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Maternal Vitamin B_{12} Status and Risk of Cleft Lip and Cleft Palate Birth Defects in Tamil Nadu State, India

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Abstract

Background and Objective: The causal role of maternal nutrition in orofacial clefts is uncertain. We tested hypotheses that low maternal vitamin B_{12} and low folate status are each associated with an increased risk of isolated cleft lip with or without cleft palate (CL ± P) in a case–control study in Tamil Nadu state, India.

Methods: Case-mothers of CL ± P children (n = 47) and control-mothers of unaffected children (n = 50) were recruited an average of 1.4 years after birth of the index child and plasma vitamin B_{12}, methylmalonic acid (MMA), total homocysteine (tHcy), and folate were measured at that time. Logistic regression analyses estimated associations between nutrient biomarkers and case–control status.

Results: Odds ratios (ORs) contrasting biomarker levels showed associations between case-mothers and low versus high plasma vitamin B_{12} (OR = 2.48, 95% CI, 1.02-6.01) and high versus low plasma MMA, an indicator of poor B_{12} status (OR = 3.65 95% CI, 1.21-11.05). Case–control status was not consistently associated with folate or tHcy levels. Low vitamin B_{12} status, when defined by a combination of both plasma vitamin B_{12} and MMA levels, had an even stronger association with case-mothers (OR = 6.54, 95% CI, 1.33-32.09).

Conclusions: Mothers of CL ± P children in southern India were 6.5 times more likely to have poor vitamin B_{12} status, defined by multiple biomarkers, compared to control-mothers. Further studies in populations with diverse nutritional backgrounds are required to determine whether poor maternal vitamin B_{12} or folate levels or their interactions are causally related to CL ± P.

Keywords

orofacial clefts, cleft lip, cleft palate, congenital anomalies, birth defects, pregnancy, maternal nutrition, vitamin B_{12}, cobalamin, methylmalonic acid, folate, homocysteine, India, Tamil Nadu

Introduction

Orofacial clefts (OFCs) are among the most common birth defects with considerable geographic, racial, ethnic, and socio-economic variation in occurrence (Mossey et al., 2009). Maternal folate nutrition is of interest, given the success of folic acid in preventing neural tube defects (NTDs; MRC Vitamin Study Research Group, 1991) and the fact that OFCs and NTDs share some developmental pathways (Kousa et al., 2017); however, the role of folate in OFCs is uncertain (Munger et al., 2004; Johnson & Little 2008; Munger et al., 2011; De-Regil et al., 2015). Vitamin B_{12} is an essential cofactor in folate-related 1-carbon metabolism, but its possible role in OFCs has

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received much less attention than folate. Experimental animal studies have provided some evidence of a causal role for poor maternal vitamin B_{12} status in the etiology of OFCs (Mann & Gautieri 1973; He et al., 2010; Zhang et al., 2011), but studies of maternal vitamin B_{12} status and OFCs in humans are few in number with varied methods and inconsistent results (van Rooij et al., 2003; Shaw et al., 2009; Vujkovic et al., 2010; Sutton et al., 2011; Wallenstein et al., 2013; Blanco et al., 2016). Further insight from human studies of the associations between maternal vitamin B_{12} and folate nutrition and OFCs may lead to global public health efforts to prevent OFCs.

The birth prevalence of OFCs in India is not well defined due to limited population-based birth defect surveillance and the high mortality of infants with OFCs may result in underascertainment of cases (Mossey & Little 2009). The high prevalence of vitamin B_{12} deficiency in India has been linked to many adverse reproductive health outcomes, including spontaneous abortion, intrauterine growth retardation, fetal adiposity, insulin resistance, and gestational diabetes (Muthayya et al., 2006; Rosenberg, 2008; Yajnik et al., 2008; Krishnaveni et al., 2009; Katre et al., 2010; Dwarkanath et al., 2013).

Investigations of the role of maternal nutrition in causing OFCs are difficult in prospective cohort studies because OFCs are rare, requiring the follow-up of 500 to 1000 pregnancies to find each birth of a child with an OFC; hence, such a study would be very costly. We and others have employed case–control studies with maternal nutrient biomarkers, based on the premise that maternal dietary habits and social environments are relatively stable before and after pregnancy and genetic and epigenetic factors that influence blood biomarkers levels are fixed (Leck et al., 1983; van Rooij et al., 2003; Munger et al., 2004; Tamura et al., 2005; Tamura et al., 2007; Munger et al., 2011); in these studies, maternal biomarker associations with birth defects have persisted for years after the affected birth and may provide clues to the etiology and prevention of OFCs.

Multiple biomarkers provide a more detailed and in-depth assessment of vitamin B_{12} and folate status, respectively, than single biomarkers (Bailey et al., 2015; Green et al., 2017). Validation studies with comparisons of assay results from independent laboratories are important but unfortunately are not reported in many publications, especially in reports on folate status (Pfeiffer et al., 2011). We employed assays of multiple biomarker indicators of maternal vitamin B_{12} status (plasma vitamin B_{12} and methylmalonic acid [MMA]) and folate status (microbiologic and liquid chromatography–tandem mass spectrometry (LC-MS/MS) assays of plasma folate) and total plasma homocysteine, an indicator of both B_{12} and folate status, and report on validation findings. With these biomarkers, we tested the hypotheses that low maternal vitamin B_{12} status and low folate status are each associated with an increased risk of isolated cleft lip with or without cleft palate (CL $\pm$ P) in a case–control study in Tamil Nadu state, India.

Methods

A case–control study of associations between maternal plasma nutrient biomarkers and risk of OFCs was conducted in the Thiruvallur, Kanchipuram, and Chennai districts of Tamil Nadu state, India. The case definition for mothers included last child born with a CL $\pm$ P, and with no other birth defect or known genetic etiology, ascertained via the surgical records of the Cleft and Craniofacial Center of Sri Ramachandra University Hospital (SRUH) in Chennai and reviewed by the Director J.M. The controls were mothers residing and giving birth in the same geographic area as case-mothers and were recruited from the Tamil Nadu Air Pollution Health Effects study, an ongoing prospective, community-based cohort study of the effects of air pollution on pregnant women and their children, thus representative of the population from which the case-mothers were drawn (Balakrishnan et al., 2015). Study participants lived within a 1 to 2 hours' drive to SRUH, and free transportation to SRUH was provided for case-mothers and control-mothers. Control-mothers had no child ever born with a birth defect and were frequency matched with a 1:1 ratio to case-mothers by maternal age-group (18-22, 23-27, 28-32 years) and age-group of their last live-born child (<6, 6-11, 12-17, 18-23, 24-29, 30-35, 36-41 months). Informed consent was obtained from all mothers via protocols approved by the institutional review boards of Sri Ramachandra University, Utah State University, the United States, and the India Council of Medical Research. Mothers were interviewed, had clinical examinations, and had blood samples collected during the period May to July 2014, and the included births of case-children and control-children were between January 2012 and September 2013. The sample size of 50 case-mothers and 50 control-mothers was determined by resource limitations.

Trained members of the research team conducted interviews in the local Tamil language to collect demographic data, lifestyle data, and health histories of study participants and family members. Information on breastfeeding (BF), including direct BF and expressing breast milk for bottle feeding at the time of blood collection, was obtained because of the concern that this may affect maternal blood nutrient levels (Jathar et al., 1970; Obeid et al., 2017). Data on the duration of BF at the time of maternal blood collection were not available. Weight was measured on a Samso Progress 150 digital weighing scale, and height was measured with a Seca 213 stadiometer. Maternal venous blood samples were collected by trained phlebotomists from the antecubital fossa in evacuated tubes with ethylenediaminetetraacetic acid anticoagulant. The blood tubes were placed in wet ice immediately upon mixing and centrifuged within 2 hours of collection. Plasma aliquots were immediately drawn off after centrifugation and then stored at -80 °C until assayed for biomarkers.

Plasma vitamin B_{12} was determined by electrochemiluminescence (Elecsys 2010, Roche Diagnostics). Methylmalonic acid, an indicator of B_{12} status related to mitochondrial metabolism, and total homocysteine (tHcy), an indicator of both B_{12} and folate status, were measured by gas chromatography–mass

spectrometry (Varian 3800) at the Division of Nutrition, St. John's Research Institute, Bangalore, India, via methods previously published (Duggan et al., 2014). Validation assays of a subsample for plasma vitamin B_{12} were performed with a microbiologic method (Kelleher & Broin 1991) at the Biomedical Sciences Institute, Trinity College, Dublin, Ireland. Plasma folate was determined by a microbiological method (Molloy & Scott 1997), and validation assays of a subsample for folate forms were performed with LC-MS/MS methods (Bjorke-Monsen et al., 2008) at Bevital (www.bevital.com), Bergen, Norway. All assays were performed without knowledge of the case–control status of the samples. Validation assays of each analyte were performed in different laboratories without knowledge of the results from the assay with which they were compared.

Studies were completed with 50 case-mothers and 50 control-mothers. Of the 55 case-mothers who were sought out, 5 (9%) could not be contacted and there were no refusals. Of the 72 control-mothers who were sought out, 12 (17%) could not be contacted and 10 (14%) refused. Two of the case-mothers were excluded from analyses because their children were found to have an isolated cleft palate without a cleft lip, a group thought to be etiologically distinct from cases with CL ± P (Mossey, Little et al. 2009) and 1 case-mother was excluded due to missing biomarker data. The remaining 47 case-children had no other birth defects.

The Statistical Package for the Social Sciences, version 25.0, was used to calculate medians and interquartile ranges of demographic and biomarker variables, and Spearman rank-order correlation coefficients between biomarkers; medians were compared with the independent samples nonparametric median test. Unconditional logistic regression models were used, as is appropriate for frequency matching by age in case–control studies (Kuo et al., 2018), to calculate odds ratios (ORs) and their 95% CIs as estimates of relative risk of CL ± P by level of maternal plasma nutrient biomarker. The restricted sample size did not allow analyses over a graded series of finer biomarker levels to evaluate dose–response relationships; hence, only 2 levels were compared, above and below the median for controls, to maximize the statistical power of comparing higher versus lower biomarker groups. Plasma vitamin B_{12} and MMA determinations are overlapping, but distinct measures of vitamin B_{12} nutritional status; hence, a cross-classification with both measures may better define adequate versus suboptimal vitamin B_{12} status (Green et al., 2017). Therefore, additional analyses were completed that compared exposure levels defined by combinations of vitamin B_{12} and MMA levels. Adjusted ORs were calculated in logistic regression models that included maternal age (due to a 2-year median difference between case and control maternal ages despite frequency matching), rural versus urban residence, and current BF status, including direct BF and expressing breast milk for infant bottle feeding, at the time of blood sample collection. A covariate for BF status at the time of maternal blood collection was included because previous publications have reported lower blood nutrient levels in BF versus nonbreastfeeding (NBF) mothers in India (Jathar et al., 1970; Obeid et al., 2017).

Results

The demographic characteristics and biomarker levels of study participants are summarized in Table 1. Case-children were slightly older than controls at the time of maternal blood collection (median 1.8 years vs 1.2 years). Fewer case-children were female (36%; 1:1.8 female-to-male ratio) than controls (52% female), which is consistent with the 1:2 female-to-male sex ratio for infants born with CL ± P in European populations (Mossey & Little 2002). Case-mothers, compared to controls, were marginally younger, taller, heavier, and had more rural residences. Fewer case-mothers than controls were BF at the time of study, consistent with the known difficulties in breastfeeding children with OFCs (Goyal et al., 2014). Median plasma vitamin B_{12} was lower in case-mothers versus controls (232.0 and 286.3 pmol/L, respectively, $P = .04$). Median MMA was higher in case-mothers versus controls (0.36 and 0.32 μmol/L, respectively, $P = .03$), also an indication of poorer vitamin B_{12} status in case-mothers. Median tHcy levels were marginally higher in case-mothers versus control-mothers (10.7 and 9.6 umol/L, respectively, $P = .54$), and median folate levels were marginally lower in case-mothers versus controls (10.7 and 12.9 nmol/L, respectively, $P = .27$). All case-mothers and control-mothers reported receiving iron-folate supplements from public health centers during pregnancy, and none reported taking these supplements at the time of interview and blood collection. Iron-folate supplements are offered free by local public health clinics to pregnant women for the purpose of prevention of anemia, intrauterine growth retardation, and maternal growth stunting rather than as an effort to prevent birth defects, and the supplements are typically taken up in the second trimester, after the period of OFC formation (Kumar, 1999; Mason et al., 2012). None of the mothers reported taking vitamin B_{12} supplements during or after pregnancy.

Spearman rank-order correlation coefficients for comparisons of biomarkers appear in Table 2 for all mothers combined. The number of validation assays was limited by the remaining sample volumes of split aliquots. Fifty-eight aliquots were assayed for plasma vitamin B_{12} with the microbiologic method (B_{12}-MB) in the laboratory of A.M., and these values were highly correlated with the assays performed with the electrochemiluminsecence method (B_{12}-ECL) used in the full sample in the laboratory of A.K. ($r = 0.89$, $P < .001$). Thirty-three aliquots were assayed for plasma folate forms with the folate-LC-MS/MS method (Ueland et al., 2007) in the Bevital laboratory and the values for total folates, computed as the sum of 5-methyltetrahydrofolate plus the oxidized form, 4-hydroxy-5-methyltetrahydrofolate, were highly correlated with the assays performed with the microbiologic method (folate-MB) used in the full sample in the laboratory of A.M. ($r = 0.87$, $P < .001$). The correlations for the validation assays were similar for cases and controls when the analyses were stratified by case–control status (data not shown).

Table 1. Characteristics of Case-Mothers and Control-Mothers and Children: Tamil Nadu, India, Orofacial Cleft Study.

Characteristic	Cases,[a] N = 47	Controls, N = 50
	Median (IQR) or percent	Median (IQR) or percent
Age of index child at blood draw (years)	1.8 (1.0-2.6)	1.2 (0.7-2.1)
Sex of index child (female, %)	36	52
Age of mother at blood draw (years)	22 (20-24)	24.0 (21-26)
Height of mother (cm)	155 (150-157)	151.0 (148-158)
Weight of mother (kg)	54.0 (48.0, 59.8)	50 (44.8-61.3)
Body mass index of mother (kg/m^2)	22.1 (20.8, 24.9)	21.0 (18.8, 24.9)
Residence (%)		
Rural	61.7	48.0
Urban	38.3	52.0
Breast feeding[b] (%)		
No	87	38
Yes	13	62
Vitamin B_{12} (pmol/L)[c]	232.0 (173.5-323.2)	286.3 (222.5-336.4)
Methylmalonic acid (μmol/L)[d]	0.36 (0.28-0.52)	0.32 (0.28-0.46)
Homocysteine (μmol/L)[d]	10.7 (8.3-12.6)	9.6 (8.7-12.3
Folate (nmol/L)[e]	10.7 (8.1-13.5)	12.9 (7.2-15.5)

Abbreviation: IQR, interquartile range.

[a]Index cases are children with cleft lip with or without cleft palate and no other birth defects.

[b]Includes direct breastfeeding and expressing breast milk for infant feeding at the time of blood collection.

[c]Determination by electrochemiluminescence method (Elecsys 2010; Duggan et al., 2014).

[d]Determination by chromatography–mass spectrometry method (Varian 3800; Duggan et al., 2014).

[e]Determination by microbiological method (Molloy & Scott 1997); sample includes 29 cases and 41 controls due to sample volume limitations.

The correlations in Table 2 also reveal expected associations between the biomarkers of 1-carbon metabolism. Both methods of plasma vitamin B_{12} assay revealed similar correlations with plasma MMA (B_{12}-ECL: $r = -0.35$, $P < .001$; B_{12}-MB: $r = -0.39$, $P = .002$). In addition, both methods of plasma vitamin B_{12} assay revealed similar correlations with plasma tHcy (B_{12}-ECL: $r = -0.25$, $P = .01$; B_{12}-MB: $r = -0.21$, $P = .11$). The results of the two vitamin B_{12} assays and the MMA assay were not significantly associated with either of the folate assays, indicating that vitamin B_{12} status in this sample was not associated with folate status. Each of the folate assays was similarly correlated with plasma tHcy (folate-MB: $r = -0.44$, $P < .001$; folate-LC-MS/MS: $r = -0.41$, $P = .02$).

The associations between nutrient biomarker levels and case–control status, estimated as ORs and 95% CIs comparing mothers above and below the median plasma biomarker levels, appear in Table 3. The logistic regression models included covariates of age of mother (due to residual differences in case and control maternal ages despite frequency matching), rural versus urban residence, and BF status. Case-mothers were 2.48 times more likely than control-mothers to have a lower plasma vitamin B_{12} level (OR = 2.48; 95% CI, 1.02-6.01) than having a higher plasma vitamin B_{12} level. Case-mothers were 3.65 times more likely than control-mothers to have a higher plasma MMA acid level (an indicator of poorer vitamin B_{12} status) than having a lower plasma MMA acid level (OR = 3.65; 95% CI, 1.21-11.05). Higher versus lower level of tHcy had an OR of 2.08, but the 95% CI (0.73-5.90) broadly included 1.0, weaker evidence of association compared to the vitamin B_{12} and MMA results. Similarly, lower versus higher level of plasma folate had an adjusted OR of 2.87, but the 95% CI (0.72-11.46) also broadly included 1.0, also weaker evidence of association compared to the vitamin B_{12} and MMA results.

The results of logistic regression models estimating the association between combined vitamin B_{12} biomarker levels and case–control status appear in Table 4. With the low-risk reference level defined as plasma vitamin B_{12} above the median and MMA below the median, the adjusted OR for low-risk level of plasma vitamin B_{12} (above median) combined with high-risk level of MMA (above median) was 0.90 (95% CI, 0.17-4.80) and the adjusted OR for high-risk level of plasma vitamin B_{12} (below median) combined with low-risk level of MMA (below median) was 0.74 (95% CI, 0.15-3.48). Only the group with combined high-risk level of plasma vitamin B_{12} (below median) and high-risk level of MMA (above median) showed a stronger association with case-mothers (adjusted OR = 6.54, 95% CI, 1.33-32.09) than the single analyses of these vitamin B_{12} status indicators. Thus case-mothers were 6.5 times more likely to have poor vitamin B_{12} status, defined by multiple biomarkers, compared to control-mothers.

The prevalence of BF was higher among the control-mothers (62%) compared to case-mothers (13%), and we found that plasma B_{12} was lower and MMA was higher in BF versus NBF mothers in both case-mothers and control-mothers when these groups were examined separately. Among BF case-mothers, vitamin B_{12} was 24% lower and MMA was 36% higher compared to NBF case-mothers. Among BF control-mothers, vitamin B_{12} was 8% lower and MMA was 6% higher compared to NBF control-mothers. To further explore the possibility that case-control differences in BF prevalence may have somewhat biased associations, we restricted the logistic regression analyses to NBF mothers only and found that OFC association with low B_{12} was similar among NBF only (OR = 2.67, 95% CI, 0.76-9.33) compared to the overall results (OR = 2.48, 95% CI, 1.02-6.01); association with high MMA among NBF only was higher (OR = 5.00, 95% CI, 1.26-19.82) compared to the overall results (OR = 3.65, 95% CI, 1.21-11.05); and association with the joint level of low B_{12} and high MMA among NBF only was higher (OR = 11.13, 95% CI, 1.45-85.79) compared to the overall results (OR = 6.54, 95% CI, 1.33-32.09). There was a 0.6-year difference between the median ages of case-children and control-children at the time of maternal blood collection, and we felt this was unlikely to confound the analyses of maternal biomarker differences; to assess this further, we repeated all logistic regression analyses with the addition of a covariate for child age and the results were unchanged.

Table 2. Spearman Rank-Order Correlations (*r*) Between Maternal Nutrient Biomarkers Related to Folate-Dependent 1-Carbon Metabolism: Tamil Nadu, India, Orofacial Cleft Study (47 Case-Mothers and 50 Control-Mothers Combined).

	Spearman rank-order correlations (*r*)					
Maternal plasma nutrient biomarker	B_{12}-ECL[a]	B_{12}-MB[b]	MMA[c]	tHcy[c]	F-MB[d]	F-LC-MS/MS[e]
Vitamin B_{12} (B_{12}-ECL)[a]	1.0					
Vitamin B_{12} (B_{12}-MB)[b]	0.89 $P < .001$	1.0				
Methylmalonic acid (MMA)[c]	−0.35 $P < .001$	−0.39 $P = .002$	1.0			
Homocysteine (tHcy)[c]	−0.25 $P = .01$	−0.21 $P = .11$	0.32 $P = .001$	1.0		
Folate (folate-MB)[d]	0.07 $P = .57$	0.09 $P = .52$	0.16 $P = .18$	−0.44 $P < .001$	1.0	
Folate (folate-LC-MS/MS)[e]	−0.02 $P = .93$	0.12 $P = .49$	0.07 $P = .71$	−0.41 $P = .02$	0.87 $P < .001$	1.0

[a]B_{12}-ECL: Electrochemiluminescence method for plasma vitamin B12 determination (Elecsys, 2010; Duggan et al., 2014).
[b]B_{12}-MB: Microbiological method for plasma vitamin B_{12} determination (58 validation samples; Kelleher & Broin, 1991).
[c]MMA: Gas chromatography-mass spectrometry method for methylmalonic acid determination (Varian 3800; Duggan et al., 2014).
[d]Folate-MB: Microbiological method for plasma folate determination (Molloy & Scott, 1997).
[e]Folate-LC-MS/MS: Liquid chromatography–tandem mass spectrometry method for plasma folate determination (33 validation assays).

Table 3. Relative Odds (Odds Ratios and 95% CIs) Estimates of Associations Between Nutrient Biomarker Levels and Mothers of Orofacial Cleft Children (Isolated Cleft Lip Only and Cleft lip and Palate): Tamil Nadu, India, Orofacial Cleft Study.

Plasma biomarker	Levels compared	Number of cases	Number of controls	Unadjusted Odds ratio (95% CI)	Adjusted[a] odds ratio (95% CI)
Vitamin B_{12}[b]	Above median (>286.3 pmol/L)	14	25	1.00 (reference)	1.00 (reference)
	Below median (≤286.3 pmol/L)	33	25	2.36 (1.02-5.44)	2.48 (1.02-6.01)
Methylmalonic acid[c]	Below median (≤0.32 μmol/L)	16	25	1.00 (reference)	1.00 (reference)
	Above median (>0.32 μmol/L)	31	25	1.94 (0.85-4.40)	3.65 (1.21-11.05)
Homocysteine[c]	Below median (≤9.6 μmol/L)	18	25	1.00 (reference)	1.00 (reference)
	Above median (>9.6 μmol/L)	29	25	1.61 (0.72-3.62)	2.08 (0.73-5.90)
Folate[d]	Above median (>12.9 pmol/L)	10	21	1.00 (reference)	1.00 (reference)
	Below median (≤12.9 pmol/L)	19	20	2.00 (0.75-5.32)	2.87 (0.72-11.46)

[a]Covariates in logistic regression model include age of mother, rural versus urban residence, and breastmilk feeding or expression at the time of blood collection (yes vs no).
[b]B_{12}-ECL: Electrochemiluminescence method determination (Elecsys 2010; Duggan et al., 2014).
[c]MMA: Gas chromatography-mass spectrometry method for methylmalonic acid determination (Varian 3800; Duggan et al., 2014).
[d]F-MB: Microbiological method for plasma folate determination (Molloy & Scott, 1997); includes fewer 9 participants due to limitations of blood sample volumes.

Table 4. Relative Odds (Odds Ratios and 95% CIs) Estimates of Associations Between Nutrient Biomarker Levels and Mothers of Orofacial Cleft Children (Isolated Cleft Lip With or Without Cleft Palate) by Combinations of High and Low Levels of Maternal Plasma Vitamin B_{12} and Methylmalonic Acid: Chennai, Tamil Nadu, India, Orofacial Cleft Study.

Vitamin B_{12}[b] level	Methylmalonic acid[c] level	Number of cases	Number of controls	Unadjusted odds ratio (95% CI)	Adjusted[a] odds ratio (95% CI)
Lower risk (> median, 286.3 pmol/L)	Lower risk (≤ median, 0.32 μmol/L)	8	14	1.0 (reference)	1.00 (reference)
Lower risk (> median, 286.3 pmol/L)	Higher risk (> median, 0.32 μmol/L)	6	11	0.96 (0.26-3.56)	0.90 (0.17-4.80)
Higher risk (≤ median, 286.3 mol/L)	Lower risk (≤ median, 0.32 μmol/L)	8	11	1.27 (0.36-4.48)	0.74 (0.15-3.48)
Higher risk (≤ median, 286.3 mol/L)	Higher risk (> median, 0.32 μmol/L)	25	14	3.13 (1.05-9.27)	6.54 (1.33-32.09)

[a]Covariates in logistic regression model include age of mother, rural versus urban residence, and breastmilk feeding or expression at the time of blood collection (yes vs no).
[b]B_{12}-ECL: Electrochemiluminescence method determination (Elecsys 2010; Duggan et al., 2014).
[c]MMA: Gas chromatography-mass spectrometry method for methylmalonic acid determination (Varian 3800; Duggan et al., 2014).

Discussion

Mothers of CL ± P children in the Tamil Nadu study in southern India were more likely to have poor vitamin B_{12} status, defined by lower maternal plasma vitamin B_{12} and elevated plasma MMA levels. An analysis with a more precise definition poor vitamin B_{12} status using a combination of plasma vitamin B_{12} and MMA levels revealed an even stronger association with poor vitamin B_{12} status.

The possible role of vitamin B_{12} in OFC prevention has received much less attention than folate, though both are interrelated cofactors in 1-carbon metabolism. Both are required for the remethylation of tHcy to form methionine, which is important for the methylation of DNA, RNA, and histone proteins involved in the epigenetic regulation of gene expression and for the synthesis of neurotransmitters, phosphatidylcholine, and other small molecules important in fetal development (Bailey et al., 2015). In vitamin B_{12} deficiency, folate is "trapped" in the unusable methyl form, resulting in perturbations of thymidine synthesis and DNA replication, resulting in genomic instability (Herbert & Zalusky 1962; Green et al., 2017). Vitamin B_{12} is an essential cofactor of methyl-malonyl CoA mutase in mitochondrial metabolism; thus, B_{12} deficiency results in elevated MMA, which disrupts mitochondrial function leading to elevated levels of inflammatory cytokines and generation of excess levels of reactive oxygen species (Fenech, 2012). The precise role of these or other molecular mechanisms involving vitamin B_{12} in the disruption of development and formation of OFCs is not known.

Experimental animal and cell culture models of OFCs induced by cortisone, retinoic acid, and dexamethasone have shown that vitamin B_{12} reduces the occurrence of chemically induced OFCs (Mann & Gautieri, 1973; He et al., 2010; Zhang et al., 2011). The relevance of these models for humans is uncertain as these studies used different methods, chemical exposures for OFC induction, and doses of vitamin B_{12}.

Studies of maternal vitamin B_{12} status and OFCs in humans are few in number with varied methods and inconsistent results. A case–control study in California found an increased risk of OFCs among mothers in the lowest quartile of self-reported dietary intake of vitamin B_{12}, but only among nonusers of vitamin supplements, and there was no association among supplement users (Wallenstein et al., 2013). A case–control study in the Netherlands initially found a lower mean maternal serum vitamin B_{12} level in case-mothers compared to control-controls (van Rooij et al., 2003), but a later report from the same study found a less significant difference (Vujkovic et al., 2010). A nested case–control study in California of midpregnancy serum vitamin B_{12} found no association between vitamin B_{12} level and risk of OFCs (Shaw et al., 2009). An Irish prospective study of maternal serum samples collected at 15 weeks' gestation found significantly higher vitamin B_{12} levels among mothers later giving birth to children with OFCs compared to controls (Sutton et al., 2011). A meta-analysis of 7 studies that collected blood specimens from case-mothers and control-mothers, all from North American and European populations with generally more sufficient vitamin B_{12} levels compared to South Asian populations, found no overall association with measured vitamin B_{12} level; however, the heterogeneity between studies in timing of blood collection, laboratory methods for analyses, ethnicity, and the nutritional backgrounds of the study populations limited the conclusions of this meta-analysis (Blanco et al., 2016). Biomarkers measured in samples taken during pregnancy are affected by pregnancy-associated hemodilution (plasma volume expansion), changes in renal function, and hormonal changes, with large interindividual and interpopulation variations (Faupel-Badger et al., 2007); thus, blood samples collected during pregnancy may not accurately reflect maternal nutritional status at the time of conception and during OFC formation in the earliest weeks of pregnancy. Blood samples in the Tamil Nadu study were collected, with a mean of 1.4 years after delivery. We are not aware of other biomarker studies of vitamin B_{12} and risk of OFCs in India or other South Asian populations with dietary patterns quite different than North American and European populations.

Plasma folate was not significantly associated with CL $\pm$ P in the present study, though larger studies may indeed show associations. A systematic review and meta-analysis that reviewed studies of dietary folate intake, folate food fortification, and genetic and biomarker indicators of folate status suggested no association between these measures and OFC risk, though substantial heterogeneity between studies was cited as a limitation (Johnson & Little, 2008). A Cochrane systematic review found no evidence from folate intervention trials of a preventive effect on OFCs, though the trials were considered low-quality evidence-based on design issues and limited power to detect OFC outcomes (De-Regil et al., 2015).

The meta-analysis by Blanco et al cited previously included 10 case–control studies with measurement of plasma folate and found no overall association with OFC risk; the conclusions were limited by the same reasons cited previously (Blanco et al., 2016). We are not aware of other biomarker studies of folate and risk of OFCs in India or other South Asian populations.

Other nutrients required in 1-carbon metabolism have been associated with OFCs including vitamin B_6 (Davis et al., 1970; Munger et al., 2004; Tamura et al., 2007) and zinc (Hurley & Swenerton, 1966; Krapels et al., 2004; Tamura et al., 2005; Hozyasz et al., 2009). A case–control study in the Philippines found that poor plasma vitamin B_6 and zinc levels, cofactors with both folate and vitamin B_{12} in 1-carbon metabolism, were each very common and each strongly associated with OFC risk. Plasma folate was inconsistently associated with OFCs in the Philippines due to an interaction with vitamin B_6 status (Munger et al., 2004). In a Utah case–control study, very few deficiencies in vitamin B_6 and zinc were found and hence neither were associated with OFC risk (Munger et al., 2009; Munger et al., 2011). These findings indicate that studies in populations with diverse dietary backgrounds and employing a wider variety of biomarkers of nutrients involved in 1-carbon metabolism and that their interactions will be important to furthering understanding of maternal nutrition and risk of OFCs.

A limitation of the Tamil Nadu study is that it was retrospective without observation of diet and maternal nutrient biomarkers at conception and in the period of lip and palate development early in the first trimester. Prospective cohort studies of birth defects would allow tracking of maternal nutritional status before and during pregnancy but would require very large sample sizes of observed pregnancies to observe an adequate number of affected infants; thus, case–control studies are a more practical study design, but not without

limitations. Our approach to nutrient biomarker analyses in the Tamil Nadu case–control study was based on the premise that maternal dietary habits and social environments in this setting are relatively stable before and after pregnancy and genetic and epigenetic factors that influence blood biomarkers levels are fixed. Prospective studies have provided evidence that dietary patterns are stable between preconceptional and postpartum periods (Devine et al., 2000; Cuco et al., 2006). Leck et al. (1983), in their early studies of NTDs in North London, found significant correlations between blood folate levels measured early in pregnancy and measured in blood samples collected from mothers 1 to 2 years after delivery and concluded that such studies of maternal samples collected after delivery "are well worth pursuing as a possible means of identifying associations of etiological significance between maternal nutritional status and malformations." In other studies of OFCs that have used this approach, maternal biomarker associations appear to have persisted for years after the affected birth (van Rooij et al., 2003; Munger et al., 2004; Tamura et al., 2005; Tamura et al., 2007; Munger et al., 2011).

A description of dose–response relationships between nutrient biomarkers and CL ± P risk was not possible due to the restricted sample size. To maximize the statistical power of comparisons, "higher" and "lower" biomarker groups were defined by median levels of biomarkers. Published standards for defining marginal levels of nutritional biomarkers have not been based on OFC risk nor are they specific for India, but they are useful to provide evidence of a generally poor status for nutrients related to 1-carbon metabolism in the Tamil Nadu area. Marginal plasma vitamin B_{12} levels defined as <221 pmol/L (Green et al., 2017) were found in 45.8% and 24.0% of cases and controls, respectively. The high prevalence of marginal plasma vitamin B_{12} status observed among Tamil Nadu control women is uncommon in North American and European populations but is common and even higher in many South Asian, African, and Latin American populations. Marginal vitamin B_{12} status defined by plasma MMA > 27 µmol/L (Green et al., 2017) was found in 76.6% of cases and 80.0% of controls. Marginal plasma folate defined as <10 nmol/L (Bailey et al., 2015) was found in 29.2% and 33.3% of cases and controls, respectively. Elevated tHcy defined as >8.0 µmol/L (Green et al., 2017) was found in 79.2% of cases and 86.0% of controls.

The validation studies that compared blinded assays of plasma vitamin B_{12} and folate from 2 independent laboratories using different analytical methods added confidence to the accuracy of the overall results. The 2 methods of assaying plasma vitamin B_{12} each showed close agreement to each other and both had similar correlations with MMA and tHcy, and no significant correlations with folate. The 2 methods of assaying folate status showed close agreement to each other and similar correlations with tHcy and no significant correlations with vitamin B_{12} or MMA. Validation studies are important to include in future publications as it has been shown that assay variations between laboratories affect the results of population-based studies, and to ensure greater comparability between studies, assay harmonization is needed (Pfeiffer et al., 2011).

In developing countries such as India, BF at the time of maternal blood sample collection may result in nutrient biomarker levels different than NF mothers (Jathar et al., 1970; Obeid et al., 2017). However in the better nourished populations of Europe and North America, there may be less of an effect of BF on blood nutrient levels. Longitudinal studies among women in Denmark found no significant changes in maternal serum cobalamins between 3 weeks and 9 months postpartum, and the means for the lactating women were similar to reference levels for nonlactating women (Ramlau-Hansen et al., 2006; Morkbak et al., 2007). We found that plasma B_{12} was lower and MMA was higher in BF versus NBF mothers in both case-mothers and control-mothers when these groups were examined separately. In analyses that were restricted to NBF mothers only compared to the total sample, the OFC association with low B_{12} was similar, association with high MMA was higher, and the association with the joint level of low B_{12} and high MMA among NBF only was also higher. These additional subgroup analyses of NBF mothers add confidence to the overall findings, but the precision of the ORs estimated was limited by the smaller sample size. A further limitation of our study was a lack of information on the duration of BF immediately preceding blood collection. Future studies should obtain more complete data on BF, especially in the setting of developing countries, and include BF as a covariate in analyses when there are differences in BF prevalence between the groups compared.

The role of maternal nutrition in OFCs in India is of global public health importance because of India's large and diverse populations, widespread marginal nutritional status of women of reproductive age (Kumar et al., 2017), and dietary patterns that are quite different from North American and European populations. Diets with low intakes of animal foods are common in Tamil Nadu and other states in India, resulting in a high prevalence of vitamin B_{12} deficiency, which has been linked to many adverse reproductive health outcomes including spontaneous abortion, intrauterine growth retardation, fetal adiposity, insulin resistance, and gestational diabetes (Muthayya et al., 2006; Yajnik et al., 2008; Krishnaveni et al., 2009; Katre et al., 2010). In a review of worldwide studies of the prevalence of marginal vitamin B_{12} levels, the South Asian populations were among the highest, ranging between 27% and 72% (Green et al., 2017). Several studies among women of reproductive age in India have found a relatively high prevalence of vitamin B_{12} deficiency combined with a low prevalence of folate deficiency, unlike trends in Western populations with much higher intakes of animal foods and lower intakes of vegetable foods (Pathak et al., 2007; Yajnik et al., 2008; Bansal et al., 2016; Sivaprasad et al., 2016). Iron-folate supplements are routinely distributed to pregnant women in India for the control of anemia, and a concern has emerged that elevated folate levels in the presence of low vitamin B_{12} levels may be associated with adverse pregnancy outcomes including increased risk of obesity, insulin resistance, and diabetes (Rosenberg, 2008; Yajnik

et al., 2008; Paul & Selhub, 2017); however, this is still somewhat controversial and requires further study.

Additional studies of maternal nutritional biomarkers in a variety of settings and using standardized laboratory methods would give a clearer view of associations between nutrients involved in 1-carbon metabolism and risk of OFCs. Further studies are also needed of specific molecular forms of folate. Blood folate concentrations in persons with vitamin B_{12} deficiency cannot be assumed to mean that their functional folate status is adequate. In the presence of vitamin B_{12} deficiency, folate may be "trapped" in the unusable methyl form (Herbert & Zalusky, 1962; Green et al., 2017), and description of the distribution of various folate molecular forms may provide additional insight into impaired folate metabolism.

In conclusion, the Tamil Nadu OFC study found that low maternal vitamin B_{12} status is widespread and approximately 6.5 times more likely among mothers of CL $\pm$ P children compared to control-mothers. The potential role of improved vitamin B_{12} status for the prevention of OFCs is of considerable interest among the large populations of India and the broader South Asia region with low intakes of animal foods containing vitamin B_{12}, an area that includes nearly one-fourth of the world's population (The_World_Bank, 2017). Further studies of dietary practices and assessment with multiple biomarkers of nutrients involved in 1-carbon metabolism in populations with diverse nutritional backgrounds are needed to establish whether there is a firm causal link between abnormal 1-carbon metabolism and risk of OFCs.

Authors' Note

RM, PM, JM, and KB designed research and provided oversight. JM and PM provided clinical expertise. RK, GT, and SS conducted research. AK, AM, and PMU conducted laboratory analyses. RM, RK, and GT analyzed data and performed statistical analysis. RM, RK, PM, AM, and PMU contributed to writing the manuscript. RM had primary responsibility for final content. All authors have read and approved the final manuscript.

The findings from this manuscript were presented in an oral presentation by Dr Munger at the 75th Annual Meeting of the American Cleft Palate-Craniofacial Association on April 13, 2018, in Concurrent Session 6: Bench to Bedside. (Abstract 482).

Acknowledgments

This publication is dedicated to the memory of our distinguished colleague Professor Jyotsna Murthy. Prof Murthy transformed the lives of many in unprecedented ways. Her extraordinary surgical skills brought back smiles on thousands of faces as she painstakingly repaired the physical damage caused by cleft disorders and rehabilitated patients and families with her boundless energy and infinite levels of compassion. As she led this crusade, she built an army of trained clinicians and students to ensure enduring impacts from the SmileTrain program that she anchored in India. She also had an insatiable thirst for scientific inquiry to understand the etiology and prevention of cleft disorders and created unique multidisciplinary research collaborations across the United States, the United Kingdom, and India. Selflessness defined her contributions to society and she will continue to be an inspiration for everyone who crossed her path.

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