little (2008) and in the Cochrane review by De-Regil *et al.* (2010). In the present study, we pooled individual participant data from studies that used similar methods in European populations, unlike the previous assessments (Johnson and Little, 2008; De-Regil *et al.*, 2010) that used aggregated data from different studies in several populations.

Genetic factors

No risk was observed for CL(P) with either the infant or maternal CT and TT genotype. These results are consistent with reports from a meta-analysis that did not find any

significant association between infant *MTHFR* C667T and CL(P) (Verkleij-Hagoort et al., 2007). This is in contrast with findings from Northern China (Zhu *et al.*, 2006) and Ireland (Mills *et al.*, 2008), reporting increased risks for CP with maternal *MTHFR* 677 TT (OR=1.50; 95% CI: 1.05–2.16; $p = 0.03$).

IPD pooled analysis

Not surprisingly, the findings of our IPD pooled-analysis are consistent with some but not all studies. The results of the IPD pooled-analysis provide further support for a protective role of folic acid in the etiology of CL(P). The IPD pooled-analysis was carried out to overcome problems that may be associated with excessive reliance on published data and aggregate-level data. To the extent the original publications are compatible for a pooled analysis; the IPD approach permits data verification, harmonization of genotype and phenotype data, use of common definitions, adjustment for the same variables across studies, and thus provides an indirect way of addressing some of the questions not answered by the original publications. The IPD has more statistical power to detect an effect because there is an increase in the amount of data available for analysis. However, it may be difficult to conduct individual-level data analysis given numerous ethico-legal issues associated with harmonizing individual level genotype/phenotype data and exposure data (Fortier et al., 2010; Fortier et al., 2011a, 2011b; Knoppers et al., 2011; Harris et al., 2012). In this IPD pooled-analysis, we observed a statistically significant association between maternal smoking and risk of having an infant with CP and CL(P). This finding is consistent with results from a meta-analysis that found a statistically significant association between maternal smoking and cleft palate (Relative Risk=1.22; 95% CI: 1.10–1.35) (Little *et al.*, 2004). The recent GWEIS reported by Beaty *et al.* (2011) confirms a role for gene-environment interaction, where a significant genome-wide signal was observed with maternal smoking in the first trimester leading to an increased risk for CP (Beaty *et al.*, 2011).

Although the participants in this study are predominantly of European origin, there may be still be heterogeneity between these groups, resulting in differences in CL(P) and CP outcomes. These differences may in part reflect distinct genetic contributions inherent to the populations studied and differences in exposure to folic acid. However, pooled data using samples from these studies suggest that folic acid and maternal smoking contribute to cleft outcomes. The result of this IPD analysis is consistent with previous reports suggesting that folic acid protects against CL(P). However, we did not observe any evidence of GEI in these data.

It will be important to investigate other populations and ethnicities in order to further dissect the role of folic acid and *MTHFR* gene variants in CL(P) and CP. The IPD approach described here will be valuable in that context.

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Table 1

Included studies in the IPD meta-analysis.

Subjects	Van Rooij et al. 2003	Boyles et al., 2008	Chevrier et al. 2007	Little et al. 2008
Country	Netherlands	Norway	France	United Kingdom
Enrollment	Hospital	Population	Hospital	Hospital
Study design	Unmatched case control parent traids	Unmatched case control parent traids	Matched case control parent traids	Matched case control parent traids
Matching criteria	none	none	Sex, birth location and month of birth	Sex, birth location and month of birth
Cases	201	434	208	210
Controls	205	591	138	243
Study period	1998–2000	1996–2001	1998–2000	1997–2001
Characteristics of the 1149 cases and 1161 controls recruited from France, Netherlands, Norway and UK.				
Variables	Cases		controls	p-values*
	CL(P)	CP		
Gender males	62.3%	46.6%	53.4%	
females	37.7%	53.4%	46.6%	
p-values	0.001	0.48		
Mean gestational age	39	39	39	0.003
Average maternal age	30	29	30	0.09
Education				
University	39.9%	46.2	46.1%	0.01
Technical school	24.7%	15.8	19.5%	
Primary and secondary	35.4%	38.0	34.4%	

* Is the p-value for the case (CL(P) and CP combined) and controls in each category of maternal variable.

Table 2

Case-control comparison using Fisher's exact test

	CL(P)		Control		Chi-square	d.f	p-value	Odd ratio (OR)	95%CI
	Yes	No	Yes	No					
Supplements containing folic acid	245	527	403	561	4.84	1	0.028 ^a	0.80	0.66-0.98
Folic acid	330	434	616	514	7.14	1	0.008 ^a	0.78	0.65-0.94
Smoking	346	422	434	696	26.71	1	<0.000 ^a	1.62	1.35-1.95
Alcohol	248	394	308	574	3.50	1	0.061	1.20	0.99-1.46
	CP		Control		Chi-square	d.f.	p-value	OR	95% CI
Supplements containing folic acid	92	527	403	561	1.12	1	0.290	1.17	0.88-1.56
Folic acid	137	91	616	514	1.28	1	0.259	1.18	0.89-1.57
Smoking	108	126	434	696	4.86	1	0.028 ^a	1.38	1.04-1.83
Alcohol	50	102	308	574	3.47	1	0.062	0.73	0.52-1.02

Comparison of genotypes between cases and controls						
Maternal <i>MTHFR</i>	CC	CT	TT	Chi-square	d.f	p-value
Control	656	391	83			
Child <i>MTHFR</i>	0	1	2			
CL(P)	434	283	60	0.07	2	0.965
Control	630	409	91			
Maternal <i>MTHFR</i>	0	1	2			
CP	123	88	23	3.06	2	0.217
Control	656	391	83			
Child <i>MTHFR</i>	0	1	2			
CP	108	108	18	8.41	2	0.015 ^a
Control	630	409	91			

The threshold for significance was set as $p < 0.05$.

^a statistically significant. Some exposure data are missing and numbers in the table do not match the total number of cases and controls included in the study.

Table 3

a. Logistic regression using the exposure variables and genotypes to predict CL(P) outcomes										
	B	S.E.	Wald	d.f.	Sig.	Exp(B)	95.0% C.I. for EXP(B)			
							Lower	Upper		
Step 1 ^a										
Supplement	-0.100	0.101	.969	1	0.325	0.91	0.74	1.10		
Folic acid	-0.450	0.096	21.941	1	<0.000 ^a	0.64	0.53	0.77		
Smoking	0.225	0.096	5.525	1	0.019 ^a	1.25	1.04	1.51		
Alcohol	0.183	0.104	3.114	1	0.078	1.20	.98	1.47		
Child MTHFR										
CT	0.041	0.186	0.048	1	0.826	1.04	.72	1.50		
TT	0.011	0.186	0.004	1	0.952	1.01	.70	1.46		
Maternal MTHFR										
CT	0.020	0.189	0.011	1	0.918	1.02	0.70	1.48		
TT	0.077	0.191	0.165	1	0.685	1.08	0.74	1.57		
Constant	0.347	0.256	1.842	1	0.175	1.42	0	0		
b. Stepwise logistic regression using folic acid and maternal smoking to predict CL(P) outcomes.										
	B	S.E.	Wald	df	Sig.	Exp(B)	95.0% C.I. for EXP(B)			
							Lower	Upper		
Step 1 ^a										
Folic acid	-0.484	0.094	26.564	1	<0.000 ^a	0.616	0.51	0.74		
Constant	0.624	0.068	83.712	1	<0.000 ^a	1.867	0	0		
Step 2 ^b										
Folic acid	-0.474	0.094	25.294	1	<0.000 ^a	0.623	0.52	0.75		
Smoking	0.230	0.095	5.833	1	0.016 ^a	1.259	1.04	1.52		
Constant	0.484	0.089	29.435	1	<0.000 ^a	1.622	0	0		
c. Logistic regression using the maternal variables to predict CP outcomes										
	B	S.E.	Wald	df	Sig.	Exp(B)	95.0% C.I. for EXP(B)			
							Lower	Upper		
Step 1 ^a										
Supplements	-0.146	0.151	0.944	1	0.331	0.86	0.64	1.16		

c. Logistic regression using the maternal variables to predict CP outcomes									
	B	S.E.	Wald	df	Sig.	Exp(B)	95.0% C.I. for EXP(B)		
							Lower	Upper	
Folic acid	-0.149	0.149	0.996	1	0.318	0.86	0.64	1.15	
Smoking	0.316	0.174	3.281	1	0.070	1.37	0.97	1.93	
alcohol	-0.320	0.146	4.786	1	0.029 ^a	0.73	0.55	0.97	
Child MTHFR									
CT	-0.001	0.289	0.000	1	0.997	1.00	.566	1.76	
TT	0.353	0.283	1.556	1	0.212	1.42	.817	2.48	
Maternal MTHFR									
CT	-0.264	0.267	0.977	1	0.323	0.77	0.46	1.30	
TT	-0.131	0.268	0.239	1	0.625	0.88	0.52	1.4820	

^a Variable(s) entered in step 1: folic acid.

^b Variable(s) entered on step 2: smoking.

^a statistically significant