Published by Oxford University Press on behalf of the International Epidemiological Association
International Journal of Epidemiology 2008;37:1041–1058
doi:10.1093/ije/dyn098

Folate intake, markers of folate status and oral clefts: is the evidence converging?

Candice Y Johnson[1,2] and Julian Little[1,3]*

Accepted 29 April 2008

Background The ability of folic acid in the periconceptional period to prevent the occurrence of neural tube defects has stimulated tremendous interest in its effects on other health outcomes. Its possible effect on oral clefts has generated considerable debate. The purpose of this systematic review and meta-analysis was to assemble evidence on the role of folate in the aetiology of cleft lip with or without cleft palate (CL/P) and cleft palate only (CPO).

Methods Medline, PubMed, Embase, Science Citation Index and the HuGE Published Literature Database were searched to February 2007 for articles related to oral clefts and multivitamin use, dietary folate, folic acid fortification, biochemical markers of folate status and polymorphisms in 5,10-methylenetetrahydrofolate reductase (MTHFR) and other genes involved in folate metabolism. Random effects meta-analysis was conducted when appropriate.

Results Maternal multivitamin use was inversely associated with CL/P [odds ratio (OR) 0.75, 95% CI 0.65–0.88, based on 5717 cases and 59 784 controls] but to a lesser extent CPO (OR 0.88, 95% CI 0.76–1.01, 2586 cases and 59 684 controls). The volume of evidence on dietary folate, fortification and biochemical and genetic measures of folate status is substantially less; in aggregate, the evidence suggests that no association exists but there is substantial heterogeneity between studies.

Conclusions The evidence is not converging and there is no strong evidence for an association between oral clefts and folic acid intake alone. Multivitamin use in early pregnancy, however, may protect against oral clefts, especially CL/P although this association may be confounded by other lifestyle factors associated with multivitamin use.

Keywords Cleft lip, cleft palate, folic acid, methylenetetrahydrofolate reductase (NADPH2), meta-analysis, review

Introduction

Oral clefts are some of the most common birth defects, affecting an estimated one of every 600 births worldwide.[1] Animal studies in the first half of the 20th-century demonstrated that vitamin deficiencies, including folate deficiency, could cause oral clefts.[2] Folate, a general term for various forms of this naturally occurring B-vitamin, and folic acid, its oxidized and more bioavailable form found in multivitamins and food supplements, play important roles in

[1] Department of Epidemiology and Community Medicine, University of Ottawa, Ottawa, Canada.
[2] Department of Epidemiology, Rollins School of Public Health, Emory University, Atlanta, USA.
[3] Canada Research Chair in Human Genome Epidemiology.
* Corresponding author. Department of Epidemiology and Community Medicine, University of Ottawa, 451 Smyth Road, Ottawa, ON K1H 8M5, Canada.
E-mail: jlittle@uottawa.ca

the synthesis and methylation of DNA as well as in the metabolism of amino acids and their by-products, such as homocysteine.[3]

Many studies have been performed in an attempt to determine the role of folate in the aetiology of the two most common types of oral clefts: cleft lip with or without cleft palate (CL/P) and cleft palate only (CPO). A number of intervention studies suggesting a protective effect of folic acid on the recurrence of oral clefts have been performed.[4–6] Since these intervention studies were not randomized and the effects of folic acid could not be separated from the effects of other vitamins in the intervention, the results of these studies are difficult to evaluate.[7] One randomized controlled trial for the prevention of a first occurrence of oral clefts where the effect of a folic acid-containing multivitamin was compared to the effect of a trace element-containing supplement did not find a difference between groups.[8] A cohort study using the same vitamin found similar results.[9] However, both the trial and cohort study were not adequately powered to detect a difference. Several case–control studies have been undertaken to investigate the use of folic acid-containing multivitamins during pregnancy, but results have been variable.[10–14]

In view of the difficulties in investigating the specific effects of folate separate from the effects of other vitamins found in supplements or multivitamins, attention has also been given to the effects of polymorphisms in genes involved in folate metabolism. One of the first folate metabolism genes studied in association with oral clefts was methylenetetrahydrofolate reductase (*MTHFR*; EC 1.5.1.20; 1p36.3). *MTHFR* catalyses the creation of 5-methyltetrahydrofolate from 5,10-methylenetetrahydrofolate, which is then combined with homocysteine to synthesize methionine.[15]

Rare mutations in *MTHFR* causing severe enzyme deficiency result in hyperhomocysteinemia, hyperhomocystinuria, mental retardation, seizures and thrombosis.[16] To date over 60 single nucleotide polymorphisms (SNPs) have been identified in *MTHFR*, with most functional amino acid changes leading to reduced enzyme activity.[15] *MTHFR* polymorphisms found at high frequencies in the population, C677T and A1298C, were first investigated in cardiovascular disease aetiology because of their impact on homocysteine metabolism, the impaired enzyme leading to increased homocysteine levels when folate availability is low.[17–20] Since the first report in 1998 that the *MTHFR* C677T variant genotype was found more commonly among CL/P cases than controls,[21] population-based and family-based studies have been undertaken to determine the role of this gene in the aetiology of CL/P and CPO, which have produced variable results.[13,22–27]

With conflicting evidence on the role of folate in oral cleft aetiology, the present systematic review and meta-analysis was undertaken to synthesize evidence from studies of associations between CL/P and CPO and folate intake and biochemical markers of folate status. Recently, two meta-analyses of folic acid-containing multivitamin use and oral clefts have shown that women taking these multivitamins during pregnancy have a decreased risk of both CL/P and CPO.[10,28] However, a recent meta-analysis of population-based studies of the association between folate metabolism gene *MTHFR* and CL/P has shown only weak evidence for an association.[22] One purpose of these systematic reviews and meta-analyses was to review other evidence not covered in the prior reviews, such as dietary folate intake, prevalence of oral clefts following folic acid fortification, polymorphisms in genes aside from *MTHFR* involved in folate metabolism and transport, and gene–environment interactions. A second purpose was to update the existing meta-analyses and reviews on oral clefts and multivitamins and *MTHFR* polymorphisms by including studies published in the interim, using adjusted as opposed to crude effect estimates in the meta-analyses and including a wider spectrum of studies that were not included in the previous reviews, for example, including family-based association studies in the review of *MTHFR* and expanding the search strategy to include non-English language studies.

Methods

The main databases used for locating studies were OVID Medline (1950–), PubMed (1950–), OVID Embase (1980–) and ISI Science Citation Index (1970–), which were searched to the end of February 2007 using search terms 'cleft lip', 'cleft palate', 'folic acid', 'vitamins', 'dietary supplements' and 'fortification'. Reference lists from included articles were searched for additional articles. For reviews of genetic variants, the HuGE Published Literature Database[29] was searched to the end of February 2007 for 'cleft lip with cleft palate', 'cleft lip without cleft palate', 'cleft palate' and 'oral clefts' to determine which genes involved in folate metabolism or transport had been investigated in association with oral clefts. Full texts were retrieved and articles were included if the authors indicated that the gene under investigation was involved in folate metabolism or transport. Reference lists from these studies were used to identify additional articles. The gene names identified using this search strategy were used for a more in-depth search in the main databases.

To be included in the review, studies were required to have information on CL/P, CPO or both types of clefts combined. There was no restriction by language. Animal studies, review articles, case reports, case series, abstracts and meeting proceedings were excluded. When two studies sampled from the same population during the same time period, the study with the most relevant primary outcome was included; if there was more than one, the study with the largest sample size or the most recent was included.

Each review topic, listed below, had specific inclusion and exclusion criteria and subgroup analyses. Articles could be included in more than one review topic, and inclusion or exclusion of the article was assessed independently for each topic.

Supplement use: observational studies

Included studies were case–control, case–cohort or cohort studies where women who took folic acid supplements at any time during the 3 months prior to pregnancy to the end of her pregnancy were compared to women who did not. Since folic acid is usually consumed as a part of a multivitamin instead of as a folic acid supplement alone, women taking multivitamins were also included. Subgroup analyses were undertaken to separate the effects of folic acid from the effects of other components of the multivitamin. Studies specifically mentioning use of folic acid supplements or folic acid-containing multivitamins were classified as 'folic acid use' and studies mentioning multivitamin use were labelled 'multivitamin use'; these two categories are not mutually exclusive. Subgroup analyses were also conducted to investigate the effect of timing of multivitamin use: the first restricted to women who had started supplementation prior to conception and had continued throughout at least the first 2 months of pregnancy, and the second restricted to women who started supplementation after the aetiologically relevant time period. The aetiologically relevant time period was defined as preconception to the end of the 3rd month for CL/P, and pre-conception to the end of the 4th month for CPO, as has been defined in other studies.[30,31]

Supplement use: randomized controlled trials

Trials using interventions of folic acid supplements or folic acid-containing multivitamins were included.

Supplement use: recurrence studies

Studies were included if the investigators attempted to prevent the recurrence of oral clefts by giving folic acid supplements or folic acid-containing multivitamins to mothers planning a pregnancy in instances where either or both of the parents were themselves born with a cleft, or where the mother had previously given birth to an affected child. In the included studies, a group of mothers receiving folic acid-containing prophylaxis was compared with a group of women not receiving folic acid. Narrative studies without sufficient quantitative data for meta-analysis were excluded from this analysis.

Dietary folate

Any study attempting to quantify maternal folate intake during pregnancy from dietary sources aside from supplements or multivitamins and to compare these values in mothers of children with oral clefts to mothers of non-cases was included. Studies which included folate from supplements and multivitamins in addition to other sources of folate in the estimate of dietary intake were also included. The highest quantile of maternal folate intake was compared with the lowest quantile to estimate risk using quantiles defined by the author of each study. *P*-values for tests of trend and for differences in mean dietary folate intake between case and control mothers were also noted.

Folic acid fortification

Included studies were those reporting prevalence ratios (PR) and 95% confidence intervals (CI) for the prevalence of oral clefts after, compared with before, implementation of folic acid fortification. For studies sampling from overlapping populations, national studies were included over regional studies. Studies were grouped based on whether the fortification was compulsory or optional for that country.

Biochemical markers of folate status

Observational studies where investigators compared the plasma (serum) or erythrocyte (red cell) folate levels of mothers of children with oral clefts with those of mothers of unaffected children were included. Comparisons were made between the highest and lowest quantiles of folate, as defined by the author of each study, to estimate risk. *P*-values from dose–response relationships were noted, as were *P*-values for differences between mean levels of folate between case and control mothers.

MTHFR C677T, A1298C, haplotypes and haplogenotypes

Studies were included if a *MTHFR* genotype, haplotype or haplogenotype (i.e. the combination of haplotypes inherited from the mother and father) frequencies in cases, case mothers and case fathers were compared with frequencies in controls or their parents. For studies of *MTHFR* polymorphisms, homozygous wild-type individuals were chosen as the reference group. For the review of haplotypes, only studies of the *MTHFR* C677T-*MTHFR* A1298C haplotype were included.

Transmission disequilibrium tests (TDT)

Articles reporting results from TDT for *MTHFR* C677T, A1298C or haplotypes were included. *P*-values for differences in transmission were extracted from the studies. Studies reporting parent-of-origin effects were considered separately.

Gene–environment interactions

Included studies were those where interactions between *MTHFR* and folate were investigated. Studies were grouped by polymorphism (C677T, A1298C), individual genotyped (infant, mother, father) and exposure (dietary folate intake, folic acid-containing supplements).

Other genes related to folate metabolism or transport

The same methods were used as for the *MTHFR* association and TDT reviews.

Separate analyses were performed for CL/P, CPO and OFC (orofacial clefts; only including studies not differentiating between CL/P and CPO). The authors independently abstracted data from articles and resolved differences by consensus. Adjusted odds ratios (OR) were extracted from included studies if available; if not, crude estimates were used. If ORs were not provided, they were calculated from data available in the article. Relative risks were assumed to be equivalent to ORs since oral clefts have a low population prevalence. For review topics where ORs were inappropriate, *P*-values were extracted from the articles when possible, again using adjusted estimates if available. Random effects meta-analysis was used to determine summary ORs and 95% CIs for each association, if applicable, and random effects cumulative meta-analysis was used to show time trends in the association. Between-study heterogeneity was detected using Cochran's *Q*-test and the I^2 statistic with 95% uncertainty intervals (UI).[32] Publication bias was assessed using Egger's test.[33] All calculations were performed in Stata 8.

Results

The characteristics of studies included in the systematic reviews and meta-analyses are shown in Supplementary Table 1. Most studies were conducted in Europe and North America. There were few studies from South America, Australia and Asia, and none from Africa.

Supplement use: observational studies

Twenty-two[9,11–14,30,31,34–48] and twenty-one[9,11–14,30,31,34–46,48] studies were included in the meta-analyses for CL/P and CPO, respectively. There were a total of 5717 cases of CL/P and 2586 cases of CPO. The predominant study type was case–control, but each meta-analysis also included one cohort[9] and one case–cohort[12] study. All articles except one were found using the search strategy; one paper in press was known to the authors and was included.[48] The majority of studies included women taking multivitamin supplements during the periconceptional period, continuing through the first trimester of pregnancy. Often the folic acid content of multivitamins was not reported.

Results of the meta-analyses are shown in Table 1. Use of any supplements before or during pregnancy was associated with a decreased risk of CL/P (OR 0.75; 95% CI 0.65–0.88) but to a lesser extent CPO (OR 0.88; 95% CI 0.76–1.01) as shown in Figures 1 and 2. Between-study heterogeneity was detected for the analysis of CL/P using the Cochran *Q* and I^2 statistics, while the analysis for CPO had low to moderate heterogeneity. Cumulative meta-analysis for CL/P showed a consistent inverse association over the past 13 studies (Figure 3). In contrast, the cumulative meta-analysis of CPO showed an inverse association moving away from the null until 2006, when the association regressed towards the null once more (Figure 4).

Restricting the analysis to those studies specifically mentioning use of folic acid[9,11–14,31,36,37,42,45–48] attenuated the association for both CL/P (OR 0.82; 95% CI 0.70–0.97) and CPO (OR 0.95; 95% CI 0.79–1.14); cumulative meta-analysis for CPO showed the same pattern as for all supplement use, with the association moving away from, and then back towards, the null. For the analysis restricted to multivitamin use, the effect estimates for CL/P and CPO were no different from those in the unrestricted analysis.

Timing of supplement use affected the risk of CL/P and CPO. For women starting supplementation prior to conception, nine studies were found for CL/P[9,30,37,39,40,42,45–47] and eight for CPO.[9,30,37,39,40,42,45,46] These women had a lower risk of having a child with CL/P (OR 0.65; 95% CI 0.50–0.86) and CPO (OR 0.70; 95% CI 0.51–0.98). There were two studies for CL/P[31,37] and one for CPO[31] where information was collected on the risk of clefts to women starting supplementation after the aetiologically relevant time period; none of these studies found an association with all effect estimates close to unity.

There were six studies where results were presented for all clefts combined (total of 849 cases).[49–54] All were found through the search strategy except one that was known to the authors and was included.[51] Overall these studies found no association between supplement use and risk of clefts (OR 0.88; 95% CI 0.55–1.40) although this meta-analysis had marked heterogeneity. Exclusion of the study by Elahi *et al.*,[54] which included interventions of other nutritional supplements besides multivitamins, produced a more homogeneous analysis and raised the effect estimate (OR 1.05; 95% CI 0.85–1.30). While restricting the analysis to the four studies specifically mentioning folate[49,51–53] resulted in a possible increased estimated risk of clefts (OR 1.18; 95% CI 0.91–1.52), restricting to the two studies of multivitamin use[49,50] showed the opposite effect (OR 0.85; 95% CI 0.63–1.13) although the CIs crossed the null in both cases. There were insufficient studies to determine the effects of timing of supplement use for all clefts.

Supplement use: randomized controlled trials

One randomized controlled trial[8,31] was conducted where women planning a pregnancy were randomized to receive folic acid-containing multivitamins periconceptionally, but oral clefts were not the primary outcome of interest and the study was not adequately powered to detect an association. The authors found a possible increased risk of CL/P and decreased risk of CPO, although CIs were wide in both cases

Table 1 Random effects meta-analyses of observational studies of supplement use and oral clefts

Meta-analysis	Number of studies	OR (95% CI)	Cochran Q *P*-value	I^2 (95% CI)	Egger's test *P*-value
CL/P					
Any supplement use	22	0.75 (0.65–0.88)	<0.01	56 (29–73)	0.38
Multivitamin use[a]	18	0.77 (0.66–0.90)	<0.01	59 (30–75)	0.53
Folic acid use[b]	13	0.82 (0.70–0.97)	0.02	49 (4–73)	0.99
Started supplements preconceptionally	9	0.65 (0.50–0.86)	0.07	45 (0–74)	0.98
Started supplements after the etiologically relevant time period[c]	2	1.11 (0.65–1.92)	0.07	69 (0–93)	–
CPO					
Any supplement use	21	0.88 (0.76–1.01)	0.13	26 (0–57)	0.81
Multivitamin use	17	0.88 (0.74–1.04)	0.11	31 (0–62)	0.98
Folic acid use	12	0.95 (0.79–1.14)	0.13	32 (0–66)	0.37
Started supplements preconceptionally	8	0.70 (0.51–0.98)	0.26	21 (0–64)	0.72
Started supplements after the etiologically relevant time period[d]	1	0.99 (0.71–1.38)	–	–	–
OFC					
Any supplement use	6	0.88 (0.55–1.40)	<0.01	76 (46–89)	0.91
Any supplement use[e]	5	1.05 (0.85–1.30)	0.49	0 (0–76)	0.42
Multivitamin use[e]	2	0.85 (0.51–1.44)	0.69	0	–
Folic acid use	4	1.18 (0.91–1.52)	0.84	0 (0–46)	0.31

[a]Use of multivitamins, regardless of folic acid content.
[b]Use of folic acid supplements or folic acid-containing multivitamins.
[c]After the third month of pregnancy.
[d]After the fourth month of pregnancy.
[e]Elahi *et al.*[54] removed.

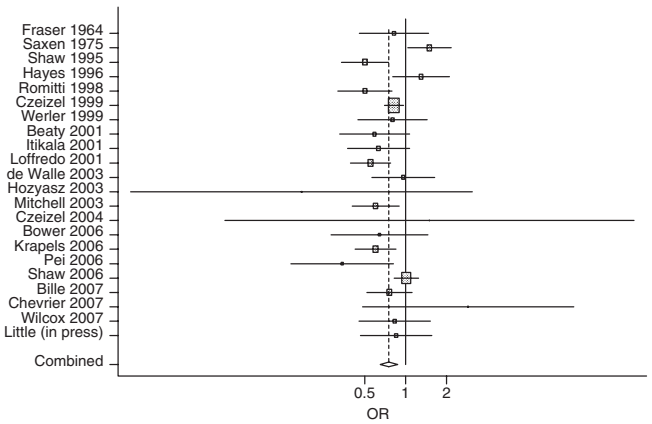

Figure 1 Random effects meta-analysis for studies of the association between supplement use before or during pregnancy and the risk of CL/P showing OR and 95% CI

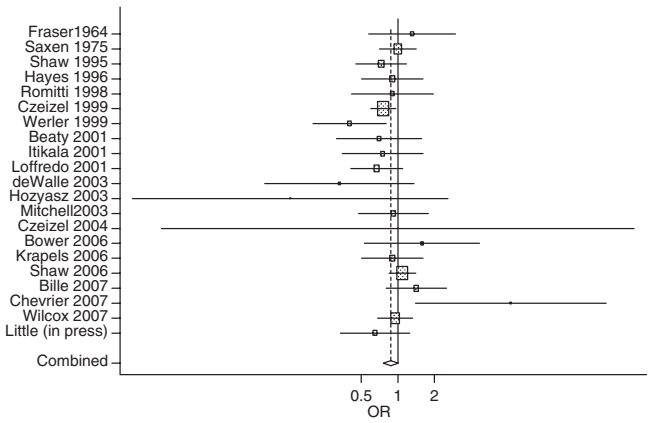

Figure 2 Random effects meta-analysis for studies of the association between supplement use before or during pregnancy and the risk of CPO showing OR and 95% CI

(OR 1.94; 95% CI 0.41–9.09 and OR 0.19; 95% CI 0.01–4.03, respectively). There were four cases among the supplemented women and two in the unsupplemented group.

One other randomized controlled trial was located where women choosing to take folic acid supplements prior to or during pregnancy were randomized to receive either high dose (2.5 mg) or low dose (1.0 mg) folic acid.[51] At the end of the trial, the prevalence of oral clefts was highest in the high-dose group, lowest in the low-dose group and intermediate among the unsupplemented women. CIs were wide as there

were less than 30 cases of oral clefts occurring during the trial.

Supplement use: recurrence studies

Three recurrence studies[4–6] were included in the meta-analysis (Table 2, Figure 5). In all three, authors included women who had previously given birth to a child with a cleft. In one study, the authors also included families where one or both of the parents had themselves been born with a cleft. All studies compared women receiving a folic acid-containing multivitamin and mineral supplement to women receiving no supplement.[6] The composition of supplements differed between studies; for example, folic acid included in the supplements ranged from 0.5 to 10 mg per day. Despite the range of folate dosages, the effect estimates were similar between studies. The meta-analysis had low heterogeneity and showed a decreased risk of CL/P (relative risk (RR) 0.33; 95% CI 0.15–0.73). Only one study presented results for CPO, which showed an increase in risk but had wide CIs (RR 1.70; 95% CI 0.47–6.11).

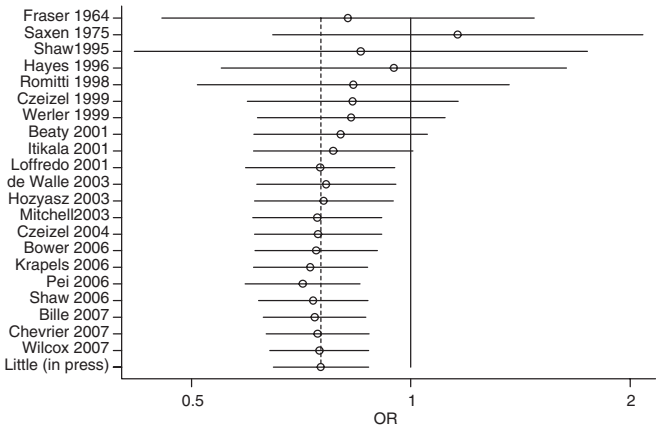

Figure 3 Random effects cumulative meta-analysis showing the association between supplement use before or during pregnancy and the risk of CL/P over time as OR and 95% CI

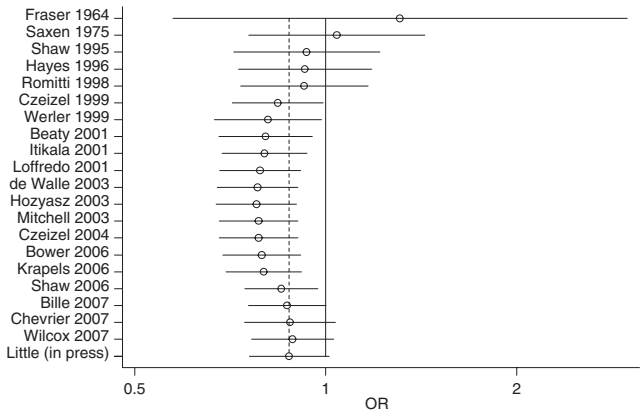

Figure 4 Random effects cumulative meta-analysis showing the association between supplement use before or during pregnancy and the risk of CPO over time as OR and 95% CI

Dietary folate intake

Six studies[11,13,14,45,48,55] were identified that measured dietary folate intake during pregnancy among CL/P case and control mothers (total of 1571 mothers of cases and 4621 mothers of controls); four of these also measured dietary folate in relation to CPO (total of 577 mothers of cases and 3655 mothers of controls).[11,13,45,48] One additional study provided information for all clefts combined.[37] No meta-analysis was performed due to differences in definitions of quantiles of exposure between studies. There was the suggestion

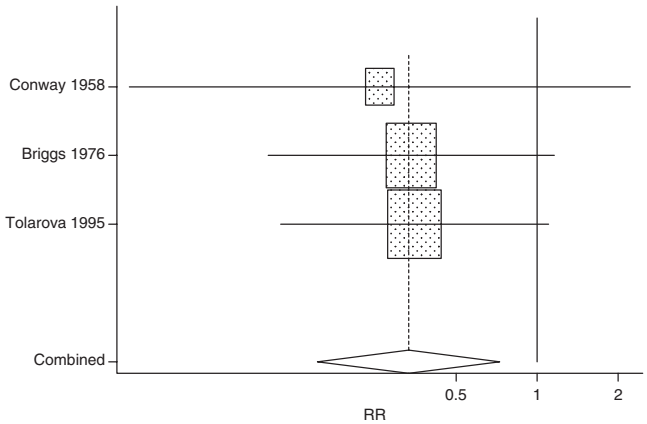

Figure 5 Random effects meta-analysis for recurrence studies of CL/P showing RR and 95% CI

Table 2 Results of recurrence studies using multivitamin and mineral prophylaxis that included folic acid

Study	Eligible women	Intervention	CL/P RR (95% CI)	CPO RR (95% CI)
Conway 1958[4]	Previous child affected	Multivitamin with 0.5 mg folic acid	0.26 (0.03–2.19)	[a]
Briggs 1976[5]	Previous child affected	Multivitamin with 5 mg folic acid	0.34 (0.10–1.16)	1.70 (0.47–6.11)
Tolarova 1995[6]	Previous child or either parent affected, no other family history of clefts	Multivitamin with 10 mg folic acid	0.35 (0.11–1.09)	–

[a]No cases observed in the intervention or control groups.

Table 3 Risk of oral clefts by quantiles of maternal dietary folate intake, as defined in each study

Study	Number of quantiles	Quantile definition (μg/day)		CL/P			CPO	
		Highest	Lowest	OR[a] (95% CI)	*P* for trend	*P* for difference[b]	OR[a] (95% CI)	*P* for trend
Hayes 1996[37]	3			0.9 (0.5–1.6)[c]				
van Rooij 2004[55]	5	Mean 242	Mean 152	0.54 (0.27–1.05)	0.06	<0.001[d]		
Bower 2006[45]	2	Above 326	Below 326	1.56 (0.67–3.63)			2.07 (0.42–10.16)	
Shaw 2006[11]	4	Above 705	Below 329.45	1.36 (0.46–4.02)			0.37 (0.07–1.88)	
Chevrier 2007[13]	3	Above 314	Below 230	0.64 (0.4–1.1)		0.03[d]	0.70 (0.3–1.4)	
Wilcox 2007[14]	4	Above 265	Below 171	0.80 (0.52–1.24)	0.21			
Little (in press)[48]	4	Median 775	Median 269	0.9 (0.44–2.03)	0.53		1.0 (0.43–2.36)	0.93

[a]Odds ratio for highest vs lowest quantiles of folate intake as defined in each study.
[b]Difference in mean folate intake between cases and controls.
[c]Estimate for CL/P and CPO combined.
[d]Higher folate intake among controls.

Table 4 Prevalence of oral clefts after, as compared with before, folic acid fortification, by type of fortification implemented

	Number of studies	PR (95% CI)	Cochran Q *P*-value	I^2 (95% CI)	Egger's test *P*-value
CL/P					
Any fortification	7	0.95 (0.91–0.99)	0.46	0 (0–69)	0.88
Compulsory fortification	5	0.93 (0.90–0.98)	0.56	0 (0–72)	0.37
Optional fortification	2	1.02 (0.93–1.12)	0.79	0	–
CPO					
Any fortification	7	1.01 (0.90–1.15)	<0.01	75 (46–88)	0.36
Compulsory fortification	5	0.92 (0.85–0.99)	0.32	15 (0–82)	0.48
Optional fortification	2	1.19 (1.03–1.38)	0.19	43	–
OFC					
Compulsory fortification	2[a]	0.94 (0.91–0.97)	0.31	2	–

[a]One study without numerical results was not included in the meta-analysis.

of an inverse association between folate intake and oral clefts, but overall the results were varied (Table 3). Sample sizes were small, creating wide CIs.

Folic acid fortification

The meta-analysis using data from studies performed in Australia, Canada and the United States[56–58] (Table 4) shows that the prevalence of CL/P was lower by a small margin after fortification was introduced (PR 0.95; 95% CI 0.91–0.99; Figure 6). This decline was not seen for CPO (PR 1.01; 95% CI 0.90–1.15; Figure 7), although both the Cochran *Q* and I^2 statistics detected between-study heterogeneity in this analysis. Upon separating the countries with optional from those with compulsory fortification, it appeared that the prevalence of CL/P and CPO remained the same or increased in Australia where there is optional fortification ($PR_{CL/P}$ 1.02; 95% CI 0.93–1.12 and PR_{CPO} 1.19; 95% CI 1.03–1.38) and decreased in the United States and Canada where there is compulsory fortification ($PR_{CL/P}$ 0.93; 95% CI 0.90–0.98 and PR_{CPO} 0.92; 95% CI 0.85–0.99). In one study of hospitalizations for CL/P and CPO in the United States, no change in hospitalizations before

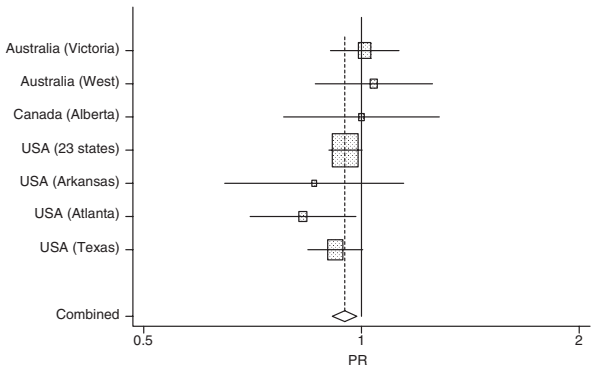

Figure 6 Random effects meta-analysis showing the change in the prevalence of CL/P following folic acid fortification as PR and 95% CI

and after introduction of folic acid fortification was observed.[59] This study was not included in the meta-analysis as it measured hospitalizations for oral clefts and not prevalence at birth.

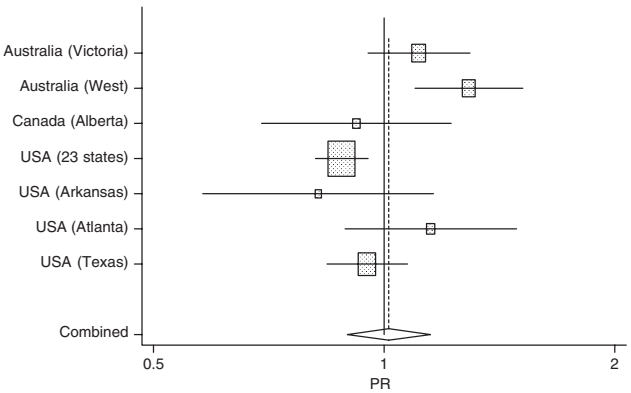

Figure 7 Random effects meta-analysis showing the change in the prevalence of CPO following folic acid fortification as PR and 95% CI

Three studies reported results for all clefts together (OFC). One study from the United States[60] found a decrease in the prevalence of oral clefts after fortification (OR 0.94; 95% CI 0.92–0.96) while one study from Canada[61] and one from Chile[62] reported no decrease in cleft prevalence; the study from Chile did not present numerical results.

Biochemical markers of folate status

Four studies were found where plasma folate or erythrocyte folate status were compared between CL/P case and control mothers (total of 270 mothers of cases and 399 mothers of controls; Table 5).[63–66] There was one study for CPO[66] and two for all clefts combined.[67,68] No meta-analysis was performed due to differences in exposure quantile definition between studies. Results were varied, with both increased and decreased risks found for individuals with lower folate status.

MTHFR C677T and A1298C

There was no association between infant or maternal *MTHFR* C677T or A1298C genotype and CL/P or CPO

Table 5 Risk of oral clefts by quantiles of maternal plasma and erythrocyte folate, as defined in each study

	Quantile definition (nmol/l)				
	Highest	Lowest	OR[a] (95% CI)	*P* for trend	*P* for difference[b]
Plasma folate					
CL/P					
Niebyl 1985[63]	–	–	–	–	NS
Stoll 1999[64]	–	–	–	–	NS
Munger 2004[65c]	Median 20.6	Median 8.3	0.89 (0.40–2.01)	0.99	NS
Munger 2004[65d]	Median 20.6	Median 8.3	2.70 (1.18–6.17)	0.02	<0.05[e]
OFC					
Wong 1999[67]	–	–	–	–	<0.01[e]
van Rooij 2003[68]	Above 7.5	Below 7.5	1.2 (0.4–3.2)	–	–
Erythrocyte folate					
CL/P					
Niebyl 1985[63]	–	–	–	–	NS
Munger 2004[65c]	Median 1189	Median 596	0.46 (0.20–1.09)	0.33	<0.05[f]
Munger 2004[65d]	Median 1189	Median 596	4.85 (2.24–10.50)	<0.001	<0.001[e]
Little (in press)[66]	584–2228 (μg/l)	103.5–323.5 (μg/l)	0.5 (0.18–1.18)	–	–
CPO					
Little (in press)[66]	607.5–2228 (μg/l)	107–355 (μg/l)	3.22 (1.14–9.10)	–	–
OFC					
Wong 1999[67]	–	–	–	–	<0.05[e]
van Rooij 2003[68]	Above 394	Below 394	0.9 (0.3–2.3)	–	0.60

NS, exact *P*-value not stated but reported to be above 0.05.
[a]Odds ratio for highest vs lowest quantiles of folate levels, as defined in each study.
[b]Difference in mean folate levels between cases and controls.
[c]Negros Occidental, Philippines.
[d]Davao, Philippines.
[e]Higher folate levels in cases than controls.
[f]Higher folate levels in controls than cases.

(Table 6, Figures 8 and 9). The association between infant *MTHFR* C677T and CL/P was the most commonly investigated, with 13 studies located including 1808 cases.[13,23–26,66,69–75] Fewer studies have been performed for maternal *MTHFR* C677T, with the nine studies totalling 1173 case mothers. Studies were conducted mostly in Europe and Asia, with few studies from North and South America. No studies were found from Australia and Africa. All but two studies were found using the search strategy: one submitted for publication was known to the authors and was included[66] and one was found by searching reference lists.[76]

Published reports have suggested a possible increased risk of CL/P for fathers with the *MTHFR* C677T TT genotype compared with the CC genotype (OR 1.63; 95% CI 1.00–2.65, Figure 10) based on the results of four studies including 343 case fathers.[70,72,73,75] Few studies of *MTHFR* and CPO have been conducted and results have been heterogeneous. There have been no studies of CPO with paternal *MTHFR* C677T or A1298C and only one of CPO with infant and maternal *MTHFR* A1298C.[24]

MTHFR C677T/A1298C haplotypes and haplogenotypes

Three studies of haplotype frequency[72,73,75] and three of haplogenotype frequency[25,72,77] were found, and none found a difference in haplotype frequencies in case infants, mothers or fathers compared with controls. There were few studies investigating haplogenotypes, and the results were too varied to determine if any haplogenotype was associated with oral clefts.

TDT

For *MTHFR* C677T, 12 studies included results from TDTs for CL/P in over 1500 families[25,26,70,72–76,78–81] and three included results for CPO in a total of 121 families.[76,80,82] Two studies found differences in transmission of the variant allele found for CL/P.[81,82] For *MTHFR* A1298C, four studies were found for CL/P totalling 382 families,[25,72,73,75] one of which found a difference in transmission.[25] No haplotype overtransmission was found for CL/P or CPO in the four studies where this information was reported.[24,72,73,75]

Gene–environment interactions

Six articles described gene–environment interactions between *MTHFR* and either supplement use (Table 7) or dietary folate intake (Table 8).[13,24,27,66,73,83] Among these six studies, 10 different associations were described: combinations of outcome (CL/P, CPO), exposure (supplements, dietary folate), genotype (*MTHFR* C677T, A1298C) and individual genotyped (infant, mother). No meta-analysis was performed due to small numbers of studies in most subgroups and differences in the definition of reference groups. Often the highest risks were found among women who had not taken folic acid supplements or who had low folate intake, and who themselves or their children carried variant genotypes.

Other genes involved in folate metabolism or transport

Genes reported to be involved in folate metabolism or transport, and investigated in association with oral clefts, aside from *MTHFR*, were: betaine-homocysteine methyltransferases (*BHMT* and *BHMT2*),[84] cystathionine beta-synthase (*CBS*),[85,86] folate receptors (*FOLR1* and *FOLR2*),[87] methylenetetrahydrofolate dehydrogenase (*MTHFD1*),[88] methionine synthase (*MTR*),[88,89] methionine synthase reductase (*MTRR*),[89] reduced folate carrier (*RFC1*)[47,80,88,90] and transcobalamins (*TCN1* and *TCN2*).[89] Few associations have been studied in more than one population. *RFC1* was the gene most often studied, but no association with oral clefts has been found in any of the four studies.[47,80,88,90] Several studies did find associations: an inverse association between CL/P and *TCN2*,[89] a positive association between CL/P and *MTR*,[88] and a difference in transmission for *CBS* (mother's allele) among CL/P cases.[85] These results have not yet been replicated in other populations.

Discussion

When considering the spectrum of evidence for an association between folate and oral clefts, including environmental, biochemical and genetic measures of exposure, there is no strong evidence that folate alone plays an important role in oral cleft aetiology. The most promising evidence for an association comes from studies on multivitamin use, but it is just as likely that a component of the multivitamin aside from folic acid is responsible for the observed protective effect. Following folic acid fortification, there appeared to be a small decrease in the prevalence of both CL/P and CPO in North America, but a marked decrease in prevalence following fortification, like that observed for neural tube defects[58] was not seen. Evidence from biochemical and genetic markers of folate status show no clear association between folate and oral clefts. Overall, the evidence is not converging and is not in favour of an association between folic acid and oral clefts.

With the knowledge that folic acid can prevent a substantial proportion of neural tube defects, conducting a randomized controlled trial to investigate the effects of folic acid against placebo to prevent oral clefts would be unethical. The one trial that has been done did not have oral clefts as the primary outcome of interest and was not adequately powered to detect an association. Currently, there is an oral cleft recurrence trial underway in Brazil where high-risk women will be randomized to receive high (4.0 mg) or low (0.4 mg) dose folic acid supplements, which may be able

Table 6 Random effects meta-analyses of *MTHFR* polymorphisms and oral clefts

Meta-analysis	Number of studies	Summary OR (95% CI)	Cochran Q *P*-value	I^2 (95% CI)	Egger's test *P*-value
***MTHFR* C677T**					
CL/P					
Infants					
TT vs CC	13	1.14 (0.88–1.48)	0.16	28 (0–63)	0.53
CT vs CC	12	1.12 (0.90–1.39)	0.01	56 (15–77)	0.04
Mothers					
TT vs CC	9	1.19 (0.77–1.82)	0.02	55 (4–79)	0.82
CT vs CC	9	0.95 (0.74–1.20)	0.04	50 (0–77)	0.23
Fathers					
TT vs CC	4	1.63 (1.00–2.65)	0.74	0 (0–64)	0.58
CT vs CC	4	0.99 (0.73–1.34)	0.69	0 (0–69)	0.79
CPO					
Infants					
TT vs CC	5	0.99 (0.45–2.16)	0.01	70 (23–88)	0.98
CT vs CC	4	1.11 (0.71–1.72)	0.07	57 (0–86)	0.72
Mothers					
TT vs CC	3	1.03 (0.56–1.89)	0.38	0 (0–89)	0.54
CT vs CC	3	0.78 (0.48–1.27)	0.15	48 (0–85)	0.47
OFC					
Infants					
TT vs CC	1	0.85 (0.53–1.38)	–	–	–
CT vs CC	1	0.95 (0.45–1.98)	–	–	–
Mothers					
TT vs CC	1	0.9 (0.2–4.0)	–	–	–
CT vs CC	1	0.8 (0.3–1.9)	–	–	–
***MTHFR* A1298C**					
CL/P					
Infants					
CC vs AA	6	0.94 (0.63–1.39)	0.48	0 (0–72)	0.44
CA vs AA	6	1.12 (0.85–1.48)	0.16	38 (0–75)	0.21
Mothers					
CC vs AA	4	0.96 (0.63–1.45)	0.62	0 (0–74)	0.95
CA vs AA	4	0.97 (0.69–1.36)	0.13	47 (0–82)	0.90
Fathers					
CC vs AA	3	0.65 (0.28–1.52)	0.20	39 (0–81)	0.56
CA vs AA	3	0.84 (0.62–1.15)	0.88	0 (0–17)	0.15
CPO					
Infants					
TT vs CC	1	0.30 (0.09–1.04)	–	–	–
CT vs CC	1	1.06 (0.62–1.82)			–
Mothers					
TT vs CC	1	0.77 (0.33–1.81)	–	–	–
CT vs CC	1	0.65 (0.38–1.10)	–	–	–
OFC					
Mothers					
TT vs CC	1	1.4 (0.4–5.2)	–	–	–
CT vs CC	1	1.2 (0.5–2.9)	–	–	–

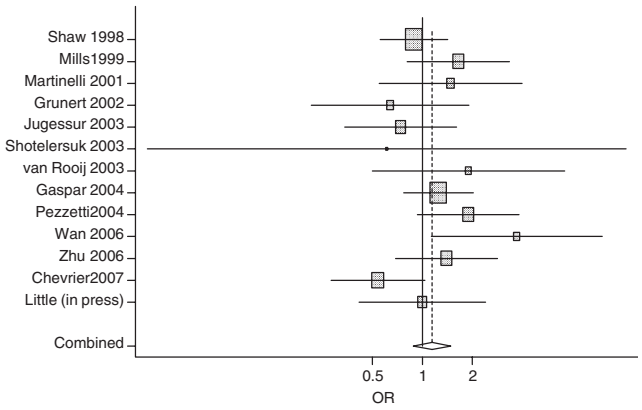

Figure 8 Random effects meta-analysis of the association between infant *MTHFR* C677T TT versus CC genotype and CL/P showing OR and 95% CI

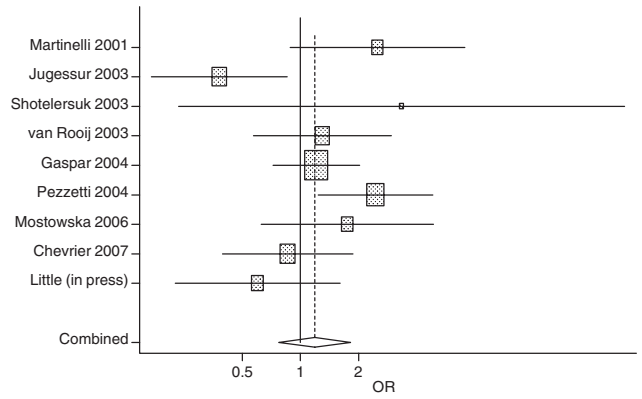

Figure 9 Random effects meta-analysis of the association between maternal *MTHFR* C677T TT versus CC genotype and CL/P showing OR and 95% CI

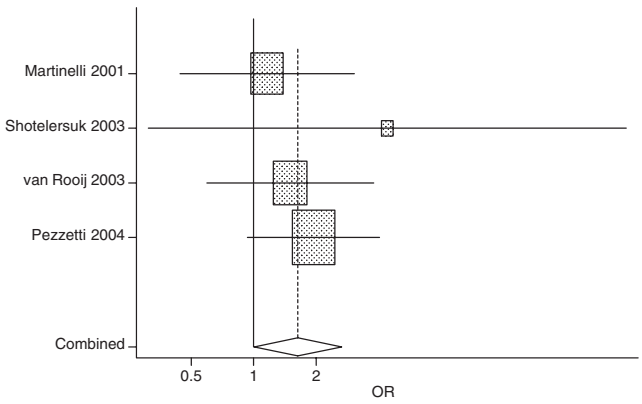

Figure 10 Random effects meta-analysis of the association between paternal *MTHFR* C677T TT versus CC genotype and CL/P showing OR and 95% CI

to provide information on the association between folate and oral clefts, as well as information on potential dose–response effects (www.clinicaltrials.gov, NCT00098319).

As there has been no trial of oral cleft prevention where women are randomized to receive multivitamins or not, most of the evidence for an association between multivitamins and clefts has come from observational studies. These studies have shown that women taking any type of multivitamin before or during pregnancy have a reduced risk of CL/P, and to a lesser extent CPO. It is difficult to determine which component(s) of the multivitamin is responsible for this reduction in risk, as the composition of multivitamins is rarely reported. The attempt made to separate the effects of folic acid and multivitamins through subgroup analyses was inadequate since the subgroups were not mutually exclusive; however, it was found that the folic acid subgroup had effect estimates closer to the null than the estimates for multivitamins for all types of oral clefts.

The subgroup analysis investigating timing of multivitamin use suggested that women starting multivitamins prior to pregnancy and continuing during early pregnancy had a lower risk of having a child with CL/P and CPO, while as expected there was no change in risk for women starting after the aetiologic time period. Although this suggests that multivitamins, particularly when started prior to pregnancy, can protect against having a child with a cleft, it is also possible that early multivitamin use is a marker of general good health practices, or may be correlated with other healthy behaviours such as not smoking or drinking alcohol during pregnancy. It may also be a marker for pregnancy planning; planning a pregnancy has recently been shown to be inversely associated with oral clefts.[91]

The stronger association observed between multivitamin use and CL/P compared with CPO suggests that this intervention may not be equally beneficial for both types of clefts. It is recognized that CL/P and CPO are aetiologically distinct entities[92] although they share some risk factors in common, such as in the case of van der Woude syndrome where mutations in the same gene, *IRF6*, cause both CL/P and CPO.[93] Results of the cumulative meta-analysis, however, suggest that the association between CPO and multivitamin use is not yet stable. At one point in time, the association between CPO and multivitamins was nearly identical to the association for CL/P. Why the association between multivitamins and CPO has recently regressed towards the null is unclear.

There was a reduction in the risk of cleft recurrence with multivitamin prophylaxis, with a larger effect estimate (i.e. greater protective effect) found in recurrence studies compared with the observational studies. The results of recurrence studies are difficult to evaluate, however, because these intervention studies were not randomized and are therefore subject to confounding and bias, and often presented results narratively without statistical analysis.[7] Small sample sizes and wide CIs also limited interpretation of the results of the recurrence studies. Although these studies varied

Table 7 Gene–environment interactions between *MTHFR* and supplement use during pregnancy

	No supplement use			Supplement use		
MTHFR C677T	TT	CT	CC	TT	CT	CC
CL/P						
Infant						
Wyszynski 2000[83]	3.0 (1.2–7.2)	1.7 (0.9–3.0)	2.1 (1.2–3.8)	0.7 (0.4–1.4)	0.8 (0.5–1.2)	1.0 (reference)
Jugessur 2003[24]	1.44 (0.73–2.82)		1.0 (reference)	4.31 (1.55–12.01)		1.0 (reference)
van Rooij 2003[73]	3.5 (0.3–42.4)	1.7 (0.7–3.9)	1.7 (0.8–3.8)	2.4 (0.5–12.3)	1.3 (0.5–3.1)	1.0 (reference)
Maternal						
Jugessur 2003[24]	1.44 (0.73–2.82)		1.0 (reference)	0.78 (0.33–1.85)		1.0 (reference)
van Rooij 2003[73]	5.9 (1.1–30.9)	1.3 (0.6–2.5)	1.7 (0.9–3.2)	1.2 (0.4–3.7)	0.8 (0.4–1.8)	1.0 (reference)
CPO						
Infant						
Shaw 1999[27]	0.9 (0.2–3.3)	–	1.0 (reference)	0.4 (0.2–1.1)	–	1.0 (reference)
***MTHFR* A1298C**	**CC**	**AC**	**AA**	**CC**	**AC**	**AA**
CL/P						
Infant						
van Rooij 2003[73]	1.7 (0.5–5.8)	1.8 (0.7–4.5)	1.9 (0.8–4.4)	2.7 (0.5–14.3)	1.0 (0.4–2.6)	1.0 (reference)
Maternal						
van Rooij 2003[73]	2.2 (0.7–6.5)	1.9 (0.9–3.9)	1.7 (0.8–3.4)	1.3 (0.5–4.5)	0.7 (0.3–1.7)	1.0 (reference)

widely in the dosage of folic acid (0.5–10 mg) included in the multivitamin, there was no difference in the effect estimates between studies, suggesting no dose–response effect.

The association between dietary folate intake and oral clefts was difficult to interpret due to differences in quantile definition between studies and differences in inclusion criteria; for example, some studies included women taking folic acid-containing supplements or multivitamins while others excluded them. Overall there was a suggestion of an inverse association between high folate intake and oral clefts although not all studies found these results. Most studies had small sample sizes and large CIs around the effect estimates meaning that chance may be responsible for some of the variability between studies. An attenuated association would be expected for studies which were conducted in regions with folic acid fortification. The American study conducted between 1997 and 2000 reported overall higher folate intake compared with other studies and found no association between dietary folate and oral clefts.[11] Similarly, no association was found in the Australian study where optional folic acid fortification is in place, although the levels of folate intake did not appear to be as high as in the American study.[45]

Numerical information on the before-and-after prevalence of oral clefts following folic acid fortification was available from North America and Australia. Only the studies from countries with compulsory fortification (United States and Canada) showed decreases in the prevalence of oral clefts whereas no decrease was shown in Australia, where there is optional fortification. Whether the decrease is due to the institution of compulsory, as opposed to optional, fortification or to other differences between North America and Australia is not clear. The observed decrease in prevalence of oral clefts may not be due to fortification, but instead to existing trends in prevalence, or due to other environmental or lifestyle factors changing over time. For example, the proportion of American women taking folic acid-containing multivitamins has increased from 28% to 33% in the 10-year period of 1995–2005,[94] which is approximately the same timeframe in which these before-and-after prevalence studies were conducted.

The small reduction in oral cleft prevalence observed following folic acid fortification of grains in North America suggests that folic acid may only play a minor role, if any, in oral cleft aetiology. Another possibility is that the effect of folic acid is dose-dependent and the amount of folate ingested through fortification, estimated at 0.1 mg daily in North America and up to 0.4 mg daily in Chile,[95] is insufficient to have a major impact on oral cleft prevalence. Possible dose–response relationships between folic acid and clefts have been described in several studies[12,14,31] and may warrant further attention. In contrast, one study from Denmark where women choosing to take folic acid supplements were randomized to receive either 1.0 mg or 2.5 mg of folic acid daily found that women randomized to the higher dose of folic acid were nearly twice as likely to

Table 8 Gene–environment interactions between *MTHFR* and maternal dietary folate intake

	Low folate intake			**High folate intake**			
MTHFR C677T	TT	CT	CC	TT	CT	CC	***P*-value for interaction**
CL/P							
Infant							
van Rooij 2003[73]	1.4 (0.3–6.1)	1.2 (0.5–2.7)	1.1 (0.5–2.4)		1.0 (0.5–2.2)	1.0 (reference)	
Chevrier 2007[13]	0.46 (0.1–1.5)	0.47 (0.2–1.1)	1.0 (reference)	0.43 (0.2–1.1)	0.66 (0.3–1.3)	1.0 (reference)	0.81
Maternal							
van Rooij 2003[73]	2.8 (0.7–10.5)	1.0 (0.5–2.0)	1.8 (0.9–3.6)	1.7 (0.6–2.9)	1.2 (0.6–2.5)	1.0 (reference)	
Chevrier 2007[13]	1.19 (0.4–3.6)	1.35 (0.6–3.3)	1.0 (reference)	0.48 (0.1–2.0)	0.92 (0.4–1.8)	1.0 (reference)	0.57
Little (in press)[66]	0.86 (0.28–2.62)	0.78 (0.38–1.60)	1.0 (reference)	0.17 (0.02–1.52)	0.37 (0.17–0.83)	0.17 (0.02–1.52)	0.32
CPO							
Maternal							
Little (in press)[66]	0.80 (0.23–2.82)	0.72 (0.32–1.63)	1.0 (reference)	0.42 (0.08–2.23)	0.41 (0.16–0.97)	0.85 (0.37–1.96)	0.72
***MTHFR* A1298C**	**CC**	**AC**	**AA**	**CC**	**AC**	**CC**	
CL/P							
Infant							
van Rooij 2003[73]	1.5 (0.4–5.3)	1.1 (0.4–2.8)	1.5 (0.7–3.3)	1.2 (0.3–5.2)	1.0 (0.4–2.3)	1.0 (reference)	
Maternal							
van Rooij 2003[73]	2.5 (0.8–7.9)	1.1 (0.5–2.4)	1.3 (0.6–2.7)	0.7 (0.2–2.7)	1.2 (0.6–2.5)	1.0 (reference)	

have a child with a cleft, although the study was underpowered to detect an association.[51] As aforementioned, in the review of recurrence studies, although folic acid dosage varied 20-fold between studies, there was no difference in the effect estimate. Likewise, in the studies of dietary folate intake no dose–response effect was observed in any study which tested for this effect.

There was marked variability in the results from studies of biochemical markers of folate status. These biochemical markers, measured as plasma and erythrocyte folate, when compared between case and control mothers gave inconsistent results. Low folate status was in some populations associated with an increased risk of oral clefts, and in others associated with a lower risk.

None of the analyses of genes involved in folate metabolism or transport provided convincing evidence for their importance in oral cleft aetiology. Of the associations found, most have only been performed in a single population and will require confirmation in further studies. Overall, there was no association between *MTHFR* C677T and A1298C and oral clefts in observational studies. Results from the TDT analyses support this, with little evidence of overtransmission of the variant allele. One exception was a possible association between the paternal *MTHFR* C677T TT genotype and CL/P, although this CI included the null value and the estimate was based on the results of only four studies.

With relatively few studies investigating gene–environment interactions between *MTHFR* and folic acid and the large number of possible combinations of outcome, environmental exposure and genetic exposure categories, it was difficult to determine if there was any true interaction. Differences in reference group assignment also made synthesis of evidence difficult. Gene–environment interaction studies require large sample sizes, and the wide CIs seen in most studies of gene–environment interactions suggest that the power was likely not high enough to detect an association. However, it appeared that there might be an interaction between *MTHFR* and multivitamin use, with higher risk among individuals with the variant genotype and who did not use multivitamins.

Polymorphisms in genes involved in folate metabolism and transport were included in this review due to the belief that individuals with variants of these genes may have impaired folate metabolism leading to suboptimal folate availability. Effect estimates for the mother's, father's and infant's genotype were included in the reviews of gene–disease associations and gene–environment interactions regardless of the biological plausibility of proposed mechanisms in order to include the greatest spectrum of evidence as possible. For example, although an interaction might be plausible between maternal folate intake and the mother's genotype, information on interactions between the mother's folate intake and infant's genotype were also included in the review even though the mechanism whereby this interaction might occur is less obvious.

Between-study heterogeneity was detected in several meta-analyses. By including adjusted instead of crude estimates in the meta-analysis it was hoped to minimize the effect of possible confounders, although one would expect differences in the covariates adjusted for in each study. There were other sources of heterogeneity in these analyses; for example, in the studies of supplements and multivitamin use the studies differed in study design, selection of participants, composition of supplements, and timing and duration of supplement use. The existence of an interaction between *MTHFR* genotype and multivitamin use may explain some of the heterogeneity observed in *MTHFR*–oral cleft association studies. In this case, one would expect an attenuated association between *MTHFR* and oral clefts in populations where multivitamin use is high.

Egger's test suggested the existence of publication bias in the meta-analysis of recurrence studies ($P = 0.02$) and in one of the meta-analyses of gene–disease association studies ($P = 0.04$). In the other reviews there was a variety of studies reporting positive and inverse associations and fairly small effect sizes, which does not suggest obvious publication bias.

The quality of the included studies was not formally assessed. With most of the evidence in these analyses coming from case–control studies, the potential impact of misclassification, selection bias, low participation rates or other sources of bias and confounding may be important but are difficult to quantify.

Several gaps in the evidence became evident from these systematic reviews. In particular, there were fewer studies of CPO compared to CL/P, perhaps due to its lower prevalence. There was no study of CPO in relation to either plasma folate status or paternal *MTHFR* genotype, and only one study each of CPO and erythrocyte folate status, *MTHFR* A1298C maternal and infant genotype, and gene–environment interactions.

There is other evidence, not reviewed here, that also suggests an association between folic acid and oral clefts. In particular, some studies have shown that women with epilepsy have an increased risk of having a child with a cleft, which has been attributed to both the epilepsy itself and the use of antiepileptic drugs during pregnancy, many of which are folic acid antagonists.[30,48,96–98] Other evidence for the involvement of folate in oral cleft occurrence comes from a recent study where it was shown that case mothers were more likely to have autoantibodies against folate receptors than control mothers.[99]

It has also been suggested that folate may be associated with oral clefts only indirectly, through its effects on homocysteine metabolism. Folate metabolite 5,10-methyltetrahydrofolate is combined with

homocysteine to produce methionine, meaning that when folate levels are high, homocysteine levels are low.[100] Elevated homocysteine levels have been found in oral cleft case mothers compared with controls in some studies[67,99] while other studies have found no association.[65,66,68]

The demonstrated importance of periconceptional folic acid supplementation in the prevention of neural tube defects means that all women of childbearing age are encouraged to consume folic acid supplements.[101–104] It has been recently proposed that use of folic acid-containing multivitamins in early pregnancy may also be effective in preventing several other types of congenital anomalies as well as certain childhood cancers.[28,105] Two recent meta-analyses on the use of folic acid-containing multivitamins and oral clefts have also produced similar results to the present meta-analysis.[10,28] None of this evidence, however, can conclusively single out folic acid as the biologically active component of the multivitamin, as multivitamins contain dozens of vitamins and minerals that might truly be responsible for the inverse associations observed with childhood cancers and congenital anomalies aside from neural tube defects.

The present systematic reviews and meta-analyses suggest that although folic acid is not strongly associated with oral clefts, multivitamin use in early pregnancy may be beneficial for reducing the occurrence of oral clefts, particularly CL/P. However, there is little information on the effects of low participation rates and self-selection on the study results and whether accounting for these would lead to a different interpretation of results. Folate was studied here as a single agent for the prevention of oral clefts. Other complex interventions such as pregnancy planning or optimization of diet during pregnancy, both of which may include folic acid or multivitamins as interventions directly or indirectly, may be more beneficial than focusing attention on a single nutrient alone.

Supplementary data

Supplementary data are available at *IJE* Online.

Acknowledgements

We would like to thank Christina Mei-Kuk Yang for assistance with translation.

KEY MESSAGES

- There is no strong evidence for an association between oral clefts and maternal intake of dietary or supplemental folate alone.
- There is evidence of a lack of association between maternal *MTHFR* C677T genotype, which has been associated with lowered red cell folate levels, and oral clefts.
- Maternal multivitamin use in early pregnancy is inversely associated with oral clefts, although whether this is due to a healthy user effect or some specific component of the multivitamin is unknown.

References

[1] Mossey PA, Little J. Epidemiology of oral clefts: an international perspective. In: Wyszynski DF (ed.). *Cleft Lip and Palate: From Origin to Treatment*. New York: Oxford University Press, 2002. pp. 127–58.

[2] Munger R. Maternal nutrition and oral clefts. In: Wyszynski D (ed.). *Cleft Lip and Palate: From Origin to Treatment*. New York: Oxford University Press, 2002. pp. 170–92.

[3] Forges T, Monnier-Barbarino P, Alberto JM, Gueant-Rodriguez RM, Daval JL, Gueant JL. Impact of folate and homocysteine metabolism on human reproductive health. *Human Reprod Update* 2007;**13:**225–38.

[4] Conway H. Effect of supplemental vitamin therapy on the limitation of incidence of cleft lip and cleft palate in humans. *Plast Reconstr Surg* 1958;**22:**450–53.

[5] Briggs RM. Vitamin supplementation as a possible factor in the incidence of cleft lip/palate deformities in humans. *Clin Plast Surg* 1976;**3:**647–52.

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

[7] World Health Organization. Global strategies to reduce the health-care burden of craniofacial anomalies: report of WHO meetings on International Collaborative Research on Craniofacial Anomalies, Geneva, Switzerland, 5–8 November 2000; Park City, Utah, USA, 24–26 May 2001. Geneva: World Health Organization; 2002.

[8] Czeizel AE, Dudas I. Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation. *N Engl J Med* 1992;**327:**1832–35.

[9] Czeizel AE, Dobo M, Vargha P. Hungarian cohort-controlled trial of periconceptional multivitamin supplementation shows a reduction in certain congenital abnormalities. *Birth Defects Res A Clin Mol Teratrol* 2004;**70:**853–61.

[10] Badovinac RL, Werler MM, Williams PL, Kelsey KT, Hayes C. Folic acid-containing supplement consumption

during pregnancy and risk for oral clefts: a meta-analysis. *Birth Defects Res A Clin Mol Teratol* 2007;**79:**8–15.

[11] Shaw GM, Carmichael SL, Laurent C, Rasmussen SA. Maternal nutrient intakes and risk of orofacial clefts. *Epidemiology* 2006;**17:**285–91.

[12] Bille C, Olsen J, Vach W *et al*. Oral clefts and life style factors – a case-cohort study based on prospective Danish data. *Eur J Epidemiol* 2007;**22:**173–81.

[13] Chevrier C, Perret C, Bahuau M *et al*. Fetal and maternal MTHFR C677T genotype, maternal folate intake and the risk of nonsyndromic oral clefts. *Am J Med Genet A* 2007;**143:**248–57.

[14] Wilcox AJ, Lie RT, Solvoll K *et al*. Folic acid supplements and risk of facial clefts: national population based case-control study. *Br Med J* 2007;**334:**464.

[15] Martin YN, Salavaggione OE, Eckloff BW, Wieben ED, Schaid DJ, Weinshilboum RM. Human methylenetetrahydrofolate reductase pharmacogenomics: gene resequencing and functional genomics. *Pharmacogenet Genomics* 2006;**16:**265–77.

[16] Sibani S, Leclerc D, Weisberg IS *et al*. Characterization of mutations in severe methylenetetrahydrofolate reductase deficiency reveals an FAD-responsive mutation. *Hum Mutat* 2003;**21:**520.

[17] Ogino S, Wilson RB. Genotype and haplotype distributions of MTHFR 677C>T and 1298A>C single nucleotide polymorphisms: a meta-analysis. *J Hum Genet* 2003; **48:**1–7.

[18] Kang SS, Wong PWK, Susmano A, Sora J, Norusis M, Ruggie N. Thermolabile methylenetetrahydrofolate reductase: an inherited risk factor for coronary artery disease. *Am J Hum Genet* 1991;**48:**536–45.

[19] Weisberg I, Tran P, Christensen B, Sibani S, Rozen R. A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity. *Mol Genet Metab* 1998;**64:**169–72.

[20] Goyette P, Pai A, Milos R *et al*. Gene structure of human and mouse methylenetetrahydrofolate reductase (MTHFR). *Mamm Genome* 1998;**9:**652–56.

[21] Tolarova MM, van Rooij IALM, Pastor 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* 1998;**63:**A27.

[22] 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 Genet A* 2007; **143:**952–60.

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

[24] Jugessur A, Wilcox AJ, Lie RT *et al*. Exploring the effects of methylenetetrahydrofolate reductase gene variants C677T and A1298C on the risk of orofacial clefts in 261 Norwegian case-parent triads. *Am J Epidemiol* 2003; **157:**1083–91.

[25] Wan WD, Wang LJ, Zhou XP *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* 2006;**22:**8–11.

[26] Zhu J, Ren A, Hao L *et al*. Variable contribution of the MTHFR C677T polymorphism to non-syndromic cleft lip and palate risk in China. *Am J Med Genet A* 2006;**140:**551–57.

[27] Shaw GM, Todoroff K, Finnell RH, Rozen R, Lammer EJ. Maternal vitamin use, infant C677T mutation in MTHFR, and isolated cleft palate risk. *Am J Med Genet* 1999;**85:**84–85.

[28] Goh YI, Bollano E, Einarson TR, Koren G. Prenatal multivitamin supplementation and rates of congenital anomalies: a meta-analysis. *J Obstet Gynaecol Can* 2006;**28:**680–89.

[29] Lin BK, Clyne M, Walsh M *et al*. Tracking the epidemiology of human genes in the literature: the HuGE Published Literature Database. *Am J Epidemiol* 2006;**164:**1–4.

[30] Werler MM, Hayes C, Louik C, Shapiro S, Mitchell AA. Multivitamin supplementation and risk of birth defects. *Am J Epidemiol* 1999;**150:**675–82.

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

[32] Higgins PT, Thompson SG. Quantifying heterogeneity in meta-analysis. *Stat Med* 2002;**21:**1539–58.

[33] Egger M, Davey Smith G, Schneider M, Minder CE. Bias in meta-analysis detected by a simple, graphical test. *Br Med J* 1997;**315:**634.

[34] Fraser FC, Warburton D. No association of emotional stress or vitamin supplement during pregnancy to cleft lip or palate in man. *Plast Reconstr Surg* 1964;**33:**395–99.

[35] Saxen I. Associations between oral clefts and drugs taken during pregnancy. *Int J Epidemiol* 1975;**4:**37–44.

[36] Shaw GM, Lammer EJ, Wasserman CR, O'Malley CD, Tolarova MM. Risks of orofacial clefts in children born to women using multivitamins containing folic acid periconceptionally. *Lancet* 1995;**346:**393–96.

[37] Hayes C, Werler MM, Willett WC, Mitchell AA. Case-control study of periconceptional folic acid supplementation and oral clefts. *Am J Epidemiol* 1996;**143:**1229–34.

[38] Romitti PA, Munger RG, Murray JC, Daack-Hirsch S, Hanson JW, Burns TL. The effect of follow-up on limiting non-participation bias in genetic epidemiologic investigations. *Eur J Epidemiol* 1998;**14:**129–38.

[39] Beaty TH, Wang H, Hetmanski JB *et al*. A case-control study of nonsyndromic oral clefts in Maryland. *Ann Epidemiol* 2001;**11:**434–42.

[40] Itikala PR, Watkins ML, Mulinare J, Moore CA, Liu Y. Maternal multivitamin use and orofacial clefts in offspring. *Teratology* 2001;**63:**79–86.

[41] Loffredo LC, Souza JM, Freitas JA, Mossey PA. Oral clefts and vitamin supplementation. *Cleft Palate Craniofac J* 2001;**38:**76–83.

[42] de Walle HEK, Reefhuis J, Cornel MC. Folic acid prevents more than neural tube defects: a registry-based study in the northern Netherlands. *Eur J Epidemiol* 2003; **18:**279–80.

[43] Hozyasz K, Milanowski A, Piwowar W, Rowicka G. Vitamin use during pregnancy in mothers of children with orofacial clefts. *Przeglad Pediatryczny* 2003;**33:**209–11.

[44] Mitchell LE, Murray JC, O'Brien S, Christensen K. Retinoic acid receptor alpha gene variants, multivitamin use, and liver intake as risk factors for oral clefts: a population-based case-control study in Denmark, 1991–1994. *Am J Epidemiol* 2003;**158:**69–76.

[45] Bower C, Miller M, Payne J, Serna P. Folate intake and the primary prevention of non-neural birth defects. *Aust N Z J Public Health* 2006;**30:**258–61.

[46] Krapels IP, Zielhuis GA, Vroom F *et al*. Periconceptional health and lifestyle factors of both parents affect the risk of live-born children with orofacial clefts. *Birth Defects Res A Clin Mol Teratol* 2006;**76:**613–20.

[47] Pei L, Zhu H, Zhu J, Ren A, Finnell RH, Li Z. Genetic variation of infant reduced folate carrier (A80G) and risk of orofacial defects and congenital heart defects in China. *Ann Epidemiol* 2006;**16:**352–56.

[48] Little J, Gilmour M, Mossey PA *et al*. Folate and clefts of the lip and palate – a UK based case-control study: Part I: Dietary and supplemental folate. *Cleft Palate Craniofac J* (in press).

[49] Hill L, Murphy M, McDowall M, Paul AH. Maternal drug histories and congenital malformations: limb reduction defects and oral clefts. *J Epidemiol Community Health* 1988;**42:**1–7.

[50] Dlugosz L, Vena J, Byers T, Sever L, Bracken M, Marshall E. Congenital defects and electric bed heating in New York State: a register-based case-control study. *Am J Epidemiol* 1992;**135:**1000–11.

[51] Ulrich M, Kristoffersen K, Rolschau J, Grinsted P, Schaumburg E, Foged N. The influence of folic acid supplement on the outcome of pregnancies in the county of Funen in Denmark Part II. Congential anomalies. A randomised study. *Eur J Obstet Gynecol Reprod Biol* 1999;**87:**111–13.

[52] Kallen BAJ, Olausson PO. Use of folic acid and delivery outcome: a prospective registry study. *Reprod Toxicol* 2002;**16:**327–32.

[53] Edwards MJ, Agho K, Attia J *et al*. Case-control study of cleft lip or palate after maternal use of topical corticosteroids during pregnancy. *Am J Med Genet A* 2003;**120:**459–63.

[54] Elahi MM, Jackson IT, Elahi O *et al*. Epidemiology of cleft lip and cleft palate in Pakistan. *Plast Reconstr Surg* 2004;**113:**1548–55.

[55] van Rooij IA, Ocke MC, Straatman H, Zielhuis GA, Merkus HM, Steegers-Theunissen RP. Periconceptional folate intake by supplement and food reduces the risk of nonsyndromic cleft lip with or without cleft palate. *Prev Med* 2004;**39:**689–94.

[56] Simmons CJ, Mosley BS, Fulton-Bond CA, Hobbs CA. Birth defects in Arkansas: is folic acid fortification making a difference? *Birth Defects Res A Clin Mol Teratol* 2004;**70:**559–64.

[57] Canfield MA, Collins JS, Botto LD *et al*. Changes in the birth prevalence of selected birth defects after grain fortification with folic acid in the United States: findings from a multi-state population-based study. *Birth Defects Res A Clin Mol Teratol* 2005;**73:**679–89.

[58] Botto LD, Lisi A, Bower C *et al*. Trends of selected malformations in relation to folic acid recommendations and fortification: An international assessment. *Birth Defects Res A Clin Mol Teratol* 2006;**76:**693–705.

[59] Robbins JM, Tilford JM, Bird TM, Cleves MA, Reading JA, Hobbs CA. Hospitalizations of newborns with folate-sensitive birth defects before and after fortification of foods with folic acid. *Pediatrics* 2006;**118:**906–15.

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

[61] Ray JG, Meier C, Vermeulen MJ, Wyatt PR, Cole DE. Association between folic acid food fortification and congenital orofacial clefts. *J Pediatr* 2003;**143:**805–7.

[62] Castilla EE, Orioli IM, Lopez-Camelo JS, Dutra Mda G, Nazer-Herrera J. Latin American Collaborative Study of Congenital Malformations (ECLAMC). Preliminary data on changes in neural tube defect prevalence rates after folic acid fortification in South America. *Am J Med Genet A* 2003;**123:**123–28.

[63] Niebyl JR, Blake DA, Rocco LE, Baumgardner R, Mellits ED. Lack of maternal metabolic, endocrine, and environmental influences in the etiology of cleft lip with or without cleft palate. *Cleft Palate J* 1985;**22:**20–28.

[64] Stoll C, Dott B, Alembik Y, Koehl C. Maternal trace elements, vitamin B12, vitamin A, folic acid, and fetal malformations. *Reprod Toxicol* 1999;**13:**53–57.

[65] Munger RG, Sauberlich HE, Corcoran C, Nepomuceno B, Daack-Hirsch S, Solon FS. Maternal vitamin B-6 and folate status and risk of oral cleft birth defects in the Philippines. *Birth Defects Res A Clin Mol Teratol* 2004;**70:**464–71.

[66] Little J, Gilmour M, Mossey PA *et al*. Folate and clefts of the lip and palate – a UK based case-control study: Part II: Biochemical and genetic analysis. *Cleft Palate Craniofac J* (in press).

[67] Wong WY, Eskes TK, Kuijpers-Jagtman AM *et al*. Nonsyndromic orofacial clefts: association with maternal hyperhomocysteinemia. *Teratology* 1999;**60:**253–57.

[68] van Rooij IA, Swinkels DW, Blom HJ, Merkus HM, Steegers-Theunissen RP. Vitamin and homocysteine status of mothers and infants and the risk of nonsyndromic orofacial clefts. *Am J Obstet Gynecol* 2003;**189:**1155–60.

[69] Shaw GM, Rozen R, Finnell RH, Todoroff K, Lammer EJ. Infant C677T mutation in MTHFR, maternal periconceptional vitamin use, and cleft lip. *Am J Med Genet* 1998;**80:**196–98.

[70] Martinelli M, Scapoli L, Pezzetti F *et al*. C677T variant form at the MTHFR gene and CL/P: a risk factor for mothers? *Am J Med Genet* 2001;**98:**357–60.

[71] Grunert RR, Braune A, Schnackenberg E, Schloot W, Krause HR. [Genetic differences in enzymes of folic acid metabolism in patients with lip-jaw-palate clefts and their relatives]. *Mund Kiefer Gesichtschir* 2002;**6:**131–33.

[72] 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* 2003;**40:**e64.

[73] van Rooij IA, Vermeij-Keers C, Kluijtmans LA *et al*. Does the interaction between maternal folate intake and the methylenetetrahydrofolate reductase polymorphisms affect the risk of cleft lip with or without cleft palate? *Am J Epidemiol* 2003;**157:**583–91.

[74] Gaspar DA, Matioli SR, de Cassia Pavanello R *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* 2004;**12:**521–26.

[75] Pezzetti F, Martinelli M, Scapoli L *et al*. Maternal MTHFR variant forms increase the risk in offspring of isolated nonsyndromic cleft lip with or without cleft palate. *Hum Mutat* 2004;**24:**104–5.

[76] Beaty TH, Hetmanski JB, Zeiger JS *et al*. Testing candidate genes for non-syndromic oral clefts using a case-parent trio design. *Genet Epidemiol* 2002;**22:**1–11.

[77] Nurk E, Tell GS, Refsum H, Ueland PM, Vollset SE. Associations between maternal methylenetetrahydrofolate reductase polymorphisms and adverse outcomes of pregnancy: The Hordaland Homocysteine Study. *Am J Med* 2004;**117:**26–31.

[78] Blanton SH, Patel S, Hecht JT, Mulliken JB. MTHFR is not a risk factor in the development of isolated nonsyndromic cleft lip and palate. *Am J Med Genet* 2002;**110:**404–5.

[79] Prescott NJ, Winter RM, Malcolm S. Maternal MTHFR genotype contributes to the risk of non-syndromic cleft lip and palate. *J Med Genet* 2002;**39:**368–69.

[80] Vieira AR, Murray JC, Trembath D *et al*. Studies of reduced folate carrier 1 (RFC1) A80G and 5,10-methylenetetrahydrofolate reductase (MTHFR) C677T polymorphisms with neural tube and orofacial cleft defects. *Am J Med Genet A* 2005;**135:**220–23.

[81] da Silva AL, Ribeiro LA, Cooper ME, Marazita ML, Moretti-Ferreira D. Transmission analysis of candidate genes for nonsyndromic oral clefts in Brazilian parent-child triads with recurrence. *Genet Mol Biol* 2006;**29:**439–42.

[82] Shi M, Caprau D, Dagle J, Christiansen L, Christensen K, Murray JC. Application of kinetic polymerase chain reaction and molecular beacon assays to pooled analyses and high-throughput gentyoping for candidate genes. *Birth Defects Res A Clin Mol Teratol* 2004;**70:**65–74.

[83] Wyszynski DF, Diehl SR. Infant C677T mutation in MTHFR, maternal periconceptional vitamin use, and risk of nonsyndromic cleft lip. *Am J Med Genet* 2000;**92:**79–80.

[84] Zhu H, Curry S, Wen S *et al*. Are the betaine-homocysteine methyltransferase (BHMT and BHMT2) genes risk factors for spina bifida and orofacial clefts? *Am J Med Genet A* 2005;**135:**274–77.

[85] Rubini M, Brusati R, Garattini G *et al*. Cystathionine beta-synthase c.844ins68 gene variant and non-syndromic cleft lip and palate. *Am J Med Genet A* 2005;**136:**368–72.

[86] Birnbaum S, Reutter H, Mende M *et al*. A family-based association study in Central Europeans: no evidence for the cystathionine beta-synthase c.844ins68 gene variant as a risk factor for non-syndromic cleft lip and palate. *Am J Med Genet A* 2007;**143:**205–7.

[87] Scapoli L, Marchesini J, Martinelli M *et al*. Study of folate receptor genes in nonsyndromic familial and sporadic cleft lip with or without cleft palate cases. *Am J Med Genet A* 2005;**132:**302–4.

[88] Mostowska A, Hozyasz KK, Jagodzinski PP. Maternal MTR genotype contributes to the risk of non-syndromic cleft lip and palate in the Polish population. *Clin Genet* 2006;**69:**512–17.

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

[90] Shaw GM, Zhu H, Lammer EJ, Yang W, Finnell RH. Genetic variation of infant reduced folate carrier (A80G) and risk of orofacial and conotruncal heart defects. *Am J Epidemiol* 2003;**158:**747–52.

[91] Mossey PA, Davies JA, Little J. Prevention of orofacial clefts: does pregnancy planning have a role? *Cleft Palate Craniofac J* 2007;**44:**244–50.

[92] Saal HM. Classification and description of nonsyndromic clefts. In: Wyszynski DF (ed.). *Cleft Lip and Palate: From Origin to Treatment*. New York: Oxford University Press, 2002. pp. 47–52.

[93] Beiraghi S, Miller-Chrisholm A, Kimberling WJ *et al*. Confirmation of linkage of Van der Woude syndrome to chromosome 1q32: evidence of association with STR alleles suggests possible unique origin of the disease mutation. *J Craniofac Genet Dev Biol* 1999;**19:**128–34.

[94] Green-Raleigh K, Carter H, Mulinare J, Prue C, Petrini J. Trends in folic acid awareness and behavior in the United States: the Gallup Organization for the March of Dimes Foundation surveys, 1995-2005. *Matern Child Health J* 2006;**10:**177–82.

[95] Oakley Jr GP, Bell KN, Weber MB. Recommendations for accelerating global action to prevent folic acid-preventable birth defects and other folate-deficiency diseases: meeting of experts on preventing folic acid-preventable neural tube defects. *Birth Defects Res A Clin Mol Teratol* 2004;**70:**835–37.

[96] Abrishamchian AR, Khoury MJ, Calle EE. The contribution of maternal epilepsy and its treatment to the etiology of oral clefts: a population based case-control study. *Genet Epidemiol* 1994;**11:**343–51.

[97] Metneki J, Puho E, Czeizel AE. Maternal diseases and isolated orofacial clefts in Hungary. *Birth Defects Res A Clin Mol Teratol* 2005;**73:**617–23.

[98] Hernandez-Diaz S, Werler MM, Walker AM, Mitchell AA. Folic acid antagonists during pregnancy and the risk of birth defects. *N Engl J Med* 2000;**343:**1608–14.

[99] Bliek JB, Rothenberg SP, Steegers-Theunissen RP. Maternal folate receptor autoantibodies and cleft lip and/or palate. *Int J Gynaecol Obstet* 2006;**93:**142–43.

[100] Lucock M, Yates Z. Folic acid – vitamin and panacea or genetic time bomb? *Nature Rev Genet* 2005;**6:**235–40.

[101] Recommendations for the use of folic acid to reduce the number of cases of spina bifida and other neural tube defects. *MMWR Recomm Rep* 1992;**41**:1–7.

[102] Folic acid for the prevention of neural tube defects. American Academy of Pediatrics Committee on Genetics. *Pediatrics* 1999;**104:**325–27.

[103] Food and Nutrition Program of the Pan American Health Organization, March of Dimes, Centers for Disease Control and Prevention. Recommended levels of folic acid and vitamin B12 fortification: conclusions. *Nutr Rev* 2004;**62:**S62–66.

[104] Wilson RD, Davies G, Desilets V *et al*. The use of folic acid for the prevention of neural tube defects and other congenital anomalies. *J Obstet Gynaecol Can* 2003;**25:**959–73.

[105] Goh YI, Bollano E, Einarson TR, Koren G. Prenatal multivitamin supplementation and rates of pediatric cancers: a meta-analysis. *Clin Pharmacol Ther* 2007;**81:**685–91.