High folate and low vitamin B-12 intakes during pregnancy are associated with small-for-gestational age infants in South Indian women: a prospective observational cohort study[1–4]

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ABSTRACT

Background: Folic acid supplementation in those with a low vitamin B-12 intake or status may have adverse effects. These effects are unknown with regard to birth outcome in pregnant Indian women who are routinely supplemented with high doses of folic acid.

Objective: The objective was to examine the association of unbalanced vitamin B-12 and total folate (folic acid supplement + dietary folate) intakes during pregnancy with outcomes in small-for-gestational-age (SGA) infants.

Design: This was a prospective observational cohort study of 1838 pregnant women in South India. Low intake of dietary vitamin B-12 in the presence of high total folate intake was examined as the ratio of vitamin B-12 intake to total folate intake.

Results: The inadequacy of vitamin B-12 intake (<1.2 μg/d) assessed by a food-frequency questionnaire in the first, second, and third trimesters of pregnancy was 25%, 11%, and 10%, respectively. Multivariate log binomial regression showed that low vitamin B-12 and folate intakes in the first trimester were independently associated with a higher risk of SGA. In a subgroup of women with high supplemental folic acid intakes in the second trimester, those with the lowest tertile of vitamin B-12:folate ratio had a higher risk of SGA outcome than did those in the highest tertile (adjusted RR: 2.73; 95% CI: 1.17, 6.37). A similar trend was observed in the analysis of blood micronutrient status in a random subset (n = 316) of the sample.

Conclusions: These findings suggest that, in addition to vitamin B-12 and folate deficiencies alone, there may be adverse birth outcomes associated with unbalanced vitamin B-12 and folate intakes or status during pregnancy. These findings have important implications for the antenatal B vitamin supplementation policy in India. This trial was registered at the Clinical Trial Registry of India as 2013/07/005342. *Am J Clin Nutr* 2013;98:1450–8.

INTRODUCTION

Studies from ~50 y ago confirmed that folic acid supplementation prevents folate deficiency and megaloblastic anemia during pregnancy and resulted in the adoption of folic acid–supplementation policies for pregnant women in many countries (1, 2). In the 1990s, the Medical Research Council Vitamin Study and other analyses showed that periconceptional folic acid supplementation also significantly reduced the occurrence of fetal neural tube defects (NTDs[5]; 3–5). Low maternal folate status has also been shown to be related to low birth weight (1, 6), small-for-gestational-age (SGA) infants (7), and maternal preeclampsia (1). Because of such risks, the Indian Ministry of Health and Family Welfare mandates that all pregnant women be given folic acid supplementation (at a dose of 0.5 mg folic acid) along with iron daily for ≥100 d, starting at 14–16 wk of gestation, and double that dose in anemic women. However, pregnant women are often prescribed much higher doses of folic acid in the first trimester in India, probably linked to reports that women planning pregnancy should take 5 mg folic acid/d (8).

About one-third of Indian infants are born with low birth weight every year, accounting for 26% of the global burden; 60% of these infants are born at term after fetal growth restriction (9). These numbers may also be related to deficiencies in vitamin B-12, because low maternal vitamin B-12 status, in addition to being associated with the risk of fetal NTD (10), was also found to be strongly associated with intrauterine growth restriction in urban South Indians (11). Vitamin B-12 deficiency is especially a cause of concern in India because of the widespread predominance of vegetarianism (12). In a vegetarian diet excluding meat, eggs, and fish, dietary vitamin B-12 intake is virtually nonexistent, except for that coming from milk products (13). Apart from vegetarianism, tropical sprue, giardiasis, and other gastrointestinal infections may lead to malabsorptive states and hence vitamin B-12 deficiency (12). In one Western India study, it was found that vegetarians had a 4-fold higher risk of low vitamin

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[5] Abbreviations used: ARR, adjusted RR; FFQ, food-frequency questionnaire; NTD, neural tube defect; RDA, Recommended Dietary Allowance; SGA, small for gestational age; SJMCH, St John's Medical College Hospital.

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B-12 concentrations compared with those who frequently ate egg and meat products (14), and evidence suggests that vegetarians should take vitamin B-12 supplements (13). However, there is currently no policy in India that recommends vitamin B-12 supplementation during pregnancy, because it has been assumed that, in the Indian context, an improvement in diet quality can meet the requirement (15).

Enthusiastic folic acid supplementation for pregnant women, leading to high plasma folate, may exacerbate the effects of existing vitamin B-12 deficiency. This has been observed in relation to cognitive impairment in vitamin B-12–deficient elderly (16) and may be related to high concentrations of unmetabolized plasma folic acid (17). It is possible that similar adverse effects may occur in folic acid–supplemented pregnant women who may be vitamin B-12 deficient, but there is no literature on these effects during pregnancy.

The current analysis was therefore undertaken in a prospective maternal cohort in Bangalore, India, to examine independent and combined associations of vitamin B-12 and total folate (folic acid supplement + dietary folate) intakes during pregnancy with outcomes in SGA infants in a setting where routine antenatal supplementation of folic acid, but not vitamin B-12, is practiced.

SUBJECTS AND METHODS

The study was conducted at St John's Research Institute and St John's Medical College Hospital (SJMCH), Bangalore, India. This 1200-bed tertiary hospital draws patients of diverse socioeconomic status, from urban slums to high-income residential areas. The institutional ethical review board of SJMCH Bangalore approved all study procedures and a written and signed consent was obtained from each study participant at enrollment.

Study design

This was a prospective observational cohort study. All pregnant women aged 17–40 y and at <13 wk of gestation, registered for antenatal screening at the Department of Obstetrics and Gynecology at the SJMCH, were invited to participate in the study. As a tertiary referral hospital, pregnant women of the obstetrics clinic are a mix of normal and referral or complicated pregnancies who register at different times during the course of their pregnancy. Therefore, exclusion criteria—such as women with multiple fetuses; with a clinical diagnosis of chronic illness such as diabetes mellitus, hypertension, heart disease, and thyroid disease; with a positive test result for hepatitis B surface antigen, HIV, or syphilis infection (venereal disease); or who anticipated moving out of the city before delivery—were applied. Although this may have resulted in a sampling bias, our intention was to study apparently healthy pregnancies from the first trimester along with a careful characterization of the pregnancy outcome. All eligible women willing to participate were recruited as early in their pregnancy as possible and were followed until delivery. Sociodemographic details were collected at baseline (11.7 ± 2.8 wk of gestation). Information on maternal anthropometric measurements, dietary intake, clinical status, and routine antenatal blood biochemistry was collected at baseline and in the second trimester (24.3 ± 1.5 wk) and third trimester (34.0 ± 1.6 wk) of pregnancy. A cohort of 1838 women was recruited at the antenatal clinic over 10 y. Initially, 2558 women consented to participate and were recruited, of whom 429 were lost to follow-up. Common reasons for loss to follow-up were mostly independent of the study protocol, such as too far a distance to the hospital from the subjects' home or the Indian cultural practice of women in their third trimester visiting their native place for delivery. A sample size of 385 live births was sufficient to observe the crude odds ratio of 1.4 for SGA with respect to vitamin B-12 intake in the first trimester with 5% level of significance and 80% power. Similarly, a sample size of 133 subjects would be sufficient to observe a significant association of folate/vitamin B-12 status on SGA at the 5% level of significance and 80% power. All sample size calculations were performed by using nMaster software version 1.0 (Department of Biostatistics, Christian Medical College, Vellore, India; http://nmaster.cmc-biostatistics.ac.in/). Based on these calculations, the sample size available for the nutrient intake and status was adequate. Nevertheless, this is an ongoing cohort study, and the data presented are based on the final set of 2001 women recruited in the cohort. From the entire cohort, a subset of 316 subjects was randomly selected for whom the proportion of SGA was fairly similar to that of the entire cohort. The flow diagram for recruitment and follow-up is provided in **Figure 1**. All data were collected by trained research assistants.

Sociodemographic and anthropometric information

At the baseline visit, trained research assistants interviewed the study subjects to obtain information on age, obstetric history, and socioeconomic status. We have found education to be a good surrogate for socioeconomic status in this cohort (T Thomas, P Dwarkanath, A Thomas, S Bhat, AV Kurpad, unpublished observations, 2011), because income would not be accurately reported. The education level was classified into 3 grades: "up to high school, maximum 10 years of formal schooling," "Pre-University/Diploma, maximum 12 years of formal education," and "University and above." Gestational age (wk) was calculated from the reported first day of the last menstrual period and confirmed through ultrasonographic measurements. If there was a discrepancy of more than a week between the gestational age judged by the last menstrual period and ultrasonographic dating, the gestational age estimated by ultrasonography was used. A digital balance (Soehnle) was used to record the weights of all mothers to the nearest 100 g during each antenatal visit. The digital weighing scale was calibrated by using standard

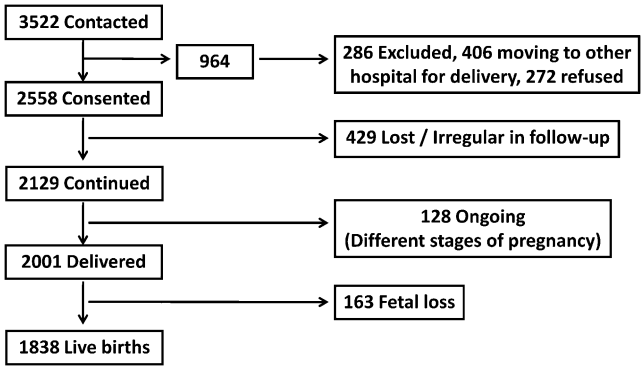

FIGURE 1. Flow diagram of recruitment and follow-up in the study cohort.

weights once every month. Height was measured with a stadiometer to the nearest 0.1 cm.

Antenatal care

Each participant was screened for routine antenatal tests (hepatitis B surface antigen, HIV, syphilis infection, and venereal disease) before enrolling in the study and was prescribed antenatal supplements of folic acid, iron, and calcium as per the antenatal schedule. At recruitment, subjects were prescribed 5 mg folic acid supplement/d until the end of the first trimester. This pattern of prescription is common in India, likely driven by the recommendation that women planning pregnancy should take 5 mg folic acid/d (8). However, this often continues into the pregnancy, where high doses of folic acid are still prescribed well after the neural tube has closed (18). Because mean gestational age at recruitment was 12 wk, which is toward the end of the first trimester, only 60% of the women reported having taken any folic acid supplements in the first trimester. From the beginning of the second trimester until delivery, 150 mg ferrous sulfate (equivalent to 45 mg elemental Fe) along with 0.5 mg folic acid/d was prescribed. In addition, during this period, the subjects were also prescribed 1000 mg Ca/d and 250 IU vitamin D_3. However, whereas 0.5 mg folic acid was prescribed in the second trimester, 47% ($n = 875$) of subjects continued with the 5 mg folic acid supplement/d for $\geq$1 wk in the second trimester. No other multivitamin or multimineral preparations were prescribed. Supplement compliance with these prescriptions was recorded during the course of pregnancy in the form of a tablet count, to sum up the daily total folic acid intake. Data regarding supplement compliance were collected by the research team, which was independent of the consulting clinicians.

Dietary intake: food-frequency questionnaire

A food-frequency questionnaire (FFQ) was administered at each trimester visit to obtain information on habitual dietary intake for the preceding 3 mo. The FFQ was adapted from one developed and validated for the urban low and middle class residing in South India (19) and administered by a trained research assistant. This questionnaire has a food list of 108 items, derived from a food database developed over a period of several years from studies at the Division of Nutrition, St John's Medical College. It consists of 4 frequency categories (daily, weekly, monthly, and yearly). Standard measures were placed before the respondent to quantify the portion size of each food item when administering the questionnaire. Recipes for the food items were tested in the laboratory and raw ingredients for each recipe were weighed, and volume to weight conversions measured for each cooked food item. Nutrient composition of the food item was calculated by using standard food conversion tables for the ingredients. Whenever available, Indian data were used (20). However, for some nutrients, for which Indian data were not available, USDA (21) data in the public domain were used. The nutrients and food groups were estimated for all the foods listed in the FFQ and summed to obtain the total nutrient or food group intake per day for an individual. Nutrient information was obtained for 27 macro- and micronutrients. Total folate intake for each subject was the sum of dietary folate intake and folic acid supplementation. The quantity of supplement consumed in each trimester was determined as the product of the dose of each supplement and the number of days taken, and an average daily consumption of folic acid in the trimester was computed. The average supplemented folic acid consumed per day in the trimester was converted into its dietary folate equivalent by a multiplicative factor of 1.7 (14), to adjust for the differential bioavailabilities of the 2 different sources, and added to the daily dietary folate intake to calculate total folate intake per day. This calculation was performed only for those subjects who provided compliance information on their supplement intake. For subjects who reported "no intake" of folic acid supplements, their dietary folate was considered as total folate intake.

Delivery and birth information

Delivery information was recorded from the medical chart. Anthropometric measurements of the infants included birth weight (g), length (cm), circumferences (midupper arm, head, and chest; cm), and skinfold thickness (biceps, triceps, subscapular, and suprailiac; mm). Infants were weighed to the nearest 10 g on an electronic weighing scale (Salter Housewares 914 Electronic Baby and Toddler Scale) immediately after birth. The outcome SGA was defined as a birth weight less than the 10th percentile for gestational age at delivery, and preterm birth was defined as delivery before 37 wk of gestation (22). The data for 1838 pregnant women with live births were considered for analysis in this study.

Blood sample collection

Blood samples were collected into EDTA-coated anticoagulant tubes (Becton Dickinson) and centrifuged at 4°C. The plasma was separated and stored at −80°C until analyzed. For red blood cell folate, the whole blood was treated with 1% ascorbic acid, and the hemolysate was stored at −80°C until analyzed. Red blood cell folate was measured by chemiluminescence (Bayer Diagnostics), with intra- and interassay variabilities of 1.9% and 5.2%, respectively. Red blood cell folate was then calculated from the measured folate and hematocrit values (23). Plasma vitamin B-12 concentrations were measured by the electrochemilumenesence method (Roche Diagnostics Mannheim), with intra- and interassay variabilities of 1.5% and 4.5%, respectively (24). Plasma total homocysteine concentrations were measured as methylchloroformate derivatives by gas chromatography–mass spectrometry (Varian), with intra- and interassay variabilities of $<$5% and $<$7%, respectively (25). Blood hemoglobin concentrations were analyzed by using an automated cyanmethemoglobin technique (ABX Pentra 60 C+ Hematology Analyzer; Horiba ABX Diagnostics). The measuring range was between 8 and 18 g/dL with a within run precision of $<$1.0%.

Statistical analysis

Continuous data are presented as means ± SDs or medians (quartile 1, quartile 3) and categorical data as number (%). The sociodemographic characteristics and dietary intakes of the subjects recruited in the study were compared with the subjects those were lost to follow-up during pregnancy. No significant differences in the first trimester were found between the 2 groups for energy, vitamin B-12, and folate intakes, indicating the representativeness of the study subjects. Nutrient intakes

from the FFQs at each trimester were adjusted for total energy intake and compared between the 3 trimesters by using repeated-measures ANOVA, and post hoc comparisons were made by using Bonferroni adjustment.

The association of tertiles of intake of vitamin B-12 and total folate in the first, second, and third trimesters with SGA outcome was examined by using log binomial regression. Potential confounders of the relation of SGA with intake were identified by using chi-square test and independent-sample *t* test. All variables that were significantly associated with SGA in the bivariate analysis at $P < 0.1$ were considered for multivariate analysis examining the association of dietary intakes. Multivariate log binomial regressions of SGA were performed with the tertiles of intake, adjusted for maternal age, education, parity, weight at recruitment, and energy intake in the trimester. The adjusted RRs (ARR) and 95% CIs are reported.

To understand the association of SGA with a low intake of vitamin B-12 in the presence of a high intake of folate, the ratio of vitamin B-12 to total folate intake (subsequently referred to as "ratio") in all trimesters was used. Because this ratio is a very small number, because the total folate intake is ~400 times that of vitamin B-12, it was multiplied by 1000. Because the interest was specifically in a low ratio arising from primarily a high folic acid intake, this ratio was examined in subgroups of subjects with high folic acid supplement intake. High folic acid intake for the subgroup analysis was defined as >1000 μg/d for the first and second trimesters (which is approximately thrice the recommended folic acid supplementation and was the median intake for these trimesters). In the third trimester, however, the average daily intakes of total folate and supplemented folic acid were much lower and close to the Recommended Dietary Allowance (RDA) of 500 μg/d. Therefore, the high folic acid subgroup analysis was not performed for the third trimester. Bivariate and multivariate log binomial regression analyses of SGA with tertiles of the ratio were performed in each trimester and within the high folic acid group. Separate analyses were performed for the first, second, and third trimesters. Log binomial regression of SGA with tertiles of plasma vitamin B-12 and red blood cell folate was also performed.

Two-sided *P* values <0.05 were considered statistically significant. All analyses were performed by using SAS (version 9.2; SAS Institute Inc). Log-binomial regression analysis was performed by using the PROC GENMOD program in SAS.

RESULTS

Baseline characteristics

The median age of the study participants (n = 1838) was 24 y. Pregnant women were recruited toward the end of the first trimester, at ~12 wk of gestation. Just >65% of the women had an education beyond high school, and ~59% of the subjects were pregnant for the first time. The mean weight and height of the subjects were 52.7 ± 9.5 kg and 1.55 ± 0.1 m, respectively; the BMI (in kg/m^2) was normal (21.8 ± 1.8) at recruitment. In all, 22% were anemic at recruitment (hemoglobin <11 g/dL). The average gestational age at birth was 38.6 ± 1.5 wk, and 52% of these were male infants. The incidences of SGA and preterm infants were 30% (n = 547) and 9% (n = 170), respectively. The mean birth weight of all newborns was 2861 g and that of SGA infants was 2523 g. Maternal education, height, and weight in first trimester were significantly associated with SGA at $P < 0.05$ (**Table 1**).

Micronutrient intake analysis

The mean micronutrient intakes in all 3 trimesters are presented elsewhere (*see* Supplemental Table 1 under "Supplemental data" in the online issue). About 10% of the subjects did not consume any nonvegetarian food items (meat, fish, poultry, and egg) during pregnancy, and the median consumption for those who consumed nonvegetarian food was 50 g/d (quartile 1 = 34, quartile 3 = 71 g/d). There was a significant increase in consumption of the nutrients and food groups from the first to the second and third trimesters (repeated-measures ANOVA, $P < 0.05$). Inadequacies in dietary vitamin B-12 intake based on the Indian RDA in pregnancy (14) of 1.2 μg/d were 25%, 11%, and 10% in first, second, and third trimesters, respectively. The RDA was used as a cutoff to define inadequate intake because the Indian dietary allowance reference does not provide an estimated average requirement (14). The reduction in inadequacy as pregnancy progressed corresponds with the increase in intake of milk from first to third trimester, because milk is a major source of vitamin B-12 in this population. A small but significant increase in nonvegetarian food intake was also found. Inadequacies in total folate intake based on the Indian RDA in pregnancy (14) of 500 μg/d were 30%, 0.2%, and 5% for first, second, and third trimesters. Calculated daily total folate intakes varied because compliance for taking supplements, recorded by

TABLE 1
Maternal and newborn characteristics of subjects by SGA and non-SGA infant status[1]

Parameters	SGA (n = 547)	Non-SGA (n = 1291)
Maternal age (y)	24.0 ± 3.7[2]	24.7 ± 4.0
Parity [n (%)][3]		
Primiparous	345 (63.1)	753 (58.3)
Multiparous		
1 or 2	184 (33.6)	491 (38.1)
3–5	16 (2.9)	46 (3.6)
Maternal education [n (%)]		
Up to high school	231 (42.2)	407 (31.5)[4]
Pre-university/diploma	143 (26.2)	328 (25.4[4]
University and above	173 (31.6)	556 (43.1)[4]
Maternal weight at recruitment (kg)	50.3 ± 9.2	53.7 ± 9.5[4]
Maternal height (m)	1.54 ± 0.06	1.56 ± 0.06[4]
Gestational age at recruitment by last menstrual period (wk)	12.0 ± 2.7	11.7 ± 2.7
Newborn gestational age at birth (wk)	39.0 ± 1.0	38.5 ± 1.7
Newborn sex, male [n (%)]	307 (56.1)	649 (50.3)
Newborn birth weight (g)	2523 ± 242	3006 ± 449[4]
Newborn birth length (cm)	48.8 ± 2.0[5]	49.8 ± 2.3[4,6]

[1] SGA, small for gestational age.
[2] Mean ± SD (all such values).
[3] Data are missing for 2 subjects in the SGA group and for 1 subject in the non-SGA group.
[4] Significantly different from SGA, $P < 0.05$ (independent-sample *t* test for continuous data and chi-square test for categorical data).
[5] n = 399.
[6] n = 933.

tablet count for each previous month, differed; 93%, 7%, and 17% of women were noncompliant (not taking tablets for ≥80% of the prescribed days) in the first, second, and third trimesters, respectively. A common reported reason for noncompliance was gastric discomfort.

Association of vitamin B-12 and total folate intake and their ratio with SGA in the entire group

Log binomial regressions of SGA were performed with tertiles of intake of total folate, vitamin B-12, and their ratio for each trimester. The third tertile (or highest intake group) was considered the reference category for the computation of ARR for these analyses. For vitamin B-12 in the first trimester, the lowest intake tertile of vitamin B-12 had a significantly higher ARR (1.20; 95% CI: 1.01, 1.43) for SGA. The median intake in this tertile was lower (1.14 μg/d) than the Indian RDA; however, in the other tertiles the intake was well above the RDA (**Table 2**). However, no significant association of vitamin B-12 intake with SGA was found in the second (**Table 3**) and third trimesters (data not shown). Similarly for total folate, a low intake was associated with SGA in the first trimester (ARR: 1.22; 95% CI: 1.02, 1.47; Table 2) but not later during pregnancy. The median vitamin B-12 intake and total folate intake in the lowest tertiles were below the Indian RDA; however, the intake was above the Indian RDA in the other tertiles (Tables 2 and 3). In the third trimester, the median (quartile 1, quartile 3) intakes of total folate were 718 (557, 830), 951 (920, 982), and 1102 (1052, 1176) μg/d and of vitamin B-12 were 1.15 (1.13, 1.71), 2.38 (2.08, 2.63), and 3.88 (3.26, 5.26) within the first, second, and third tertiles of folate and vitamin B-12 intakes, respectively.

Whole cohort analyses of the ratio, in the first trimester alone, showed that the subjects in the second tertile had a significantly lower risk of SGA in comparison with the reference third tertile (ARR: 0.77; 95% CI: 0.64, 0.92; Table 2). This was because the median vitamin B-12 intake in that particular tertile of the ratio was about twice the RDA along with a high folate intake (Table 2). On the other hand, the ARR of the ratio for SGA was not significant in the second and third trimesters (Table 3). The latter is likely because either a high folate or a low vitamin B-12 intake, or both, can create a low ratio. Therefore, low ratios caused specifically by a high folic acid intake were examined (*see* below).

Association of the vitamin B-12:folate ratio with SGA in the high folic acid supplement intake subgroup

To examine the association of vitamin B-12 intake with SGA, specifically at a high level of folate intake, log binomial regression analyses relating the ratio to SGA were performed within subgroups of subjects with a high folic acid supplement intake in each trimester. Subjects were identified as belonging to the high folic acid intake group, as described above. Within these subgroups, the tertiles of ratios as defined above were examined.

No association of the ratio in the first trimester with SGA was found. In the second trimester, vitamin B-12 intake increased 2-fold between tertiles of the ratio, in contrast with the folate intake, which was high and comparable between the tertiles. In this trimester, those in the first and second tertiles had a significantly higher risk of SGA (ARR tertile 1: 2.73, 95% CI: 1.17, 6.37; ARR tertile 2, 2.68, 95% CI: 1.13, 6.36) than did those in the third tertile. The median ratio in the third (reference) tertile was 2.27, which is comparable with a reference intake ratio of 2.9 obtained from the ratio of the Indian RDA of vitamin B-12 and folate for pregnant women (Table 3). As previously stated, in the third trimester—because of a very low intake of folic acid supplements—only 5% of subjects took a folic acid supplement

TABLE 2
RR of SGA infants based on total vitamin B-12 intake, total folate intake, and the ratio of vitamin B-12 to folate intake in the first trimester[1]

	Tertile 1	Tertile 2	Tertile 3
Vitamin B-12 intake[2]			
Median (IQR) intake (μg/d)	1.14 (0.80, 1.58)	1.71 (1.30, 2.10)	2.92 (2.27, 4.01)
Univariate RR (95% CI)[3]	1.24 (1.04, 1.48)	1.12 (0.93, 1.34)	1
Adjusted RR (95% CI)[3]	1.20 (1.01, 1.43)	1.09 (0.91, 1.30)	1
Total folate intake[4]			
Median (IQR) intake (μg/d)	271 (215, 323)	1968 (1461, 2273)	3625 (3293, 4225)
Univariate RR (95% CI)[3]	1.35 (1.08, 1.68)	1.15 (0.91, 1.44)	1
Adjusted RR (95% CI)[3]	1.22 (1.02, 1.47)	1.08 (0.89, 1.31)	1
(Vitamin B-12:total folate intakes) × 1000[5]			
Median vitamin B-12 intake (μg/d)	1.35	2.32	1.85
Median folate intake (μg/d)	3471	2203	282
Median (IQR) ratio value	0.44 (0.31, 0.57)	1.16 (0.87, 1.82)	6.39 (4.62, 8.62)
Univariate RR (95% CI)[3]	0.86 (0.70, 1.06)	0.74 (0.59, 0.92)	1
Adjusted RR (95% CI)[3]	0.92 (0.78, 1.10)	0.77 (0.64, 0.92)	1

[1] Adjusted RR from a log binomial regression model adjusted for maternal age, education, parity, weight at recruitment, and energy intake in the first trimester. SGA, small for gestational age.

[2] For tertiles 1, 2, and 3, n = 611, 612, and 612, respectively, and the SGA percentages are 36.3%, 25.7%, and 22.7%, respectively.

[3] RR at each level of the variable significant, $P < 0.05$.

[4] For tertiles 1, 2, and 3, n = 542, 541, and 543, respectively, and the SGA percentages are 34.5%, 29.4%, and 25.6%, respectively.

[5] For tertiles 1, 2, and 3, n = 542, 542, and 542, respectively, and the SGA percentages are 29.7%, 25.3%, and 34.4%, respectively.

TABLE 3
RR of SGA infants based on total vitamin B-12 intake, total folate intake, and the ratio of vitamin B-12 to folate in the second trimester[1]

	Tertile 1	Tertile 2	Tertile 3
Vitamin B-12 intake[2]			
Median (IQR) intake (μg/d)	1.45 (1.11, 1.76)	2.19 (1.89, 2.53)	3.66 (3.03, 4.63)
Univariate RR (95% CI)[3]	1.30 (1.05, 1.60)	1.06 (0.84, 1.33)	1
Adjusted RR (95% CI)[4]	1.20 (0.97, 1.48)	0.98 (0.79, 1.23)	1
Total folate intake[5]			
Median (IQR) intake (μg/d)	1183 (1061, 1290)	1740 (1525, 1991)	3082 (2704, 3789)
Univariate RR (95% CI)	1.00 (0.81, 1.24)	0.99 (0.80, 1.22)	1
Adjusted RR (95% CI)	1.05 (0.85, 1.30)	1.01 (0.82, 1.25)	1
(Vitamin B-12:total folate intakes) $\times$ 1000			
All subjects[6]			
Median intake (μg/d)			
Vitamin B-12	1.58	2.12	3.28
Folate	2768	1646	1320
Median (IQR) ratio value	0.61 (0.45, 0.74)	1.26 (1.10, 1.43)	2.36 (1.92, 3.08)
Univariate RR (95% CI)[3]	1.27 (1.02, 1.57)	1.14 (0.91, 1.43)	1
Adjusted RR (95% CI)[4]	1.14 (0.92, 1.42)	1.08 (0.86, 1.35)	1
Subjects with high folic acid supplement intake (>1000 μg/d)[7]			
Median intake (μg/d)			
Vitamin B-12	1.76	3.15	6.24
Folate	3076	2685	2561
Median (IQR) ratio value	0.58 (0.42, 0.72)	1.19 (1.02,1.34)	2.27 (1.83, 3.02)
Univariate RR (95% CI)[3]	2.67 (1.15, 6.17)	2.36 (0.99, 5.62)	1
Adjusted RR (95% CI)[3]	2.73 (1.17, 6.37)	2.68 (1.13, 6.36)	1

[1] Adjusted RR from a log binomial regression model adjusted for maternal age, education, parity, weight at recruitment, and energy intake in the second trimester. SGA, small for gestational age.
[2] For tertiles 1, 2, and 3, n = 406, 405, and 408, respectively, and the SGA percentages are 33.7%, 27.5%, and 26.0%, respectively.
[3] RR at each level of the variable significant, $P < 0.05$.
[4] Overall significant association of the variable, $P < 0.05$.
[5] For tertiles 1, 2, and 3, n = 402, 404, and 402, respectively, and the SGA percentages are 29.4%, 29.0%, and 29.4%, respectively.
[6] For tertiles 1, 2, and 3, n = 401, 403, and 402, respectively, and the SGA percentages are 32.2%, 29.3%, and 25.6%, respectively.
[7] For tertiles 1, 2, and 3, n = 315, 135, and 42, respectively, and the SGA percentages are 32.4%, 29.3%, and 25.6%, respectively.

>500 μg/d. Therefore, a meaningful analysis of risk of SGA could not be performed with reference to the high folic acid intake subgroup in this trimester.

In contrast, when the ratio was similarly examined in a subgroup of women with low vitamin B-12 intakes, no significant results were observed, which suggests that the risk of SGA increases only when the maternal vitamin B-12 intake is low in the presence of a high folic acid intake, particularly in the second trimester.

Association of micronutrient status with SGA

A weak but significant correlation was found between vitamin B-12 status (plasma concentration) and intake and between folate status and total folate intake (r = 0.16, $P < 0.01$ for vitamin B-12; r = 0.18, $P < 0.05$ for folate) in the 3 trimesters. In the first trimester, 32% of the subjects were vitamin B-12 deficient (<150 pmol/L) and 14% had low folate status (<283 nmol/L). A significant but low correlation was observed between hemoglobin and vitamin B-12 status in the second trimester (r = 0.18, $P < 0.05$), but not with folate status. Log binomial regression analysis of SGA with tertiles of vitamin B-12 status and folate status in first and second trimesters was examined (**Table 4**). The total number of subjects with vitamin B-12 and folate status in the third trimester was only 81; therefore, third trimester status was not considered for statistical analysis.

SGA was significantly negatively associated with vitamin B-12 status in the first and second trimesters. A similar significant negative association was seen with folate status in the second trimester (Table 4). The relation between low vitamin B-12 status and SGA in the presence of high folic acid supplement intake was examined within the previously defined subgroups of high folic acid intake in the first and second trimesters. Owing to the low numbers available (Table 4), vitamin B-12 status was not classified into tertiles; instead, it was considered as a continuous variable in this analysis. Because vitamin B-12 status was positively correlated with birth weight, its reciprocal was used to examine the increased risk of SGA with increasing value of vitamin B-12 status. In the high folic acid intake group, in the second trimester, vitamin B-12 status was significantly associated with SGA (Table 4), such that with every unit decrease in vitamin B-12 status, there was a 1% increased risk of having an SGA infant.

Homocysteine concentrations in the second trimester were compared between the low (<500 μg/d), medium (500–1000 μg/d), and high (>1000 μg/d) folic acid supplement intake groups. The median homocysteine values in the low, middle, and high folic acid groups were 7.0 (4.8, 10.6), 6.6 (5.0, 8.0), and 6.4 (4.6, 8.5) mmol/L, respectively. However, this decrease was not significant and was not associated with SGA.

DISCUSSION

The effort to reduce the rate of NTDs worldwide has resulted in an upswing in folate intake among pregnant women, unaccompanied by a parallel increase in the intake of other

TABLE 4
Association of plasma vitamin B-12 tertiles and red blood cell folate and percentage SGA in the first and second trimesters[1]

	Tertile 1	Tertile 2	Tertile 3
Trimester 1			
Plasma vitamin B-12 (pmol/L)[2]			
Median (IQR) vitamin B-12 status (pmol/L)	118.00 (86.37, 136.97)	186.00 (170.85, 202.51)	284.06 (253.00, 348.00)
Univariate RR (95% CI)[3]	1.31 (0.80, 2.13)	1.18 (0.71, 1.95)	1
Adjusted RR (95% CI)[4]	1.43 (1.02, 2.17)	1.34 (0.90, 1.99)	1
Red blood cell folate (nmol/L)[5]			
Median (IQR) red blood cell folate (nmol/L)	325.12 (127.82, 408.12)	582.90 (539.43, 640.50)	890.00 (805.03, 1025.00)
Univariate RR (95% CI)	1.38 (0.85, 2.24)	1.29 (0.78, 2.11)	1
Adjusted RR (95% CI)	1.30 (0.80, 1.92)	1.05 (0.72, 1.53)	1
Trimester 2			
All subjects[6]			
Median (IQR) vitamin B-12 status (pmol/L)	108.38 (90.15, 127.86)	172.00 (156.11, 192.75)	244.54 (230.17, 283.98)
Univariate RR (95% CI)[4]	1.55 (0.88, 2.72)	1.85 (1.07, 3.19)	1
Adjusted RR (95% CI)[3]	1.45 (0.92, 2.27)	1.52 (0.98, 2.35)	1
Red blood cell folate (nmol/L)[7]			
Median (IQR) red blood cell folate (nmol/L)	449.49 (342.91, 549.64)	725.95 (664.67, 776.90)	1027.84 (953.81, 1226.75)
Univariate RR (95% CI)[4]	1.94 (1.16, 3.24)	1.77 (1.05, 3.00)	1
Adjusted RR (95% CI)[3]	1.54 (0.97, 2.44)	1.61 (1.02, 2.52)	1
Subjects with high folic acid supplement intake (>1000 μg/d) (n = 121)			
Median (IQR) vitamin B-12 status (pmol/L)	175.57 (139.08, 224.46)		
Univariate RR of 1/vitamin B-12 status (95% CI)[3]	1.006 (0.999, 1.002)		
Adjusted RR of 1/vitamin B-12 status (95% CI)[3]	1.009 (1.001, 1.017)		

[1] Adjusted RR from a log binomial regression model adjusted for maternal age, education, parity, weight at recruitment, and energy intake in the corresponding trimester. SGA, small for gestational age.
[2] For tertiles 1, 2, and 3, n = 107, 103, and 103, respectively, and the SGA percentages are 35.5%, 32.0%, and 27.2%, respectively.
[3] Overall significant association of the variable, $P < 0.05$.
[4] RR at each level of the variable significant, $P < 0.05$.
[5] For tertiles 1, 2, and 3, n = 106, 105, and 105, respectively, and the SGA percentages are 36.8%, 34.3%, and 26.7%, respectively.
[6] For tertiles 1, 2, and 3, n = 96, 96, and 96, respectively, and the SGA percentages are 32.3%, 38.5%, and 20.8%, respectively.
[7] For tertiles 1, 2, and 3, n = 76, 77, and 76, respectively, and the SGA percentages are 40.8%, 37.3%, and 21.1%, respectively.

regulatory elements of the methionine cycle, such as vitamin B-12 (26), which is emerging at the forefront of pregnancy nutrition concerns linked to NTD, preeclampsia, placental abruption, pregnancy loss, hyperhomocysteinemia, or intrauterine growth restriction (9, 27). This study therefore set out to examine the importance of the intakes or plasma concentrations of vitamin B-12 and folate during pregnancy in relation to SGA and imbalances between these intakes, as a ratio. It appeared first that low vitamin B-12 and folate intakes, as well as blood concentrations, were associated with risk of SGA in early to midpregnancy and that early supplementation of both vitamins may be relevant in India. Second, however, it appeared that there was an additional risk of SGA in the presence of low vitamin B-12 and high folate intakes or a low vitamin B-12:folate ratio in the second trimester, which gives more credence to the wisdom of combined supplementation of these vitamins. This ratio analysis was performed specifically in a high folic acid intake subgroup, because a ratio could vary with its numerator, denominator, or both; the interest was specifically in SGA risk associated with low vitamin B-12 intake in the presence of high folic acid intake. It was possible to do this analysis because of the varying compliance of the pregnant women with their prescribed folic acid supplement intake. The low ratio was not simply a proxy for a low vitamin B-12 intake in this subgroup, because the increased ARR for SGA was observed only with a low vitamin B-12:folate ratio and not with a low vitamin B-12 intake alone in the second trimester. Although the first trimester analysis yielded no significant result in terms of the ratio, in the presence of high folic acid intake, this may have been because the duration of this exposure was small—for 4 wk at most. In addition, this study supports the institution of folic acid supplementation early in pregnancy, because low folate intakes were related to the risk of SGA, in agreement with an earlier report (6). Socioeconomic status was identified as a confounder in this association, but this was sufficiently adjusted for by including maternal education in the model. In addition, whereas the intake of vitamin B-12 through microbial sources (contamination) was possible, we could not adjust for this.

The association of low vitamin B-12 status could be compounded by an unbalanced high folic acid intake, and the current study showed this to be possible in the second trimester. Adverse effects of folate supplementation may be related to unmetabolized plasma folic acid rather than tetrahydrofolate, because plasma folic acid in the presence of low vitamin B-12 status has been related to poorer cognitive performance in the elderly (16). Furthermore, concentrations of homocysteine and methylmalonate are reported to increase with folate supplementation in those who are vitamin B-12 deficient (28), and a high folic acid intake (10 times the recommended intake) has been related to impaired gestational development in the rat fetus (29). Dihydrofolatereductase, which can be saturated by an excessive intake of folic acid and the appearance of unmetabolized folic acid in the plasma, occurs at relatively low dose intakes of >200 μg (30).

An independent association of low vitamin B-12 status with the risk of SGA is also possible, perhaps by increasing plasma

homocysteine concentrations (31). Although the association of a low vitamin B-12 intake and plasma concentration with SGA outcome was confirmed in this population, this relation was not strong or monotonic as reported before (10). This may have been because the odds in the earlier smaller study sample, which was a preliminary analysis of the first 150 women in this cohort, could have been unduly influenced by smaller variations in SGA occurrence. Vitamin B-12 deficiency can also act by impairing the methylation of myelin basic protein, which leads to neuropathy in the form of subacute combined degeneration of the spinal cord and peripheral nerves (32, 33). Maternal vitamin B-12 deficiency may also affect dendritic arborization and synaptic connectivity, which occur early in fetal development, and tissue concentrations of neurotransmitters (34). In a recent Indian study, lasting effects on cardiac autonomic function were observed in children born to vitamin B-12–deficient mothers (35). A low folate intake has also been shown to be related to SGA (6), as confirmed in the current analysis.

A lowered vitamin B-12 intake or an unbalanced vitamin B-12: folate ratio may act through a lower methionine synthase activity, which also leads to the folate trap. This could further affect DNA methylation by slowing the transmethylation rate in the methionine cycle and potentially, result in changes in methylation of key regions of the genome. However, this may only be relevant in severe vitamin B-12 deficiency, because methionine cycle flux measurements in mildly vitamin B-12–deficient, but folate-replete, pregnant Indian women, showed no reduction in the remethylation or transmethylation rate in the methionine cycle in the first and third trimesters (36). In addition, mathematical modeling of drivers of the methyl transfers also showed that simulating severe vitamin B-12 deficiency did not substantially change DNA methyltransferase activity (37). Nevertheless, these micronutrient imbalances are important, as borne out by the finding of global hypomethylation in rats with a vitamin B-12: folate ratio imbalance (38). In general, a balanced diet (and supplementation) is perhaps most likely to define a balanced fetal nutrient environment; for example, prudent diets (including more fruit, vegetables, and whole and unprocessed foods) in mothers have been shown to be associated with better bone mass accretion patterns in their offspring (39).

Strengths and limitations of this study

The strengths of the current study included the large sample size made possible by collecting 10 y of cohort data, the availability of a wide range of variables, and an analysis of the ratio of intake of vitamin B-12 to folate that allowed for comparison of the ratios among subgroups with different levels of folate. The limitations relate to the use of an FFQ for dietary intake measurements and the smaller sample size for biochemical estimations.

In conclusion, this analysis draws attention to the risk of SGA in pregnant Indian women first those with low vitamin B-12 and folate intakes and second those with unbalanced intakes of folate and vitamin B-12. While confirming the need for vitamin supplementation, it also suggests that a low vitamin B-12 intake in the presence of a high folate intake in the first and second trimesters is associated with an increased risk of SGA. To confirm the effects of a vitamin B-12:folate imbalance on birth outcome, it would be necessary to conduct a prospective controlled trial with vitamin B-12 supplementation during pregnancy, alongside the preexisting folic acid supplementation. However, defining the control arm in this kind of study raises ethical questions; furthermore, large sample sizes would be needed to perform subsample analyses in high, middle, and low folate intake groups. If these findings are confirmed, it might be time to consider either folic acid and vitamin B-12 supplementation through pregnancy or the education of women about the need for a better diversity of foods during maternity and of the public and health care personnel regarding optimal use of folic acid supplements during pregnancy. One way to do so in a largely vegetarian diet is by increasing the intake of milk and milk products; this may have additional beneficial effects related to the protein or other components in milk (40).

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