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Maternal biomarkers of methylation status and non-syndromic orofacial cleft risk: a meta-analysis

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Abstract. Animal models have shown evidence of the role of maternal methyl donor status and its metabolism (one-carbon metabolism) in normal embryonic maxillofacial development. Nevertheless, studies in humans have shown conflicting results for the association of maternal methylation status biomarkers in the aetiology of the main craniofacial birth defects: non-syndromic orofacial clefts (NSOFCs). The aim of this study was to perform a meta-analysis assessing the relationship between maternal levels of methylation status biomarkers (plasma and erythrocyte folates and plasma vitamin B12 and homocysteine) and the risk of NSOFCs. A literature search of the conventional and grey medical–scientific databases identified 12 studies considering these variables. Based on standardized differences between means among cases and controls (Cohen's d test), evidence was found of an association only with high plasma homocysteine ($d = 0.37$; $P = 0.026$) when single effects were pooled. In addition to its usefulness as a marker of poor methyl-donor intake and/or metabolism, homocysteine appears to have a teratogenic effect. Although the results are based on a relatively small number of reports and/or studies of small sample sizes showing between-study heterogeneity, these problems were resolved by including an additional analysis. Therefore these findings constitute a real contribution towards explaining the complex aetiology of orofacial clefts.

Orofacial clefts (OFCs) are the most common birth defects affecting the craniofacial structures. The average prevalence of OFCs varies according to ethnic origin, geographic location, and socioeconomic status, among other factors.[1]

OFCs are generally classified as cleft palate only (CPO), cleft lip only (CL), or cleft lip with cleft palate (CLP). The latter two categories are historically referred to as cleft lip with or without cleft palate (CL/P).[2] Approximately 70% of

OFCs are non-syndromic (NSOFCs), occurring as isolated conditions without any other apparently structural or cognitive abnormality. The remaining 30% are defects found as part of more than 300 recognizable genetic syndromes.[1,2] The

prevalence rates, complexity of their rehabilitation plus medical costs, and the emotional burden on patients and their families make OFCs a worldwide public health problem. In this context, OFC patients present a wide variety of medical complications in early processes such as feeding, speaking, and hearing and in their social integration. These problems can be corrected totally or in part by maxillofacial and plastic surgery and by dental, audiologist, and psychological therapies beginning in the first months of life and extending beyond 18 years of age, according to the severity of the malformation.[3]

The aetiology of NSOFCs can be explained by the interaction between functionally altered genes and environmental factors, with the relationship with folate/one-carbon metabolism being probably the best example of this genetic–environmental interaction.[3,4] It has been reported that maternal periconceptional use of folic acid supplementation has beneficial effects in preventing the occurrence of NSOFCs.[5,6] Additionally, the interaction between foetal and maternal genotypes of functional variants within the folate metabolism genes and maternal intake of folic acid has also been demonstrated in the risk of NSOFCs.[7,8]

Folates are involved in the transfer of one-carbon units (methyl groups) to molecules involved in several biological processes, such as DNA synthesis and methylation, an epigenetic mechanism of genetic expression control.[9] Through evidence found in animal models, it has been postulated that a maternal methyl group deficiency can alter the DNA methylation status of the offspring, thereby playing a role in the aetiology of birth defects.[10] A deficit of methyl groups affects embryo and foetal cells with high proliferation rates, including those from the neural tube and neural crests.[4] The latter cell population, after neural tube closure, migrates to the ventral area and differentiates through processes regulated by epigenetic mechanisms. Notably, they contribute to bone and cartilage development of the craniomaxillofacial structures.[11] In addition, murine models have shown the importance of epigenetic mechanisms in secondary palate development.[9]

Consequently, some authors have evaluated the association of maternal methylation status and the risk of NSOFCs in the offspring, based on plasma and erythrocyte folate levels as direct biomarkers. Some studies have reported that mothers of cases have significantly lower levels of plasma folate in comparison to mothers of controls.[12–14] The same relationship has been described for erythrocyte folate levels.[12,13] However, associations between both of these biomarkers and cleft risk are not found in other reports.[7,15,16] The plasma level of vitamin B12 is another biomarker of maternal methylation status. This vitamin is closely related to one-carbon metabolism and is considered as another dietary methyl donor; it acts as an enzymatic co-factor for enzymatic reactions in methyl group transfer.[17] Positive and negative results for the association between maternal plasma levels of vitamin B12 and NSOFCs are described in the literature.[7,15,16,18]

S-adenosylmethionine is the principal methyl group donor in humans, which, after demethylation, is converted to homocysteine in a two-step enzymatic reaction.[17] Elevated plasma levels of this latter molecule are considered a good and sensitive marker of an impaired folate/one-carbon metabolism status, being associated with low levels of methyl donors such as folates and vitamin B12.[17,19] Consequently, some reports have shown significantly higher plasma levels of homocysteine in mothers of NSOFC cases in comparisons to control mothers, while other studies have not found significant differences between the two groups of mothers.[13–15,19,20]

Thus, the results of studies on the relationship between maternal levels of folate, vitamin B12, and homocysteine in the plasma and folate within erythrocytes and the risk of NSOFC in the offspring are controversial. In order to contribute towards resolving this controversy, it was decided to perform meta-analyses for these variables based on a literature search of several databases, with a comparison of the mean values among case mothers and control mothers.

Materials and methods

Literature search and study quality assessment

A search of the following scientific literature databases was conducted: PubMed, EMBASE, Cochrane Library, Web of Science, SpringerLink, and Scielo. In addition, grey literature databases were also searched (GreyNet, GreyLit, OpenGrey, LILACS, and POPLINE) (Fig. 1). The literature search was conducted through 1 October 2015 with no date restrictions for early studies and including the ''Related articles'' option, based on the terms ''cleft lip palate'' OR ''cleft palate'' OR ''orofacial clefts'' AND ''maternal homocysteine'' OR ''maternal folate'' OR ''maternal vitamin B12'' and restricted to English and Spanish languages. This search was performed independently by two authors (RB and JS), who identified the authors, year of publication, sample sizes, and mean values and standard deviations (SD) or median values and interquartile range (IQR) for the four selected biomarkers in NSOFC mothers and control mothers (offspring without any structural birth defect). After discarding reports according to the criteria described in the flowchart (Fig. 1), a quality assessment was performed on the selected studies using the Newcastle–Ottawa Scale, which considers three categories: selection of cases and controls, comparability of these groups, and the ascertainment of either the exposure or outcome. The maximum score that can be awarded is 9 points. A study with a poor quality score (<5 points) will have limitations for inclusion in a meta-analysis, with a high risk of bias.[21]

Statistical analyses

The meta-analyses were performed comparing the mean values of four folate/one-carbon metabolism biomarkers in mothers of NSOFC cases and control mothers. When data were expressed as the median and IQR, the mean value and SD were estimated using the formulae described by Hozo et al.[22] In order to pool the results of the individual studies, Cohen's *d* test (with 95% confidence interval, CI) was used to standardize differences between means for each determination among cases and controls, especially when the groups presented different variances for a measurement.[23] This statistic represents the effect size of a variable on a trait even when different scales have been used in the two groups. Its calculation and interpretation are relatively simple and it can be considered as the standard for effect size estimation.[23,24]

The presence of heterogeneity among the selected studies was assessed based on the Cochran Q statistic, which is calculated by summing the squared deviations for the effect of each study related to the pooled effect.[25] In addition, heterogeneity was quantified by means of the I^2 statistic, which represents the percentage of between-study variability explained by heterogeneity.[25] Thus, the combined effect was estimated using the fixed-effects or random-effects method according to the absence ($I^2 < 50$) or presence of heterogeneity ($I^2 > 50$), respectively.[26] In the presence of between-study heterogeneity, it was attempted to detect the source by
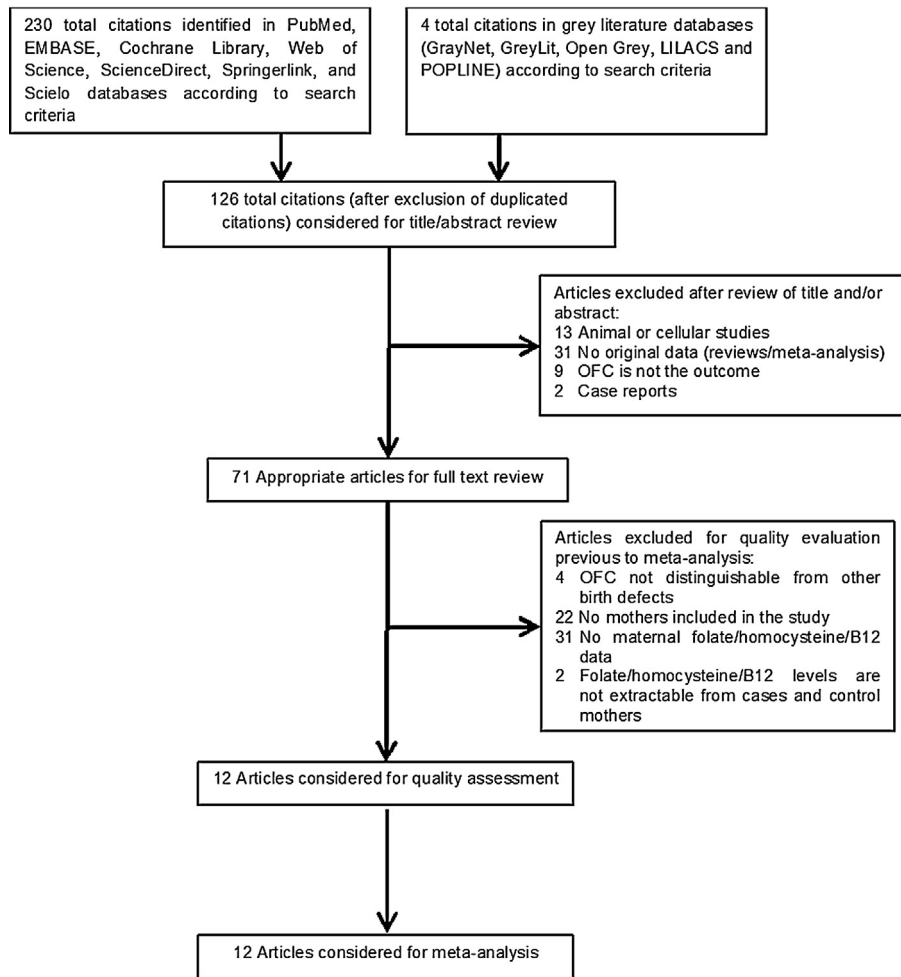
Fig. 1. Flowchart of the literature search, showing the study selection criteria..

applying a univariate meta-regression model, evaluating the relationship between the single study effect size and quantitative covariates universally described in all reports (sample sizes and maternal age mean of participants) and the date of sample collection relative to delivery (during pregnancy, between delivery date and 12 months, and >12 months).[26] The results of this analysis were recorded as the regression coefficient value (RC) with 95% CI.

Publication bias was evaluated by visual inspection of the Begg's funnel plot, in which each trail is represented around a central estimator (Napierian logarithm of the pooled effect in the ordinate) versus the standard error (as an estimator of study size). If reports are distributed symmetrically (as a funnel), one can conclude the absence of publication bias (i.e., studies have been published independently of their sample size and of their positive or negative effect).[26] In addition, visual inspection of the funnel plot was complemented by the

computation of Egger's test, which detects asymmetry from this plot based on a regression model of precision (inverse of standard error).[26] For a meta-analysis including mainly small sample size studies and/or a low number of studies (≤10), publication bias was assessed by cumulative meta-analysis by precision, which is more powerful than the funnel plot method in this situation.[27] For this purpose, one study is added at a time, recalculating the overall effect size. Studies are added from the most precise (i.e., the largest sample size) to the least precise (i.e., the smallest sample size). An important change observed in the effect (or drift) when small studies are added is considered a sign of publication bias.[27]

Finally, the robustness of the results was proved by means of a sensitivity analysis in which the meta-analysis is performed discarding one study at a time (one-factor-at-a-time analysis) and determining whether the pooled effect suffers significant change related to the overall effect.[26]

All tests were performed using the statistical package Epidat 3.1 (Pan American Health Organization, PAHO).

Results

The scientific literature database search (including grey literature) resulted in a total of 234 reports. After the exclusion of duplicated citations, 126 studies were considered for title and/or abstract review. This analysis ruled out a further 55 citations (for reasons see Fig. 1), and 71 papers were considered appropriate for full-text review. Fifty-nine citations were then excluded for the reasons listed in Fig. 1. After this full-text review, 12 articles remained and were included in the quality assessment. All of them showed a low or moderate risk of bias according to the Newcastle–Ottawa Scale. Thus these 12 reports, all of them only considering NSOFCs (i.e., OFC cases excluding recognized syndromes and chromosomal anomalies), were included in the current meta-analysis.[7,12–16,18–20,28–30] All of the

samples considered in these 12 articles were independent, with the exception only of the samples of van Rooij et al.[7] and Vujkovic et al.[16] These samples were partially common, but it was decided to include them due to their different sample sizes, means for all determinations, and effect sizes (see Table 1 and Figs 2–5). The following information was extracted from all of the studies: first author, year of reference, country of origin of the samples, cleft type, maternal biomarker values (mean ± SD) for case mothers and control mothers, and the sample sizes (Table 1).

Maternal plasma folates

Among the 12 reports included for meta-analysis, 10 included the measurement of plasma folates in mothers of cases and controls. A high heterogeneity was found among these reports ($Q = 391.3$, $I^2 = 97.3\%$, $P < 0.0001$), thus the random-effects method was used to pool the results. The meta-analysis of these 10 reports showed that mothers of NSOFC cases did not show a significant difference in plasma folate levels in comparison to control mothers (Cohen's $d = -0.24$, 95% CI -0.75 to 0.26, $P = 0.357$; Fig. 2).

Univariate meta-regression analysis showed possible sources of heterogeneity. A negative association between effect and sample size for case mothers was detected (RC -0.010, $P = 0.039$). The other variables (number of controls/number of cases ratio, age of mothers, and date of sample collection) did not show evidence of playing a role in heterogeneity (Table 2).

For all four markers analyzed, a low number of studies and/or small sample sizes were considered. Consequently, a cumulative meta-analysis by precision was applied in all situations in order to evaluate publication bias. When the most precise studies were added (45.8% of the total sample), the estimated effect was -0.23, which was not dramatically different from the global effect (-0.24). An important change was observed when the intermediate precision samples were added (-0.04 from 57.4% to 66.8% of the total sample), but this returned to the initial point when the smaller sample sizes were incorporated (-0.56 for 75.1% and -0.35 for 97.6% of the total sample) (Fig. 6A). Therefore, it was concluded that this analysis revealed an absence of publication bias.

The sensitivity analysis demonstrated that the exclusion of one study at a time did not alter the significance of the global effect, with the exception of the report by Bezerra et al.[14]; the exclusion of this report led to the overall difference between case mothers and control mothers becoming non-significant (Cohen's $d = 0.02$, 95% CI -0.23 to 0.26, $P = 0.93$; data not shown).

Maternal erythrocyte folates

Eight of the 12 reports selected showed values for maternal erythrocyte folates in cases and controls. Due to the evidence of heterogeneity ($Q = 34.9$, $I^2 = 75.4\%$, $P = 0.0001$), the random-effects method was considered for pooling. Similar values of red blood cell folates were detected among mothers of cleft cases and mothers of controls (Cohen's $d = 0.00$, 95% CI -0.19 to 0.18, $P = 0.977$; Fig. 3).

Searching for sources of heterogeneity, meta-regression did not show a significant relationship between individual Cohen's d and any variable considered (Table 2).

According to cumulative meta-analysis by precision, the addition of the two most precise samples (39.9% of the total sample size) showed an effect of 0.21. When the rest of the studies were considered, the effect suffered a considerable change, ranging between 0.01 at 57% of the total sample and 0.00 at 100% of the total sample (Fig. 6B). Therefore, it was concluded that there was a publication bias scenario by suppression of publications of studies with a small sample size. Although the exclusion of some studies changed the direction of the global effect, the sensitivity analysis showed robustness of the results, as the overall d did not become significant in any of the cases (data not shown).

Maternal plasma vitamin B12

Among the 12 reports included, only seven considered measurements of maternal plasma vitamin B12 levels (Table 1). Although inter-study heterogeneity appeared lower than for the other biomarkers, it was considered significant ($Q = 12.7$, $I^2 = 54.3\%$, $P = 0.0486$). Thus, the random-effects model was applied for meta-analysis. This showed no evidence of a significant pooled effect of this maternal variable on the risk of NSOFC (Cohen's $d = -0.01$, 95% CI -0.22 to 0.18, $P = 0.902$; Fig. 4).

Meta-regression to evaluate possible sources of heterogeneity showed a negative association between individual Cohen's d and the sample size of case mothers (RC -0.006, $P = 0.012$); no relationships were detected for the other variables (Table 2).

The sensitivity analysis demonstrated a lack of robustness of the results, as the elimination of the report by Sutton et al.[18] led to the overall effect becoming significant (Cohen's $d = 0.47$, 95% CI 0.05 to 0.89; $P = 0.0281$; data not shown). The assessment of publication bias by cumulative meta-analysis revealed that when the three most precise studies (70.6% of the total sample) were added, the effect reached 0.10, but when the rest of the studies were considered, the effect became negative (range -0.05 to -0.01) showing clear evidence of publication bias (Fig. 6C).

Maternal plasma homocysteine

Nine of the 12 studies selected in the literature search investigated maternal plasma homocysteine to evaluate its possible association with the risk of NSOFC in the offspring (Table 1). Significant heterogeneity was demonstrated among these reports ($Q = 130.5$; $I^2 = 93.4\%$; $P < 0.0001$), thus the meta-analysis was based on a random-effects model. Combining the individual effects of these nine studies, it was found that case mothers showed significantly higher plasma homocysteine values than control mothers (Cohen's $d = 0.37$, 95% CI 0.04 to 0.70, $P = 0.026$; Fig. 5).

On sensitivity analysis it was concluded that the results are robust due to no significant changes being observed when any of the reports was dropped from the meta-analysis (data not shown). Univariate meta-regression did not detect a causal relationship between heterogeneity and the variables associated with sample size, age of the mothers, or date of sample collection relative to child delivery (Table 2).

Cumulative meta-analysis by precision suggested a publication bias scenario. When the three most precise studies (51.3% of the total sample) were added, the effect reached a similar value to the final overall effect (0.31), but when the five smaller studies were considered (66.4% to 100% of the total sample), the effect moved between 0.74 and 0.37 (Fig. 6D).

Discussion

Evidence from animal models has demonstrated the relevance of a proper maternal methylation status for normal embryonic development, which has been linked to processes such as DNA methylation, an epigenetic mechanism of genetic expression control.[9,10] With regard

Table 1. Plasma folates, erythrocyte folates, plasma vitamin B12, and plasma homocysteine of mothers of NSOFC cases and mothers of controls in the 12 studies selected for meta-analysis; mean ± SD values.

Study reference	Country	Cleft type[a]	Quality (points)[b]	Plasma folates (nmol/l)		Erythrocyte folates (nmol/l)		Plasma vitamin B12 (pmol/l)		Plasma homocysteine (μmol/l)	
				Cases (n)	Controls (n)	Cases (n)	Controls (n)	Cases (n)	Controls (n)	Cases (n)	Controls (n)
Niebyl et al.[28]		CL/P	7	13.6 ± 12.4 (59)	14.3 ± 9.1 (56)	324 ± 140 (55)	313 ± 154 (56)				
Stoll et al.[29]	France	CL/P	7	8.6 ± 2.3 (14)	8.5 ± 2.7 (340)			310 ± 44 (14)	325 ± 148 (340)		
Wong et al.[19]	Netherlands	OFC	8	23.3 ± 13.1 (35)	14.5 ± 5.3 (56)	658 ± 259 (35)	573 ± 205 (56)	312 ± 164 (35)	303 ± 141 (56)	13.5 ± 3.6 (35)	10.8 ± 3.9 (56)
van Rooij et al.[7]	Netherlands	OFC	6	12.9 ± 7.0 (87)	13.3 ± 7.6 (88)	632 ± 296 (87)	636 ± 306 (88)	246 ± 131 (87)	289 ± 131 (88)	12.5 ± 4.2 (87)	11.7 ± 4.7 (87)
Munger et al.[12]	Philippines	CL/P[c]	6	16.3 ± 9.9 (73)	14.5 ± 8.4 (194)	993 ± 386 (73)	1099 ± 480 (194)				
		CL/P[d]		18.9 ± 18.8 (46)	14.3 ± 7.4 (395)	952 ± 306 (46)	777 ± 266 (395)				
Chávez-Corral et al.[30]	Mexico	CL/P	6	16.3 ± 8.6 (16)	14.3 ± 9.5 (141)	999 ± 616 (16)	870 ± 627 (141)	204 ± 124 (16)	192 ± 205 (141)	7.3 ± 1.9 (16)	7.2 ± 2.4 (141)
Shaw et al.[15]		CL/P	8	38.2 ± 21.9 (89)	36.5 ± 20.9 (409)			292 ± 101 (89)	323 ± 165 (409)	6.7 ± 2.9 (89)	6.9 ± 3.7 (409)
Vujkovic et al.[16]	Netherlands	OFC	6	15.9 ± 8.2 (70)	26.6 ± 18.5 (68)	676 ± 163 (70)	780 ± 341 (68)	307 ± 164 (70)	288 ± 103 (68)	13.9 ± 4.8 (70)	11.5 ± 3.0 (68)
Munger et al.[13]		CL/P	8	51.9 ± 26.3 (212)	64.6 ± 33.4 (468)	1991 ± 826 (211)	2119 ± 826 (465)			4.3 ± 1.4 (212)	4.1 ± 1.3 (426)
		CP		53.4 ± 28.5 (87)		1918 ± 803 (87)				4.2 ± 1.3 (87)	
Sutton et al.[18]	Ireland	OFC	6			589 ± 219 (36)	614 ± 336 (568)	298 ± 123 (23)	240 ± 123 (471)	7.5 ± 1.3 (22)	7.6 ± 1.6 (463)
Kumari et al.[20]	India	CL/P	7							14.5 ± 2.3 (98)	10.8 ± 1.4 (109)
Bezerra et al.[14]	Brazil	CL/P	7	31.3 ± 4.8 (140)	43.3 ± 2.5 (175)					6.7 ± 2.9 (140)	6.1 ± 2.3 (175)
Total n				928	2390	716	2031	334	1573	856	1934

NSOFC, non-syndromic orofacial cleft; SD, standard deviation.
[a] CL/P, cleft lip with or without cleft palate; CP, cleft palate only; OFC, CL/P and CP with no separated data.
[b] For a maximum of 9 points according to the Newcastle–Ottawa scale.
[c] Negros Occidental Province, Philippines.
[d] Davao Province, Philippines.

| Study (Reference)	d (95% CI)	Weight (%)
Niebyl et al. (28)	-0.06 (-0.43; 0.30)	5.1
Stoll et al. (29)	0.04 (-0.49; 0.57)	5.3
Wong et al. (19)	0.97 (0.52; 1.41)	2.3
Van Rooij et al. (7)	-0.07 (-0.36; 0.23)	7.7
Munger et al. (12)(A)	0.20 (-0.07; -0.36)	9.3
Munger et al. (12)(B)	0.49 (0.19; 0.80)	7.2
Chavez-Corral et al. (30)	0.21 (-0.31; 0.73)	2.5
Shaw et al. (15)	0.08 (-0.15; 0.31)	12.9
Vujkovic et al. (16)	-0.75 (-1.09; -0.41)	5.7
Munger et al. (13) (CL/P)	-0.40 (-0.56; -0.24)	25.2
Munger et al. (13) (CP)	-0.34 (-0.57; -0.11)	12.8
Bezerra et al. (14)	-3.24 (-3.57; -2.90)	5.9
Overall (Random-effects)	*-0.24 (-0.75; 0.26)*	*100*
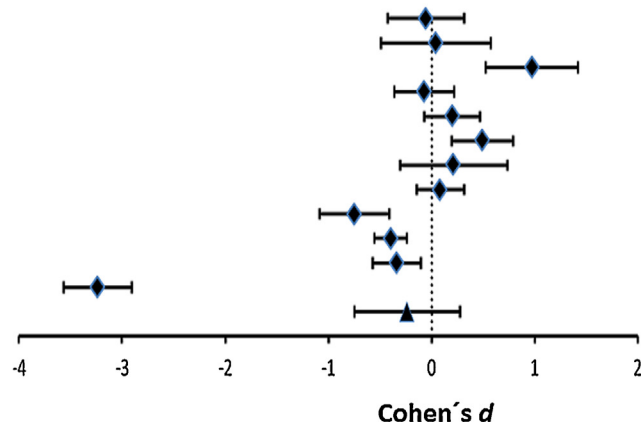
Fig. 2. Forest plot for the meta-analysis assessing the association between maternal plasma folate levels and the risk of NSOFC. Pooled effect based on the random-effects model ($Q = 391.3$, $I^2 = 97.3\%$, $P < 0.0001$) ('A', Negros Occidental Province, Philippines; 'B', Davao Province, Philippines; CP, cleft palate only; CL/P, cleft lip with or without cleft palate).

| Study (Reference)	d (95% CI)	Weight (%)
Niebyl et al. (28)	0.08 (-0.30; 0.45)	5.7
Wong et al. (19)	0.37 (-0.05; 0.80)	4.4
Van Rooij et al. (7)	-0.01 (-0.31; 0.28)	9.0
Munger et al. (12)(A)	-0.23 (-0.50; 0.04)	10.8
Munger et al. (12)(B)	0.65 (0.34; 0.96)	8.3
Chavez-Corral et al. (30)	0.21 (-0.31; 0.72)	2.9
Vujkovic et al. (16)	-0.39 (-0.73; -0.05)	6.9
Sutton et al. (18)	-0.16 (-0.32; 0.01)	9.86
Munger et al. (13)(CL/P)	-0.24 (-0.47; -0.02)	15.0
Munger et al. (13) (CP)	-0.08 (-0.41; 0.26)	6.9
Overall (Random effects)	*0.00 (-0.19; 0.18)*	*100*
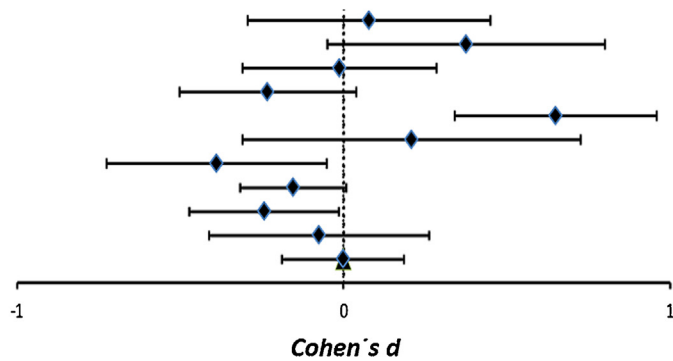
Fig. 3. Forest plot for the meta-analysis assessing the association between maternal erythrocyte folate levels and the risk of NSOFC. Pooled effect based on the random-effects model ($Q = 39.4$, $I^2 = 75.4\%$, $P = 0.0001$) ('A', Negros Occidental Province, Philippines; 'B', Davao Province, Philippines; CP, cleft palate only; CL/P, cleft lip with or without cleft palate).

to craniomaxillofacial development, epigenetic changes seem to modulate both early (neural crest cell migration and differentiation) and late processes (secondary palate morphogenesis).[9,11] However, for human populations, results for the association between maternal methylation status (measured by plasma and erythrocyte folates and plasma vitamin B12 and homocysteine) and OFC risk in the offspring are controversial (see individual effects in Figs 2–5). Therefore, it was considered necessary to perform a meta-analysis to resolve the question about the role of maternal methylation status and OFC expression.

The current meta-analyses showed common characteristics for the four maternal methylation status biomarkers. First, a low number of studies were included and many of them had small sample sizes. This common feature meant that an alternative method for the analysis of publication bias had to be found. In this situation, conventional

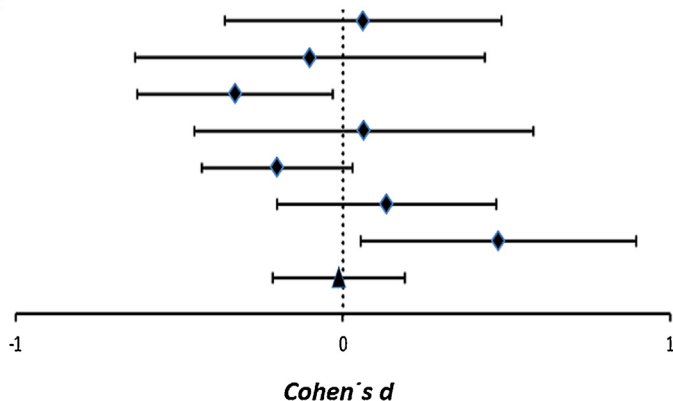| Study (Reference)	d (95% CI)	Weight (%)
Wong et al. (19)	0.06 (-0.36; 0.48)	9.7
Stoll et al. (29)	-0.10 (-0.63; 0.43)	6.1
Van Rooij et al. (7)	-0.33 (-0.63; -0.03)	19.5
Chavez-Corral et al. (30)	0.06 (-0.45; 0.58)	6.5
Shaw et al. (15)	-0.20 (-0.43; 0.03)	32.9
Vujkovic et al. (16)	0.13 (-0.20; 0.47)	15.5
Sutton et al. (18)	0.47 (0.05; 0.89)	9.8
Overall (Random Effects)	*-0.01 (-0.22; 0.18)*	*100*

Fig. 4. Forest plot for the meta-analysis assessing the association between maternal plasma vitamin B12 levels and the risk of NSOFC. Pooled effect based on the random-effects model ($Q = 12.7$, $I^2 = 54.3\%$, $P = 0.0486$).
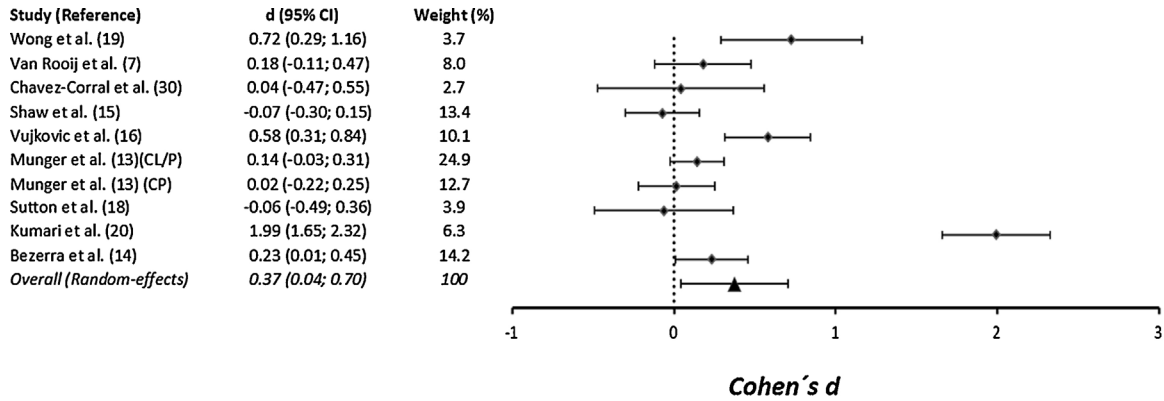
Study (Reference)	d (95% CI)	Weight (%)
Wong et al. (19)	0.72 (0.29; 1.16)	3.7
Van Rooij et al. (7)	0.18 (-0.11; 0.47)	8.0
Chavez-Corral et al. (30)	0.04 (-0.47; 0.55)	2.7
Shaw et al. (15)	-0.07 (-0.30; 0.15)	13.4
Vujkovic et al. (16)	0.58 (0.31; 0.84)	10.1
Munger et al. (13)(CL/P)	0.14 (-0.03; 0.31)	24.9
Munger et al. (13) (CP)	0.02 (-0.22; 0.25)	12.7
Sutton et al. (18)	-0.06 (-0.49; 0.36)	3.9
Kumari et al. (20)	1.99 (1.65; 2.32)	6.3
Bezerra et al. (14)	0.23 (0.01; 0.45)	14.2
Overall (Random-effects)	*0.37 (0.04; 0.70)*	*100*

Cohen´s d

Fig. 5. Forest plot for the meta-analysis assessing the association between maternal plasma homocysteine levels and the risk of NSOFC. Pooled effect based on the random-effects model ($Q = 130.5$, $I^2 = 93.4\%$, $P < 0.0001$) (CP, cleft palate only; CL/P, cleft lip with or without cleft palate).

inspection of the funnel plot and its associated statistics would exhibit a low power for the assessment of publication bias, which is why it was decided to include the more powerful method of cumulative meta-analysis by precision.[27] A second common characteristic for all of the four methylation markers was the between-study heterogeneity, which established the use of a random-effects model for the evaluation of pooled effects. In this context, some authors advise finding the sources of the heterogeneity instead of discarding the results, with the use of univariate meta-regression between effects

and quantitative covariates (based on sample size and maternal age).[25]

Another source of general heterogeneity could be the period when blood samples were obtained. For example, Stoll et al.[29] and Shaw et al.[15] considered samples from pregnant women (at 6–13 weeks and 15–18 weeks, respectively), while the rest of the studies were based on samples obtained from women at 8 months to 5 years after delivery (with great variability), a period during which several mothers are in lactation. Markers of methylation status present different range values depending on the trimester of pregnancy

and are also different to the values registered in non-pregnant women.[31] Some authors recommend that these kinds of measurement should be performed at least 12 months after delivery in order to totally avoid the effects of pregnancy and lactation.[13]

It has been documented extensively that epigenetic changes during the early embryonic stages are critical for proper development and even for adulthood.[32] It is precisely during these early stages that the embryo facial midline begins its formation (from the fourth week).[3] The fact that the great majority of the studies considered for

Table 2. Univariate meta-regression for covariates and individual effects.

Covariate	Coefficient (95% CI)	*P*-value
Maternal plasma folates		
Number of case mothers	−0.010 (−0.019; −0.000)	0.039
Number of control mothers/number of case mothers	0.035 (−0.052; 0.123)	0.430
Total sample size	−0.001 (−0.004; 0.002)	0.699
Mean case mothers age (years)	0.066 (−0.226; 0.358)	0.656
Mean age difference case mothers − control mothers (years)	−0.040 (−0.653; 0.573)	0.899
Date of sample (relative to delivery)	−0.428 (−1.299; 0.443)	0.336
Maternal erythrocyte folates		
Number of case mothers	−0.002 (−0.005; 0.001)	0.172
Number of control mothers/number of case mothers	0.017 (−0.022; 0.056)	0.403
Total sample size	0.000 (−0.001; 0.001)	0.623
Mean case mothers age (years)	0.009 (−0.079; 0.097)	0.841
Mean age difference case mothers − control mothers (years)	0.053 (−0.139; 0.244)	0.589
Date of sample (relative to delivery)	−0.029 (−0.245; 0.187)	0.110
Maternal plasma vitamin B12		
Number of case mothers	−0.006 (−0.010; −0.001)	0.012
Number of control mothers/number of case mothers	0.015 (−0.005; 0.035)	0.151
Total sample size	0.000 (−0.001; 0.001)	0.830
Mean case mothers age (years)	0.008 (−0.083; 0.098)	0.866
Mean age difference case mothers − control mothers (years)	−0.028 (−0.217; 0.160)	0.767
Date of sample (relative to delivery)	0.146 (−0.420; 0.187)	0.294
Maternal plasma homocysteine		
Number of case mothers	0.001 (−0.006; 0.008)	0.831
Number of control mothers/number of case mothers	−0.041 (−0.009; 0.017)	0.168
Total sample size	−0.002 (−0.003; 0.000)	0.082
Mean case mothers age (years)	−0.030 (−0.219; 0.159)	0.757
Mean age difference case mothers − control mothers (years)	0.031 (−0.122; 0.185)	0.688
Date of sample (relatively to delivery)	0.360 (−0.139; 0.858)	0.157

CI, confidence interval.

A

N	Cumulative %	Effect
680	18.0	-0.40
1235	32.6	-0.38
1733	45.8	-0.23
2174	57.4	-0.06
2528	66.8	-0.04
2843	75.1	-0.56
3110	82.1	-0.45
3285	86.8	-0.40
3442	90.9	-0.34
3580	94.6	-0.38
3695	97.6	-0.35
3786	100.0	-0.24

B

N	Cumulative %	Effect
676	21.0	0.37
1280	39.9	0.21
1832	57.0	0.01
2273	70.8	0.04
2540	79.1	0.02
2715	84.5	-0.03
2872	89.4	0.09
3010	93.7	0.04
3121	97.2	0.02
3212	100.0	0.00

C

N	Cumulative %	Effect
498	26.1	0.06
992	52.0	0.11
1346	70.6	0.10
1521	79.8	-0.05
1678	88.0	-0.06
1816	95.2	0.04
1907	100.0	-0.01

D

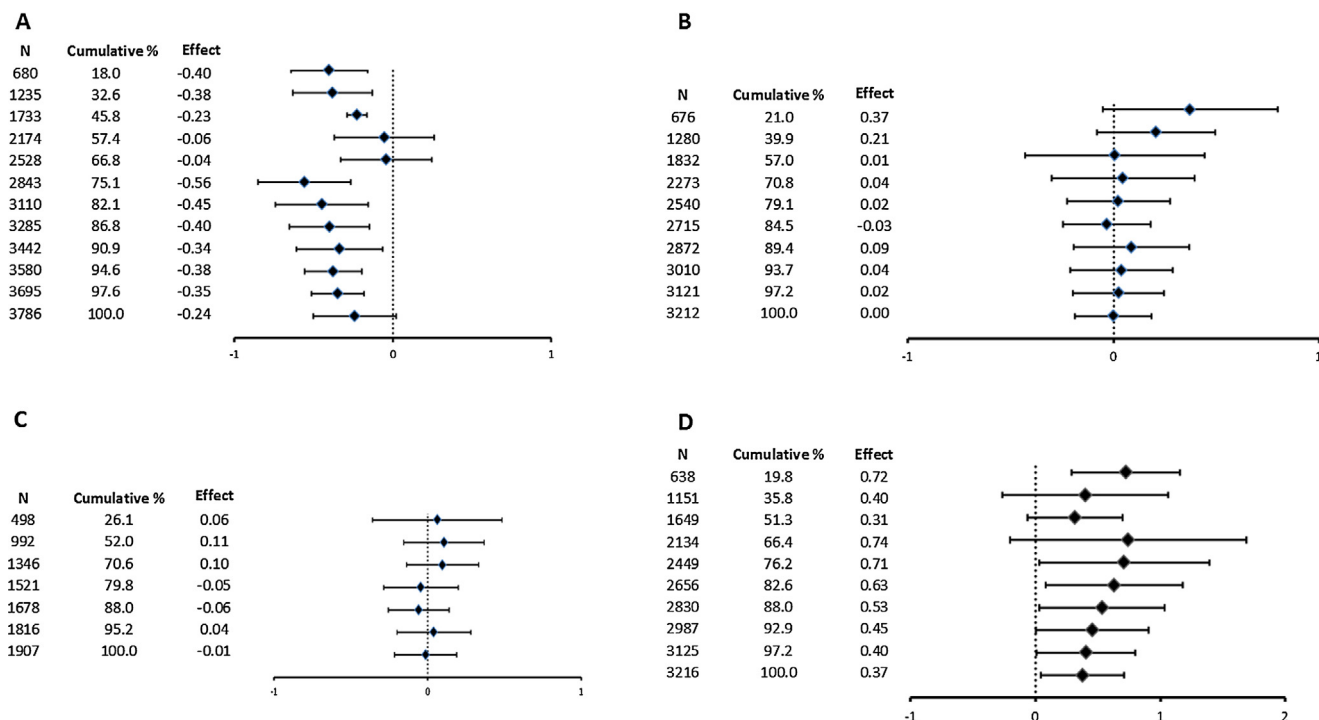| N	Cumulative %	Effect
638	19.8	0.72
1151	35.8	0.40
1649	51.3	0.31
2134	66.4	0.74
2449	76.2	0.71
2656	82.6	0.63
2830	88.0	0.53
2987	92.9	0.45
3125	97.2	0.40
3216	100.0	0.37

Fig. 6. Forest plots for cumulative meta-analysis by precision for NSOFC risk associated with (A) maternal plasma folates, (B) maternal erythrocyte folates, (C) maternal plasma vitamin B12, and (D) maternal plasma homocysteine..

the current meta-analysis were retrospective (after delivery), may explain the absence of significant results for three of the four maternal methylation biomarkers evaluated here.

Notwithstanding this biological evidence, no evidence of the effect of date of sample collection on between-study heterogeneity was detected when this was included as a covariate in the meta-regression analysis (see Table 2). These results may be explained by the fact that three groups were formed for meta-regression computation: studies including pregnant mothers, studies including mothers of children aged <12 months, and a third group including mothers of children aged >12 months, where each study was assigned to only one of these groups. However, the great inter-study variability meant that some reports could be classified into two groups simultaneously. Thus, it would be necessary to count the specific data for each mother in each study in order to generate an adequate number of groups for a proper analysis.

Heterogeneity could also be explained by ethnicity (see Table 1). In this context, significant differences have been observed among diverse ethnic groups. For example, there is variation in the frequency of the main genetic variant affecting the activity of MTHFR (p.Ala222Val or c.C677T), a key enzyme that catalyzes

the reaction that provides both the main circulating form of folates and the availability of methyl donors.[4,33] With a greater number of studies it would be possible to replicate these meta-analyses, stratifying by period of sample collection or by ethnic origin, in order to avoid the sources of heterogeneity mentioned.

Despite the above characteristics of the analyses, which could be considered as weaknesses, this meta-analysis showed strengths such as the use of diverse databases. In order to find the greatest number of published studies in this area, several grey literature databases were included (see Materials and methods) in addition to the conventional medical–scientific literature databases. Another strength is the quality of the reports included here. Based on the Newcastle–Ottawa Scale, all of the reports considered had an appropriate score, avoiding the risk of bias.[21] In summary, it is considered that this search and its results are robust.

Maternal plasma folates

Pooling the effects of 10 studies considering maternal folates in NSOFC cases and controls, this meta-analysis did not show evidence of an association of this variable with the risk of having a child with a NSOFC (Fig. 2). This result is supported by the cumulative meta-analysis by

precision, which allowed it to be inferred that there was no publication bias (Fig. 6A). High between-study heterogeneity was found, the sources of which were investigated by means of a meta-regression assessment (Table 2). Variations among studies related to the sample size of case mothers were detected, which had implications for the statistical power. It has been reported that sample size variation (i.e., the precision of each study) is always an important factor in heterogeneity in a meta-analysis.[34] The heterogeneity could also be explained by non-quantitative variables, such as the different methods used for folate measurement: four techniques, probably with different sensitivities and detection ranges, were used in the 10 studies included.

Maternal erythrocyte folates

Meta-analysis showed no significant pooled effect for this variable; therefore maternal erythrocyte folates could not be assigned a role as a risk factor for OFC (Fig. 3), in accordance with the results for maternal plasma folate. The a priori expectation was that the findings for these variables would be discordant as a result of the clinical significance of the two parameters: red blood cell folates appear to reflect tissue folate status, while low plasma folates do not necessarily indicate

a tissue deficiency.[35] Furthermore, the studies considering plasma and erythrocyte folates were not the same (10 and eight reports, respectively), with three of them considering only plasma levels and one reporting only red blood cell levels.[14,15,18,29] A high between-study heterogeneity with no apparent source (see Table 2) and the detection of publication bias (Fig. 6B) led to the conclusion that the results of the meta-analysis for maternal folate erythrocyte levels and NSOFC risk should be viewed with caution.

Maternal plasma vitamin B12

Although vitamin B12 is closely related to one-carbon metabolism as another dietary methyl donor,[7] this third biomarker of maternal methylation status showed no association with the risk of NSOFCs (Fig. 4). This result is possibly related to the low number of studies considering this variable (seven reports), the heterogeneity between studies (possibly related to the sample size of cases; Table 2), and clear evidence of publication bias (by suppression of small sample size studies; Fig. 6C). Thus this result should be interpreted with caution.

Maternal plasma homocysteine

The pooled effect of the nine studies selected for this meta-analysis showed that higher levels of maternal plasma homocysteine are associated with an increased risk of the appearance of OFC in the child (Fig. 5). The sensitivity analysis exhibited robustness. There are two possible explanations for this association. The first is the fact that an increase in the circulating levels of homocysteine is a marker of folate deficiency.[31] Normal early embryological development seems to depend on the periconceptional intake of folate, among other micronutrients.[17] Second, a toxic effect of high levels of homocysteine during embryogenesis has been reported in animal models, with alterations seen in neural tube closure and heart development.[36] Homocysteine produces a dose-dependent apoptosis in cultures of human embryonic palatal shelf cells by oxidative stress, while a high dose of this molecule alters the *in vitro* fusion of murine palatal shelves.[37]

Only one previous meta-analysis assessing the risk of maternal hyperhomocysteinemia on OFCs could be found.[38] This analysis was based on only two studies[7,19] and reported an increased risk of NSOFC in the children of mothers with hyperhomocysteinemia, but not reaching statistical significance. The difference in results between the present meta-analysis and the previous one may be explained by (1) the fact that the present analysis focused on plasma homocysteine as a quantitative trait, and (2) the significantly increased number of studies and consequently total sample size in the present analysis.

The selected studies on maternal plasma homocysteine proved to be quite heterogeneous. According to the univariate meta-regression, none of the covariates considered showed evidence of being a source of heterogeneity (Table 2). In this context, a non-quantitative source of between-study variation may be the methods used for the measurement of homocysteine. However, unlike what was observed for plasma folates, these methods for homocysteine were more homogeneous (six of the nine studies used high-performance liquid chromatography (HPLC) plus fluorometric detection). In addition to heterogeneity, this final meta-analysis showed evidence of publication bias (Fig. 6D), demonstrating that reports with small sample sizes showed a no-publication decision trend. Therefore, these results must be interpreted with caution.

In summary, the four meta-analyses performed in the current study mainly showed that a high level of maternal plasma homocysteine is a risk factor for NSOFC in the child. Although the results should be treated with caution due to the relatively low number of reports and/or small sample sizes and the high heterogeneity associated with variations in inter-study precision in some cases and with publication bias in others, additional analyses were included to help resolve these common problems for systematic reviews. Therefore, it is concluded that these results are robust and constitute a real contribution towards explaining the complex aetiology of orofacial clefts. The absence of significance for three of the four biomarkers evaluated here may be a reflection of the timing of sample collection (mainly after delivery), with the possibility that the maternal methylation status at the time that maxillofacial development was occurring was not reflected. There appear to be no reports showing biological evidence for an association of the level of global DNA methylation or for specific promoters with orofacial clefts in humans, which would be useful in order to confirm or reject the present statistical findings.

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Competing interests

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Patient consent

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