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Vitamin B-12 status in infancy is positively associated with development and cognitive functioning 5 y later in Nepalese children[1]

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ABSTRACT

Background: Poor vitamin B-12 (cobalamin) status is widespread in South Asia. Insufficient vitamin B-12 status has been linked to poor neurodevelopment in young children.

Objective: We measured the associations between vitamin B-12 status in infancy (2–12 mo) and the development and cognitive functioning in Nepalese children 5 y later.

Design: Vitamin B-12 status was assessed in infancy with the use of plasma cobalamin, total homocysteine (tHcy), and methylmalonic acid (MMA). At 5 y of age, we measured development with the use of the Ages and Stages Questionnaire, 3rd edition (ASQ-3), and cognitive functioning by using the Developmental Neuropsychological Assessment, 2nd edition (NEPSY II), in 320 children. In regression models, we estimated the associations between vitamin B-12 status, including a combined indicator of vitamin B-12 status (3cB12) and scores on the ASQ-3 and NEPSY II subtests.

Results: All markers of vitamin B-12 status with the exception of plasma cobalamin were significantly associated with the total ASQ-3 scores in the multiple regression models. A 1-unit increase in the 3cB12 score was associated with an increase in the total ASQ-3 score of 4.88 (95% CI: 2.09, 7.68; $P = 0.001$). Increases in both plasma tHcy and MMA (indicating poorer status) were associated with a decrease in scores on the NEPSY II affect recognition and geometric puzzle subtests. Each unit increment in 3cB12 scores was associated with increases of 0.82 (95% CI: 0.49, 1.14; $P < 0.0005$), 0.59 (95% CI: 0.10, 1.09; $P = 0.020$), and 0.24 (95% CI: 0.02, 0.47; $P = 0.035$) in the affect recognition, geometric puzzle, and block construction scores, respectively.

Conclusions: Vitamin B-12 status in infancy is associated with development and performance on social perception tasks and visuospatial abilities at 5 y of age. The long-term effects of poor vitamin B-12 status in infancy need further investigation in randomized controlled trials. *Am J Clin Nutr* 2017;105:1122–31.

Keywords: vitamin B12, neurodevelopment, children, cognition, low-income countries

INTRODUCTION

The number of children that fail to reach their developmental potential is large, and efforts to identify the specific causes of poor developmental outcomes are necessary (1). Deficits of key micronutrients early in life can lead to impairments in the central nervous system (2, 3), and vitamin B-12 (cobalamin) may be one such critical micronutrient (4, 5). Together with folate, vitamin B-12 is required for cell division (6). Vitamin B-12 is also important for intracellular energy production and the generation of methionine, which is needed to produce neurotransmitters and myelin (7). Early in life, vitamin B-12 deficiency is usually secondary to maternal deficiency (8). Insufficient maternal cobalamin status in pregnancy and during exclusive breastfeeding can put children at risk for deficiency in a critical period for the development of the central nervous system. In South Asia, the risk for cobalamin deficiency is high as a result of widespread poverty, low consumption of animal products because of cultural and religious reasons, and intestinal malfunction as a consequence of frequent gastrointestinal infections (8, 9). Studies in Nepal and India have revealed that poor cobalamin status is prevalent in both women and children in the general population (10–12).

Plasma or serum cobalamin concentration is the most commonly used marker for vitamin B-12 status. However, the functional markers total homocysteine (tHcy)[13] and methylmalonic acid (MMA) have been established as useful indicators

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[13] Abbreviations used: ASQ-3, Ages and Stages Questionnaire, 3rd edition; MMA, methylmalonic acid; NEPSY II, Developmental Neuropsychological Assessment, 2nd edition; tHcy, total homocysteine; 3cB12, combined indicator of vitamin B-12 status.

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of vitamin B-12 status (13) and may be more sensitive indicators of mild vitamin B-12 deficiency (14, 15). An increase in these markers may indicate that poor B-12 status has reached a level that has negative consequences for neurodevelopment (16, 17).

There are several studies that have linked vitamin B-12 status and neurodevelopment in children (17–21), including among young children in the general population in North India (17, 18) and in special groups such as young infants, with evidence of poor vitamin B-12 status referred to an outpatient clinic in Norway (19, 20). These studies do not, however, provide information of possible long-term effects of vitamin B-12 deficiency. Results from a previous Dutch study in adolescents raised on a vegan diet showed associations between early vitamin B-12 status and later cognitive performance (19, 21). This study has limited generalizability, however, given the small sample size and specific characteristics of the population.

In our study, we randomly selected children from Bhaktapur, Nepal, and measured vitamin B-12 status in infancy (2–12 mo) and the development and cognitive functioning with the use of performance on neuropsychological tests at ∼5 y of age. We hypothesized that the cobalamin markers would be associated with later developmental scores overall and—based on earlier findings—that specifically gross, fine, sensorimotor, and visuospatial functioning would be associated with early vitamin B-12 status. We included tests on executive functioning and social perception because these are considered sensitive measures in preschoolers.

METHODS

Participants

From January 2008 to February 2009, we enrolled 500 lactating women between 15 and 45 y of age and their infants from 2 to 12 mo of age from Bhaktapur, Nepal, in a cross-sectional survey on nutritional status and intake in the population. We used a 2-stage cluster sampling procedure whereby 66 neighborhoods (toles) were randomly selected as the primary sampling unit from a total of 160. We listed all women living in these toles and randomly selected women and infant pairs (11). The inclusion criteria for the study were that both mothers and children had no ongoing clinically assessed infections, resided in the selected clusters, were willing to provide their household information, and consented to participation. Details on the random selection procedure for the original study have been published elsewhere (11, 22). In 2012 and 2013, ∼5 y after the first study, we searched for and were able to locate 330 children of the initial 500 enrolled women-child pairs for follow-up developmental and neuropsychological assessments. The main constraint in identifying the children at 5 y was frequent migration because of political instability. In addition, we did not include measures in the original study to ensure long-term follow-up, such as alternative addresses, phone numbers, and contact information of relatives because the study was originally a cross-sectional design. A new written consent form from the mothers was collected for the follow-up assessments. The original study obtained ethical clearance from the institutional review board at the Institute of Medicine in Kathmandu, Nepal. The new ethical approval was obtained for the follow-up study from the same Nepalese review board as well as from the Regional Committee for Medical and Health Research Ethics in Norway.

Procedure

Anthropometric measures and biochemical markers at baseline

Upon selection at baseline, mothers were asked to bring their children to Siddhi Memorial Hospital for the administration of a household questionnaire, 24-h dietary recall, physical examination, anthropometric measurement, and blood collection. Information on the families and their socioeconomic situation was gathered in the household questionnaire as well as whether folic acid supplements were taken during pregnancy and for how many months. The length of the infants was measured with locally made wooden boards that were periodically calibrated. Approximately 3 mL whole blood was taken from the cubital vein with the use of polypropylene tubes with lithium heparin. We measured hemoglobin with the use of HemoCue (HemoCue Hb, 201+ system) immediately after blood collection. The HemoCue was calibrated on a regular basis. The samples were then centrifuged (760 × *g*; 10 min; room temperature), and plasma was allocated into polypropylene vials (Eppendorf). Samples were stored at −20°C at the field-site laboratory until they were transported with an ice pack to the central laboratory in Kathmandu at the end of each day. Samples were stored at −80°C until transported on dry ice to Norway. Blood samples were analyzed at Bevital in Bergen, Norway (www.bevital.no). Plasma vitamin B-12 concentrations were determined with the use of a microbiological assay based on the growth support of *Lactobacillus leichmannii* (23). These assays were adapted to a microtiter plate format and carried out by a robotic workstation; the within-day CVs were 5%. MMA and tHcy were analyzed with the use of gas chromatography–mass spectrometry based on methylchloroformate derivatization (24). A detailed description of the data collection, laboratory, technical equipment used, and treatment have been presented elsewhere (11).

Maternal dietary intake

Information on maternal dietary intake was collected through 3 repetitive 24-h recalls over 1 y to reflect seasonal dietary variations. Usual estimated energy intake was calculated with the use of the multiple source method (25), and Black's adaptation of the Goldberg approach was used to identify under- and over-reporters (26). A thorough description of the dietary methods has been published elsewhere (27).

Cognitive assessments

A local pediatrician and psychologist were trained to perform the assessments. The psychologist performed most of the assessments under close supervision. Assessments were conducted at the study clinic in a well-lit room free from distractions. The children were followed to the clinic by their caregivers, and the caregivers had the opportunity to sit in the back of the room during the assessments. The sessions lasted for ∼1 h.

The Ages and Stages Questionnaire, 3rd edition (ASQ-3), is a comprehensive developmental checklist standardized for children aged 1–66 mo with age-appropriate questionnaires (28). The questionnaires contain 30 items that sum ≤5 subscales—communication, gross motor, fine motor, problem solving, and

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personal-social (possible score range for each subscale: 0–60)—and a total score (total possible score range: 0–300). The ASQ-3 is designed to be answered by caregivers but can also be assessed directly with the child by a trained professional (29), as we did in this study. The examiner used a collection of standardized material (e.g., large and small balls, pens, paper, and scissors) in the assessments and completed the questionnaire based on observations of the child during the sessions and questions to the caregivers.

The 60-mo questionnaire (range: 57–66 mo) was used for this study. Of the 321 participants, 160 were aged >66 mo at the time of the assessment. We decided to perform the ASQ-3 for all children to secure a full assessment for the total sample. We translated, back-translated, and culturally adapted the questionnaire particularly for the Nepalese setting according to standardized procedures. The original and back-translated versions were then compared and discussed by the pediatrician and a neuropsychologist, and final adjustments were made. In the translated questionnaire, there was a strong correlation between the total ASQ-3 scores and the separate ASQ-3 subscale scores (range: 0.62–0.81). As expected, the correlations among the subscales were weaker (range: 0.12–0.49). The standardized α values for the 5 subscales ranged from 0.34 to 0.69. We identified 2 constant items that all children could perform; these items were in the gross motor subscale that assessed the ability to throw a small ball overhand while standing and in the problem-solving subscale that assessed the understanding of the concept of "smallest." These results were comparable to the results from a previous randomized control trial conducted in North India (29).

The Developmental Neuropsychological Assessment, 2nd edition (NEPSY II), is a comprehensive neuropsychological test battery that consists of 32 subtests in 6 functional domains for children aged 3–16 y (30). The battery is flexible and allows for individual administration. The following 6 age-appropriate subtests were administered in this study: inhibition, statue, visuomotor precision, affect recognition, geometric puzzles, and block construction. We used raw and scaled scores calculated based on US norms. The NEPSY II scaled scores have a total possible range from 1 to 19 (30). Local norms were not available. See **Table 1** for details of the domain and functions measured for each subtest.

Data management and statistics

All forms were manually checked for inconsistencies, and the data were entered twice. Infants' weight-for-age, height-for age, and weight-for-height z scores were calculated based on WHO growth standards (31). We used multiple linear and logistic regression models to estimate the association between vitamin B-12 status in infancy and the scores on the ASQ-3 and NEPSY II subtests. The ASQ-3 subscales were highly skewed, and we used logistic regression for the subscale analyses. We calculated a combined indicator of vitamin B-12 status (3cB12) based on the 3 biomarkers (cobalamin, MMA, and tHcy) according to the method suggested by Fedosov et al. (32) in which age and folate status are taken into account. Plasma log (base 2) cobalamin, tHcy, and MMA concentrations and the 3cB12 score were used as the main exposure variables in the different regression models. In the logistic regression models, the ASQ-3 scores were categorized on the 25th percentile. An OR >1 indicated increased odds of being in the lowest 25th percentile for each increment of 1 biomarker unit. Because of the lack of local norms for NEPSY II, we used raw scores in the regression models. We undertook the multiple regression analyses by adjusting for the variables listed in **Table 2**. Only variables that changed the estimates by >15% were included in the final models. We present both crude and adjusted estimates, and the variables included in the multiple regression models are shown in the tables. All models were adjusted for clustering. The effect estimates are expressed as linear regression coefficients and ORs, and $P \leq 0.05$ was considered to be statistically significant. Statistical analyses were performed with the use of Stata version 14 (StataCorp).

RESULTS

We included 321 children for the follow-up assessments. One child was excluded from the analysis because a medical condition

TABLE 1
Overview of the domains, NEPSY II subtests, and functions measured[1]

Domain	NEPSY II subtests	Functions measured
Attention and executive functioning	Inhibition	Ability to inhibit automatic responses in favor of a new one—in the naming condition the child names forms; in the inhibition condition the child is required to give the opposite name
	Statue	Assesses inhibitory control and motor persistence—the child maintains a position without being disturbed by distractions
Sensorimotor	Visuomotor precision	Fine motor ability and visuospatial integration—a timed task in which the child draws between 2 lines
Social perception	Affect recognition	Ability to recognize emotions in a matching task
Visuospatial processing	Block construction	Visuomotor and visuospatial abilities—the child produces 3-dimensional figures with the use of blocks from 2-dimensional drawings
	Geometric puzzles	Ability to recognize and mental rotation abilities—the child identifies and matches geometric shapes of increasing complexity

[1] NEPSY II, Developmental Neuropsychological Assessment, 2nd edition.

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TABLE 2
Variables assessed in the multiple regression models to measure the association between vitamin B-12 markers and ASQ-3 and NEPSY II scores[1]

	Continuous	Categorical
Sex	—	Male or female
Age at enrollment	mo	—
Exclusively breastfed at enrollment	—	Yes or no
Iron status at enrollment	μg/L	—
Height for age at enrollment	*z* score	—
Weight for height at enrollment	*z* score	—
Weight for age at enrollment	*z* score	—
Parity	—	1–2 or >2
Energy intake of mother at enrollment	kcal	—
Folic acid supplementation in pregnancy	—	Yes or no
Living in joint family	—	Yes or no
Family owns land	—	Yes or no
Rooms in the home	*n*	—
Mother's age	y	—
Parents' educational status	—	<10th grade or ≥10th grade
Parents' occupation	—	No work/agricultural or other work

[1] ASQ-3, Ages and Stages Questionnaire, 3rd edition; NEPSY II, Developmental Neuropsychological Assessment, 2nd edition.

was suspected that may have affected the validity of the assessment. Thus, the final number of valid assessments was 320. One child was tested with ASQ-3 only, and the total number of NEPSY II measurements was 319. We were not able to obtain sufficient blood samples at baseline for 14 children; thus, the number of children in the ASQ-3 and NESPY II analyses were 306 and 305, respectively (**Figure 1**).

Baseline features of the children in the total sample and the subsamples in this study are shown in **Table 3**. There were no major differences in the demographic characteristics between the initial sample and the children in the follow-up substudy. Among the 306 children, 45 (14.7%) had vitamin B-12 concentrations below the cutoff of 148 pmol/L for B-12 deficiency. Furthermore, 174 children (56.5%) had elevated tHcy (>10 μmol/L), 242 children (77.1%) had elevated MMA (>0.28 μmol/L), and 198 children (64.7%) had 3cB12 scores below the cutoff of −0.5, indicating low vitamin B-12 status (32). None of the children in the study had poor folate status (<10 nmol/L).

ASQ-3 and NEPSY II scores

The median ASQ-3 total score was 270 (IQR: 255–285) of a possible 300 (**Table 4**), whereas the median scores for the subtests ranged from 50 to 60, with 60 being the highest possible score.

Table 5 shows the mean ± SD raw and scaled scores of the 6 NEPSY II subtests for the 319 children and the range for the scaled scores. Seven of the scaled scores had the total range possible (1–19). The statue subtest was the subtest with the lowest range of scaled scores (5–14).

ASQ-3 scores and cobalamin status

The relation between markers of cobalamin status and the total ASQ-3 scores are shown in **Table 6**. In the multiple-adjusted linear regression analyses, each 2-fold increase in MMA and tHcy was associated with a change in the total ASQ-3 score of −3.12 (95% CI: −5.35, −0.90) and −6.78 (95% CI: −11.90, −9.95), respectively. A 1-unit increase in the 3cB12 score was associated with an increase in the total ASQ-3 score of 4.88 (95% CI: 2.09, 7.68). Similar associations as in the linear regression analyses were observed in the logistic models for the total ASQ-3 scores (Table 6).

In the ASQ-3 subscale analyses, we found associations in the problem-solving subscale but not in the communication, gross

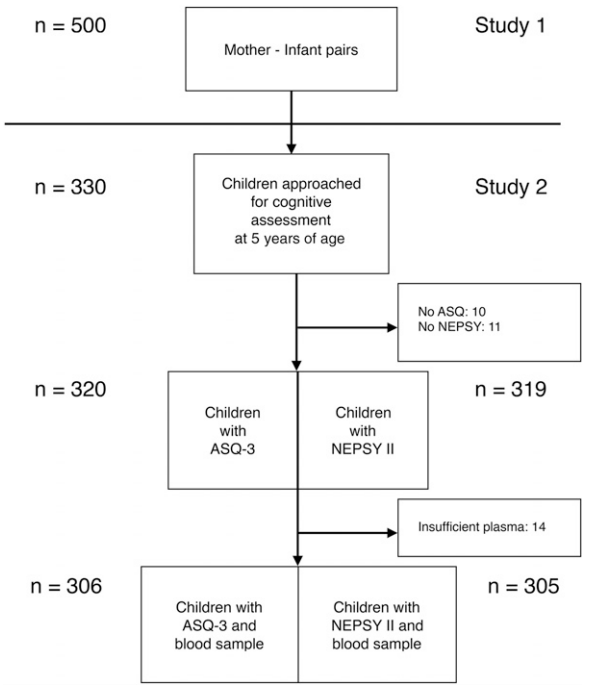

FIGURE 1 Flowchart for a study on the association between early vitamin B-12 status and the development and cognitive functioning 5 y later in Nepalese children. ASQ, Ages and Stages Questionnaire; ASQ-3, Ages and Stages Questionnaire, 3rd edition; NEPSY, Developmental Neuropsychological Assessment; NEPSY II, Developmental Neuropsychological Assessment, 2nd edition.

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TABLE 3
Demographic characteristics of the subsample and total sample[1]

	Subsample[2]	Total sample
Child characteristics at baseline		
Total, *n*	320	500
Males, *n* (%)	179 (55.9)	277 (55.7)
Age, mo	7.0 ± 2.9	6.9 ± 3
Birth weight, g	2872 ± 475.8	2891.5 ± 491.8
Exclusively breastfed at enrollment, *n* (%)	43 (13.6)	72 (14.9)
Growth status in infancy		
Weight-for-age *z* score	−0.3 ± 1	−0.3 ± 1.0
Weight-for-height *z* score	0.0 ± 1.0	0.0 ± 1.1
Length-for-age *z* score	−0.4 ± 1.3	−0.5 ± 1.3
Folate status		
Plasma folate, nmol/L	71.8 ± 33.7	73.1 ± 35.3
Iron status		
Plasma ferritin, μg/L	54.1 ± 70.5	57.9 ± 94
Cobalamin status from 2 to 12 mo of age		
Plasma cobalamin, pmol/L	264.0 ± 133.2	261.2 ± 131.4
Plasma total homocysteine, μmol/L	11.8 ± 5.0	12.5 ± 5.5
Plasma methylmalonic acid, μmol/L	0.8 ± 0.7	0.8 ± 0.9
Combined indicator of vitamin B-12 status	−0.9 ± 0.8	−0.9 ± 0.8
Family situation at baseline		
Maternal characteristics		
Age, y	26.1 ± 4.2	25.8 ± 4.2
Educational status, *n* (%)		
<10th grade	147 (48.2)	243 (52.5)
≥10th grade	158 (51.8)	220 (47.5)
Mothers who work, *n* (%)	80 (26.2)	122 (26.3)
Paternal characteristics		
Educational status, *n* (%)		
<10th grade	94 (30.3)	165 (35.6)
≥10th grade	216 (69.7)	298 (64.4)
Fathers who work, *n* (%)	281 (93.1)	430 (93.5)
Household characteristics, *n* (%)		
Joint family	170 (53.8)	250 (50.7)
Own land	182 (57.6)	270 (54.6)
Follow-up at 5 y		
Total tested at follow-up, *n*	321	—
ASQ-3	321	—
NEPSY-II	320	—
Age at testing, mo	66.7 ± 3.4	—

[1] Values are means ± SDs unless otherwise indicated. ASQ-3, Ages and Stages Questionnaire, 3rd edition; NEPSY II, Developmental Neuropsychological Assessment, 2nd edition.
[2] Refers to the 320 of 500 children who were available for follow-up developmental and neuropsychological assessments.

and fine motor, and personal-social subscales (Table 6). Increases in the MMA and tHcy concentrations and a decrease in the 3cB12 score were associated with increased odds of being in the lower quartile of the problem-solving score (3cB12 OR: 0.64; 95% CI: 0.47, 0.88). There were no significant associations between the cobalamin concentration and ASQ-3 total and subscale scores.

TABLE 4
ASQ-3 total and subscale scores in 320 Nepali preschoolers[1]

	Median (IQR)
Total ASQ-3	270 (255–285)
Subscales	
Communication	50 (40–55)
Gross motor	60 (60–60)
Fine motor	50 (45–55)
Problem solving	60 (55–60)
Personal-social	60 (50–60)

[1] The total possible ranges for the total and subscale scores are 0–300 and 0–60, respectively. ASQ-3, Ages and Stages Questionnaire, 3rd edition.

NEPSY II scores and cobalamin status

The crude and adjusted associations between the plasma cobalamin, MMA, tHcy, and 3cB12 and NEPSY II subtests are shown in **Table 7**. In the adjusted linear regressions, we found associations in the affect recognition, geometric puzzle, and block construction subtests. An increase in cobalamin concentration was associated with increased scores in the affect recognition and geometric puzzle subtests. For each increase in the log-transformed MMA concentration there was a significant decrease in scores on the affect recognition subtest, and for each log increase in the tHcy there was a decrease in both the affect

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TABLE 5
Raw and scaled NEPSY II scores in 319 Nepali preschoolers[1]

	Raw scores	Scaled scores	Range of scaled scores
Attention and executive functioning			
Inhibition-naming completion time total scores	95.0 ± 23.9	10.2 ± 2.9	1–19
Errors	4.9 ± 4.1		
Self-corrected errors	2.8 ± 2.1		
Uncorrected errors	2.1 ± 2.4		
Inhibition-naming combined scaled score		9.9 ± 3.5	1–19
Inhibition-inhibition completion time total scores	138.8 ± 38.6	9.8 ± 2.8	1–19
Errors	15.8 ± 14.8		
Self-corrected errors	4.9 ± 5.1		
Uncorrected errors	10.9 ± 14.8		
Inhibition-inhibition combined scaled score		8.8 ± 3.3	1–19
Inhibition-naming compared with inhibition contrast scaled score		8.8 ± 3.3	1–19
Inhibition total errors	20.8 ± 16.3	8.7 ± 4.3	1–19
Statue total scores	27.9 ± 2.8	12.5 ± 1.7	5–14
Body movement	1.0 ± 1.4		
Eye opening	0.9 ± 1.5		
Vocalization	0.1 ± 0.5		
Sensorimotor functioning			
Visuomotor precision total completion time	184.3 ± 56.3	5.9 ± 2.8	1–19
Total errors	24.7 ± 27.5		
Pencil lift total	13.9 ± 12.5		
Visuomotor precision combined scaled score		10.3 ± 2.2	4–19
Social perception			
Affect recognition total scores	14.5 ± 3.3	7.6 ± 3.3	1–15
Happy errors	1.1 ± 1.2		
Sad errors	2.8 ± 1.2		
Neutral errors	0.8 ± 0.9		
Fear errors	0.7 ± 0.8		
Angry errors	1.3 ± 1.0		
Disgust errors	1.3 ± 1.0		
Visuospatial processing total scores			
Block construction	7.4 ± 1.7	7.9 ± 2.5	1–15
Geometric puzzles	13.3 ± 3.0		

[1] All values are means ± SDs unless otherwise indicated. The NEPSY II scaled scores were calculated based on US norms. The scaled scores have a total possible range from 1 to 19. NEPSY II, Developmental Neuropsychological Assessment, 2nd edition.

recognition and geometric puzzle subtests. For each 1-unit increase in the 3cB12 score there was an increase of 0.82 (95% CI: 0.49, 1.14) in the affect recognition raw score, 0.59 (95% CI: 0.10, 1.09) in the geometric puzzle raw score, and 0.24 (95% CI: 0.02, 0.47) in the block construction raw score. There were no consistent associations between the vitamin B-12 markers and test performance of the children in the attention and executive domain and in sensorimotor functioning. None of the associations were altered, and all remained significant when adjusting for breastfeeding, socioeconomic variables, growth, iron status, and maternal energy intake and folate supplementation.

DISCUSSION

The results from this study show that vitamin B-12 concentrations in infancy were associated with development and performance on neuropsychological tests after 5 y and after adjusting for important confounders in a representative sample of children in which only a small proportion had cobalamin concentrations below the cutoff for deficiency (<148 pmol/L). The markers of vitamin B-12 status were associated with the total ASQ-3 scores and with the social perception abilities and a visuospatial processing skill as measured by the NEPSY II subtests. For the ASQ-3 subscales, we noted that lower 3cB12 scores and higher MMA and tHcy concentrations (indicative of poor vitamin B-12 status) were associated with increased odds of being in the lower quartile of the problem-solving scores. There were no associations between the B-12 markers and performance in the ASQ communication, gross motor, and fine motor abilities and personal-social skills. We found no significant associations in the NEPSY II domains of attention and executive and sensorimotor functioning.

Our results are in line with previous findings that have linked cobalamin and development both in population-based studies and in high-risk groups (17–21, 33). The associations between poor vitamin B-12 status and problem-solving scores are comparable to a previous study in North India (18). In contrast to previous findings (18–20, 33), however, we did not find any associations between early vitamin B-12 status and later gross motor functioning, as measured by ASQ-3. This may be because of measurement issues related to the low variability of responses and the risk of a ceiling effect in the gross motor subscale. Early

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TABLE 6
Associations between $\log_2$-transformed markers of cobalamin status and the total and subscale scores of the ASQ-3 in Nepali preschoolers[1]

		Cobalamin[2]		MMA[2]		tHcy[2]		3cB12[3]	
Variables	*n*	Value[4]	*P*	Value	*P*	Value	*P*	Value	*P*
Linear regression									
Total ASQ-3									
Crude	306	2.44 (−0.24, 5.12)	0.074	−2.87 (−5.01, −0.74)	0.009	−8.13 (−13.07, −3.19)	0.002	4.94 (2.33, 7.55)	0.001
Adjusted[5]	305	2.43 (−0.12, 4.97)	0.062	−3.12 (−5.35, −0.90)	0.007	−6.78 (−11.90, −9.95)	0.007	4.88 (2.09, 7.68)	0.001
Logistic regression									
Total ASQ-3 subscales									
Crude	306	0.82 (0.58, 1.16)	0.260	1.32 (1.07, 1.62)	0.010	2.45 (1.46, 4.10)	0.001	0.62 (0.45, 0.83)	0.002
Adjusted	305	0.79 (0.55. 1.13)	0.198	1.34 (1.06, 1.70)	0.014	2.25 (1.26, 4.03)	0.006	0.61 (0.43, 0.86)	0.005
Communication									
Crude	306	0.93 (0.68, 1.28)	0.662	1.24 (0.96, 1.60)	0.094	2.31 (1.28, 4.17)	0.006	0.69 (0.49, 0.95)	0.025
Adjusted	305	0.89 (0.63, 1.26)	0.508	1.25 (0.96, 1.64)	0.096	1.88 (0.92, 3.83)	0.083	0.70 (0.49, 1.01)	0.053
Gross motor									
Crude	306	0.82 (0.54, 1.23)	0.328	0.97 (0.74, 1.26)	0.799	1.57 (0.80, 3.06)	0.188	0.87 (0.57, 1.33)	0.519
Adjusted	305	0.79 (0.53, 1.21)	0.284	1.00 (0.76, 1.322)	0.977	1.59 (0.80, 3.18)	0.187	0.83 (0.54, 1.28)	0.406
Fine motor									
Crude	306	0.80 (0.61, 1.05)	0.112	1.23 (0.98, 1.55)	0.073	1.55 (0.86, 2.80)	0.144	0.71 (0.50, 0.10)	0.047
Adjusted	305	0.81 (0.62, 1.06)	0.118	1.23 (0.98, 1.58)	0.092	1.56 (0.81, 3.02)	0.187	0.71 (0.49, 1.03)	0.068
Problem solving									
Crude	306	0.96 (0.67, 1.39)	0.817	1.32 (1.05, 1.66)	0.018	2.32 (1.38, 3.89)	0.001	0.66 (0.47, 0.93)	0.017
Adjusted	305	0.93 (0.65, 1.33)	0.692	1.36 (1.08, 1.71)	0.009	2.33 (1.33, 4.08)	0.003	0.64 (0.47, 0.88)	0.006
Personal-social									
Crude	306	0.81 (0.50, 1.32)	0.404	1.09 (0.82, 1.46)	0.554	0.64 (0.30, 1.35)	0.245	0.92 (0.59, 1.42)	0.707
Adjusted	305	0.79 (0.49, 1.28)	0.331	1.14 (0.87, 1.51)	0.344	0.57 (1.17, 1.27)	0.127	0.89 (0.57, 1.39)	0.613

[1] Values are coefficients (95% CIs) for linear regression models and ORs (95% CIs) for logistic regression models, adjusted for clustering. ASQ-3, Ages and Stages Questionnaire, 3rd edition; MMA, methylmalonic acid; tHcy, total homocysteine; 3cB12, combined indicator of vitamin B-12 status.

[2] Log-transformed base 2.

[3] Calculated based on cobalamin, MMA, and tHcy concentrations.

[4] An OR >1 indicates the increased odds of being in the lowest 25th percentile for each increment of 1 biomarker unit; e.g., for each doubling of the tHcy units there was an increased odds of 2.25 of being in the lowest quartile of the total ASQ-3 score.

[5] Adjusted for sex, age at baseline, and weight-for-age *z* scores at baseline.

in life, motor development may to a larger extent rely on biological factors than in later childhood when a caregiver's encouragement and qualities in the environment may play a greater role (34). As a result, additional variability in later childhood may make it more difficult to observe associations between nutritional factors and motor behavior.

The impaired performance in the NEPSY II visuospatial processing domain was comparable to the results from a Dutch follow-up study that indicated long-term consequences of marginal cobalamin status on visuospatial abilities (21). In the Dutch study, there were findings in the block construction test, which depends upon motor skills and visuomotor integration. In our study, both the block construction and geometric puzzle visuospatial processing subscales yielded findings. The geometric puzzles test is a purer visuospatial test in which cognitive abilities as visuospatial analysis and mental rotation are key elements. The affect recognition subtest demonstrated robust associations with early vitamin B-12 status. Although identifying emotions such as sadness and happiness is the main task in this subtest, identification and matching are also required, and thus the performance could also be affected by visuospatial processing abilities (35). To our knowledge, this is the first study to examine the association between cobalamin status and affect recognition and social processing. More studies are needed to confirm these findings, preferably with the use of a wider test battery.

Contrary to our hypothesis, we did not observe a link between early vitamin B-12 status and executive functioning, as tested by the inhibition and statue subtests in the preschoolers. One possibility is that early cobalamin deficiency does not affect executive functioning at this age, as measured by the NEPSY II subtests. This may be because of the young age of the participants as rapid development in executive functioning starts from ∼5 y and continues to ∼9 y (36). Thus, a later test age may be preferable for measuring the potential association of cobalamin status with executive function.

The mean cobalamin concentration at enrollment was 264 pmol/L, which is within the normal range, and only 45 children (14.7%) had concentrations below the cutoff for vitamin B-12 deficiency. Many consider MMA and tHcy to be more sensitive markers for functional vitamin B-12 deficiency than plasma cobalamin concentration (13–15, 37). This is reflected in our results, in which plasma cobalamin concentration was not associated with any ASQ-3 outcomes in the logistic regressions models, whereas MMA and in particular tHcy were associated with these outcomes. Furthermore, tHcy is a better marker in infancy for vitamin B-12 deficiency than MMA (38), which was also reflected in our results. It should be noted that none of the children in our sample had poor folate status, and thus the elevated tHcy values in these children could not be caused by folate deficiency but rather poor cobalamin status (14, 15). The impact of poor vitamin B-12 status on early brain development can act

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TABLE 7
Associations between $\log_2$-transformed markers of cobalamin status and the NEPSY II subtests in Nepali preschoolers[1]

		Cobalamin[2]		MMA[2]		tHcy[2]		3cB12[3]	
Variables	*n*	Coeff. (95% CI)	*P*	Coeff. (95% CI)	*P*	Coeff. (95% CI)	*P*	Coeff. (95% CI)	*P*
Attention and executive functioning									
Inhibition-naming completion time total									
Crude	305	2.93 (−0.59, 6.44)	0.101	−0.71 (−3.33, 1.90)	0.586	−5.71 (−11.52, 0.10)	0.054	2.87 (−1.10, 6.85)	0.153
Adjusted[4]	304	2.79 (−0.83, 6.40)	0.128	−0.68 (−3.39, 2.04)	0.619	−5.22 (−11.03, 0.59)	0.077	2.71 (−1.48, 6.90)	0.200
Inhibition-inhibition completion time total									
Crude	305	5.35 (0.24, 10.45)	0.040	0.36 (−4.84, 5.57)	0.889	−7.95 (−16.05, 0.14)	0.054	3.42 (−3.27, 10.11)	0.310
Adjusted	304	5.30 (0.33, 10.26)	0.037	0.48 (−4.88, 5.83)	0.859	−7.6 (−16.05, 0.94)	0.080	3.26 (−3.66, 10.17)	0.346
Inhibition total errors									
Crude	305	−0.63 (−2.56, 1.30)	0.515	1.81 (−0.8, 3.70)	0.060	−0.22 (−3.31, 2.87)	0.887	−1.75 (−3.91, 0.40)	0.109
Adjusted	304	−0.68 (−2.59, 1.24)	0.483	1.87 (0.02, 3.71)	0.048	−0.42 (−3.59, 2.74)	0.789	−1.77 (−3.91, 0.36)	0.102
Statue total score									
Crude	305	0.16 (−0.17, 0.49)	0.334	−0.19 (−0.47, 0.10)	0.189	−0.31 (−1.11, 0.49)	0.440	0.28 (−0.15, 0.71)	0.203
Adjusted	304	0.19 (−0.14, 0.52)	0.247	−0.23 (−0.53, 0.08)	0.140	−0.39 (−1.15, 0.38)	0.313	0.34 (−0.10, 0.77)	0.131
Sensorimotor functioning									
Visuomotor precision total completion time									
Crude	305	−3.37 (−11.72, 4.98)	0.422	−3.41 (−8.80, 1.97)	0.209	−1.76 (−13.45, 9.93)	0.764	1.75 (−6.76, 10.26)	0.682
Adjusted	304	−3.49 (−11.54, 4.57)	0.389	−3.60 (−8.81, 1.62)	0.172	−0.26 (−12.97, 12.46)	0.968	1.50 (−6.62, 9.62)	0.712
Social perception									
Affect recognition total score									
Crude	305	0.50 (0.20, 0.80)	0.002	−0.52 (−0.87, −0.18)	0.004	−1.33 (−1.92, −0.75)	0.001	0.90 (0.55, 1.25)	0.001
Adjusted	304	0.46 (0.15, 0.77)	0.005	−0.50 (−0.83, −0.18)	0.003	−1.10 (−1.68, −0.52)	0.001	0.82 (0.49, 1.14)	0.001
Visuospatial processing									
Geometric puzzles									
Crude	305	0.60 (0.16, 1.04)	0.009	−0.30 (0.06, 0.01)	0.054	−0.78 (−1.39, −0.18)	0.013	0.65 (0.15, 1.15)	0.012
Adjusted	304	0.57 (0.13, 1.02)	0.013	−0.27 (−0.56, 0.01)	0.061	−0.66 (−1.28, −0.5)	0.036	0.59 (0.10, 1.09)	0.020
Block construction									
Crude	305	0.21 (−0.03, 0.46)	0.085	−0.18 (−0.32, −0.04)	0.015	−0.39 (−0.72, −0.06)	0.020	0.31 (0.10, 0.53)	0.006
Adjusted	304	0.17 (−0.06, 0.41)	0.147	−0.13 (−0.27, 0.01)	0.064	−0.31 (−0.67, 0.06)	0.095	0.24 (0.02, 0.47)	0.035

[1] Linear regression models adjusted for clustering. Coeff., coefficient; MMA, methylmalonic acid; NEPSY II, Developmental Neuropsychological Assessment, 2nd edition; tHcy, total homocysteine; 3cB12, combined indicator of vitamin B-12 status.
[2] Log-transformed base 2.
[3] Calculated based on cobalamin, MMA, and tHcy concentrations.
[4] Adjusted for sex, age at baseline, and weight-for-age *z* score at baseline.

through several mechanisms. Improvements in vitamin B-12 status may lead to advances in development and cognitive functions both as a consequence of enhanced intracellular energy production and neuroanatomic changes from enhanced myelination of the brain (39). Another mechanism may be through functional isolation in which the effect of vitamin B-12 on neurodevelopment may be mediated by factors such as enhanced activity concentrations in children and consequently enhanced stimulation and learning possibilities provided by the caregivers (40).

To our knowledge, this is the first study to investigate the associations between early vitamin B-12 status and later neurodevelopment and cognitive functioning in a general population at risk for poor vitamin B-12 status. Strengths include the prospective design, large sample size, and a thorough assessment protocol. One limitation to our study is that we only had follow-up data for 320 of the 500 children from the original study. It should be noted that the original study was designed as a cross-sectional study in which the aim was to describe the micronutrient status in the population not limited to those who would be available for a cohort lasting for years. The subsample did not differ from the original sample in terms of key demographic characteristics. Another limitation is that ASQ-3 and NEPSY II have not formally been validated in a Nepalese population. ASQ-3 has been previously used in several studies in low- and middle-income countries (29, 41, 42), and the psychometric qualities of ASQ-3 in this study were comparable to studies performed in similar settings (29). NEPSY II has also been used in low- and middle-income countries (43, 44). Table 3 indicates that there was acceptable variability in several NEPSY II subtests compared with the US norm-scaled scores. It should be noted that limited validation might result in the misclassification of child development status. This is unlikely to be differential with respect to vitamin B-12 status and as such would add noise and a loss of statistical power rather than create associations that are not real. The statistical power of our study to detect differences was also affected by the fact that 160 of the children assessed were over the maximum recommended age range of 66 mo for ASQ-3. This was also unlikely to bias our results but rather lead to a nondifferential outcome misclassification. Furthermore, because of multiple comparisons with 28 analyses with ASQ-3 and 32 analyses with NEPSY II, there is a chance for type I errors in that the reported associations were caused by chance findings. Although we adjusted for potential confounders, we cannot rule out residual confounding. Finally, c3B12 includes holotranscobalamin (32). The lack of this variable in our study, both as an individual marker and in calculating c3B12, is a limitation to our study. It should also be noted that c3B12 has only been validated in populations of adults aged ≥18 y (32) and that the use of MMA as a marker in infancy is particularly challenging because of the physiologic changes in this biomarker at this age.

The observed associations in our study suggest that early vitamin B-12 deficiency is one potential cause of adverse developmental outcomes in this Nepalese population. The number of children in Nepal that fail to meet their developmental potential has been shown to be large (1), and correcting vitamin B-12 status early may be one measure for securing healthy development. Supplementation trials and studies that explore the long-term effects of vitamin B-12 on neurodevelopment are needed to confirm our results.

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