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Vitamin B-12 Supplementation during Pregnancy and Early Lactation Does Not Affect Neurophysiologic Outcomes in Children Aged 6 Years

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ABSTRACT

Background: Deficiency of vitamin B-12 is common in pregnant Indian women. Assessment of neurophysiological measures using event-related potentials (ERPs) may yield additional information on the effects of maternal B-12 supplementation on child brain function.

Objectives: The objective of the study was to evaluate the effects of vitamin B-12 supplementation (50 μg daily orally) during pregnancy on the childhood ERP measures of positive waveform ~300 ms after stimulus (P300) and mismatch negativity.

Methods: This study was a follow-up of children born to pregnant women who received oral vitamin B-12 supplements ($n = 62$) compared with children of pregnant women who received placebo ($n = 70$) from a randomized controlled trial. The mean ± SD child age was 72 ± 1 mo. We used the Enobio system to assess the ERP measures P300 and mismatch negativity.

Results: There were no significant differences in the primary outcomes, amplitudes, and latencies of the P300 results and the mismatch negativity between children in the supplementation and placebo groups. We combined the intervention and placebo groups for secondary analyses. On multiple variable regression analysis after adjusting for treatment group, intrauterine growth restriction, and home environment, P300 amplitude in children was significantly higher in the lowest tertile of third-trimester maternal methylmalonic acid (MMA) concentrations ($\beta = 3034.04$; 95% CI: 923.24, 5144.83) compared with the highest MMA tertile ($\beta = 1612.12$; 95% CI: −258.86, 3483.10, $P = 0.005$).

Conclusions: While no significant effects of maternal vitamin B-12 supplementation on children's ERP measures were seen at 72 mo, elevated maternal MMA concentrations in the third trimester were negatively associated with P300 amplitude in children. It may be worthwhile to study the impact of maternal and infant vitamin B-12 supplementation on childhood brain structure and function in longer and larger trials. The parent trial was registered at clinicaltrials.gov as NCT00641862. *J Nutr* 2020;150:1951–1957.

Keywords: Vitamin B-12 supplementation, maternal, child ERP outcome, P300, mismatch negativity

Introduction

Recent decades have seen considerable interest in examining the impact of antenatal nutritional status on brain and cognitive development in children (1, 2), because the developing brain is particularly vulnerable to nutritional deficiencies (3). Vitamin B-12 plays a critical role in brain development during the fetal and neonatal periods (4). Vitamin B-12 acts as a cofactor in the conversion of homocysteine to methionine, and *S*-adenosyl methionine is involved in the methylation of DNA and RNA. Impaired methylation can result in deficient synthesis of myelin and consequently impact central nervous system (CNS) myelination (5). Myelination in the CNS is involved in cognition (6), and neuroimaging studies have shown an association between myelination and specific cognitive functions (7).

Electrophysiological measures can be valuable in the assessment of the effects of nutrients on brain functions (8) and are sensitive to changes with nutrient interventions (9). Event-related potentials (ERPs) represent the electrical response of the cortex to cognitive, sensory, or emotional events that are stimulus derived and time locked to particular stimuli. ERPs are noninvasive and robust, have excellent temporal resolution, and

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provide a direct measure of the underlying electrical activity of the brain (10). The ERP measures positive waveform ~300 ms after stimulus (P300) and mismatch negativity (MMN) are well-characterized cognitive potentials that represent endogenous functions of attention and memory (11). ERP-based studies are now being increasingly used to study alterations in brain functions linked to nutrient status and have shown impaired recognition memory in infants born to mothers with gestational diabetes and prenatal iron deficiency (12, 13). With the use of ERPs, delays in attention and recognition and memory were observed in 9- to 12-mo-old infants with iron deficiency anemia (14). In an iron supplementation study of 201 young children from Mexico, the ERP variable P300 was severely reduced in iron-deficient children, and a significant improvement was observed after iron supplementation (15). However, others have noted that even after correction of iron deficiency anemia during infancy, persistent delays in auditory brain responses were observed in children tested at 4 y of age (16).

To the best of our knowledge, no previous studies using ERPs have examined the effects of maternal vitamin B-12 supplementation on brain functions in children. We previously performed a double-blind randomized controlled trial of oral vitamin B-12 supplementation during pregnancy and early lactation in South Indian women (17). The children from this cohort were followed up with periodic assessment of cognitive functions, and at 30 mo, children of women who received oral vitamin B-12 supplementation had significantly higher scores of expressive language compared with children of women who received placebo (18). In the present research, we aimed to study the long-term effects of maternal vitamin B-12 supplementation on brain function in the same cohort of children at 6 y of age, using ERP measures of P300 and MMN. We hypothesized that children whose mothers were assigned to receive vitamin B-12 supplementation (50 μg daily) would have better neurophysiologic outcomes, as reflected in higher P300 amplitude and shorter P300 latency, at age 72 mo, than children of mothers who were assigned to receive placebo.

Methods

This is a follow-up study of a double-blind randomized controlled trial of oral vitamin B-12 supplementation (50 μg daily) beginning at <14 wk of gestation through 6 wk postpartum in pregnant women in Bangalore. The parent trial was registered at clinicaltrials.gov as NCT00641862. A total of 366 women were recruited from December 2008 to December 2010 from a government-administered maternal facility. At the time of recruitment a, significant proportion of pregnant women (51.1%) had vitamin B-12 deficiency (<150 pmol/L) (19).

Details of the original sample and study design have been previously published (17). Briefly, pregnant women were randomly assigned to receive either a daily oral dose of vitamin B-12 (50 μg cyanocobalamin) or identical placebo beginning at or before 14 wk of gestational age and throughout pregnancy and early lactation (6 wk postpartum). Women with multiple gestations, with chronic medical conditions (diabetes mellitus, hypertension, heart disease and or thyroid disease), those who tested positive for hepatitis B, HIV or syphilis and those who were already taking vitamin B-12 supplements were excluded. All women received iron and folic acid supplements as per routine care. Compliance with the daily regimen was measured by research nurses counting unused supplements. The mean ± SD of compliance rate among the women who were administered vitamin B-12 was 69 ± 17%, and among those who were administered placebo, it was 70 ± 13% ($P = 0.74$) (17).

Participants

Parents who participated in the original study were approached when their child was 6 y ± 1 mo and their consent was obtained for participation in the present study. Verbal assent was obtained from the participating children. The study was approved by the Institutional Ethics Committee, St John's National Academy of Health Sciences, Bangalore and the Institutional Review Board of the Harvard TH Chan School of Public Health and was conducted in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Data collection

Demographic details that included age of the parents and children, gestational age, household income, religion, parity, and educational background of both parents were collected. Trained research personnel recorded height, weight, BMI (kg/m^2), head circumference, waist circumference, hip circumference, chest circumference, mid–upper arm circumference, biceps skinfold, triceps skinfold, subscapular skinfold, and supra-iliac skinfold of the participating children. The home environment was assessed using Bradley's home inventory for early childhood (20), which has been used across cultures to study the effects of home environment on childhood cognitive abilities. Details of biochemical assays have been published in previous reports (17). We drew 10 mL of venous blood from the women at 12 (baseline), 24, and 33 wk of pregnancy. Cutoff points for vitamin B-12 and MMA were <150 pmol/L and >0.26 μmol/L, respectively (21). A 24-h diet recall (22) was administered to the children at 72 mo.

ERP Measurements

The participants were examined between breakfast and lunch in the midmorning. The testing was carried out in a soundproof room located at St John's Research Institute. where the background noise was maintained at <40 decibel throughout the experimental session. The protocol for ERP measurements was published in a previous report (23). Briefly, we used a novel wireless system, Enobio (Neuroelectrics), to acquire ERP signals. The ERP experiments were performed using presentation software (V.18.0, Neurobehavioral Systems). The Enobio Neuroelectrics instrument controller records continuous EEG data wirelessly using a Bluetooth device connected to 32 dry electrodes using an electrode cap per the international 10/20 system (24). The data were stored in a European data format file (25). The ERP-related tasks were performed on a Lenovo ThinkPad with a 64-bit operating system. The children were seated in a comfortable, height-adjustable chair, such that the computer screen was at the subject's eye level and ~60 cm from the subject.

P300 experiment

The P300 is a positive wave occurring at approximately 300 ms post-stimulus presentation (10) and is prominent over the fronto/central region of the brain in standard/discrimination tasks (26). We used visual stimuli to test P300, with a previous study demonstrating that both auditory and visual stimuli elicited an identical topography across the 2 modalities (27). The task was described to the participant and they were instructed to keep their eyes fixed on the stimuli and respond to the target stimulus by pressing a button with the index finger from their dominant hand. The experiment is described in a previous paper (23).

MMN experiment

The MMN represents a neural response of "pre-attentive" memory-based comparison of incoming deviant auditory stimulus to that of

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Address correspondence to CD (e-mail: christopher.duggan@childrens.harvard.edu).
Abbreviations used: CNS, central nervous system; ERP, event-related potentials; IUGR, intrauterine growth restriction; P300, positive waveform ~300 ms after stimulus; MMN, mismatch negativity; tHcy, total homocysteine.

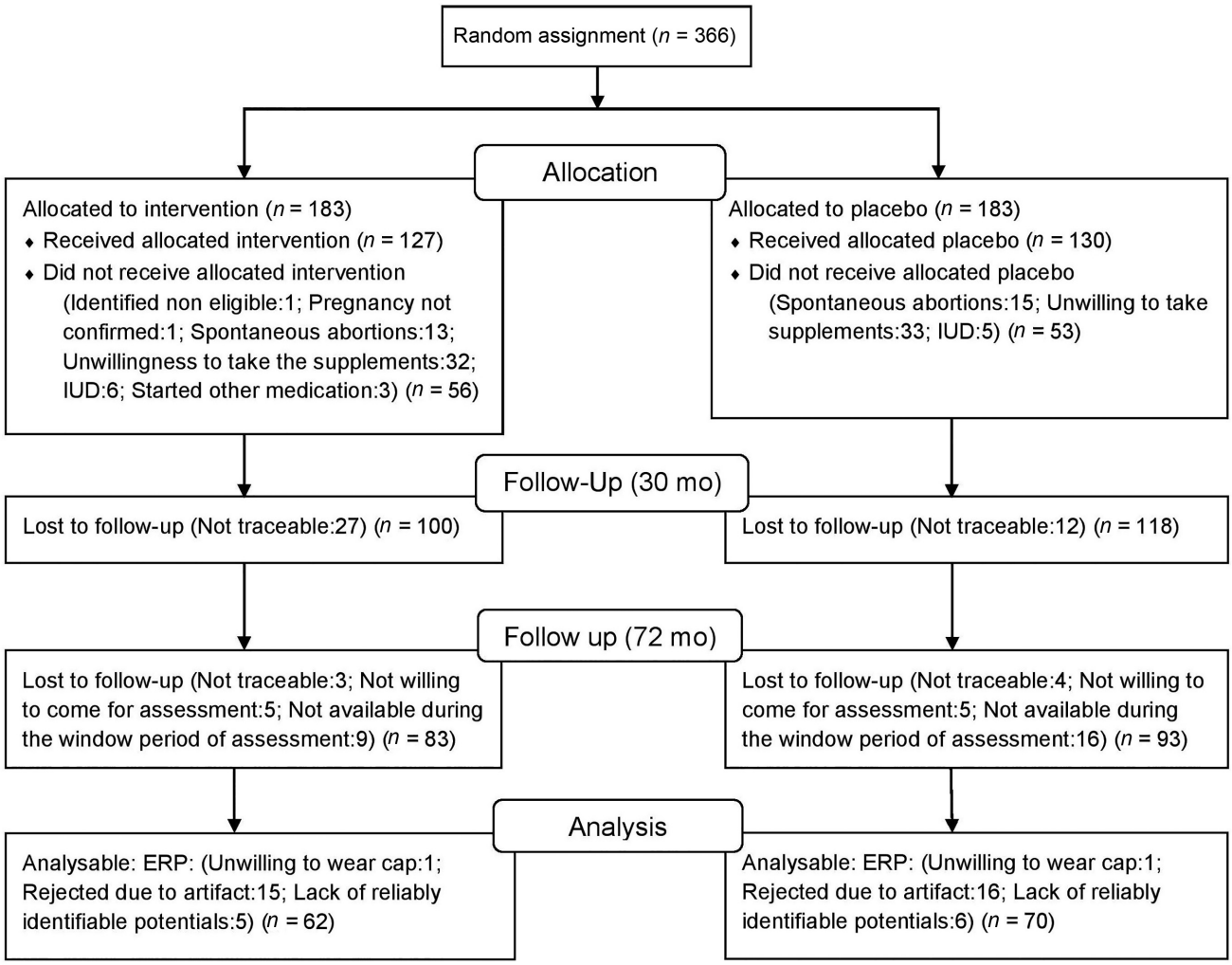

FIGURE 1 Flow chart of subject recruitment and random assignment.

previous standard stimuli (28). MMN peaks at 100–300 ms following the stimulus and reflects the cognitive state of early auditory attention in children. MMN is elicited when the participant is inattentive to the auditory stimuli but engaged in a cognitive task in another modality (29). Detailed methodology has been previously published (23).

EEG and ERP data analysis

The amplitude (μV) and latency (ms) of the ERP signals were measured. The P300 data were analyzed from the frontal midline electrode (Fz). Peak amplitudes and latencies of P300 were estimated using predetermined time windows (−100 ms to 600 ms). Similar to the P300 analysis, the MMN data from the frontal region represented by Fz was used for further analysis. After the removal of artifacts, ERPs were created by averaging the epochs (−100 ms to 600 ms) determined from the standard and the deviant stimuli, respectively. The difference waves (standard stimulus ERP subtracted from the deviant stimuli ERP) were created and peak amplitudes and latencies were estimated for MMN. A time window of −100 to 350 ms for the MMN data was chosen, and the amplitudes and latencies for the most negative peak in the time window were determined. Detailed procedures for the analysis of P300 and MMN have been previously published (23).

Statistical analysis

The primary outcome was amplitude of P300 and MMN at 72 mo compared across the 2 treatment arms. The sample size of the study was determined by the number of participants in the parent trial who were eligible for participation in this follow-up study. Eligibility for the parent trial included women aged 18 y and older who had registered for prenatal care at or before the 14-wk gestational age (as judged by the date of the last menstrual period). Mothers were excluded who had multiple gestations or chronic medical conditions (diabetes mellitus, hypertension, heart disease, or thyroid disease), who anticipated moving out of the area before study completion, and who tested positive for hepatitis B (hepatitis B surface antigen), HIV, or syphilis (Venereal Disease Research Laboratory test) infections, and those who were already taking daily vitamin supplements in addition to folate and iron. A sample size of 60 per arm provided 90% power to detect a standardized effect size of 0.6 or higher in P300 amplitude with a 2-sided 2-sample t test at a 0.05 significance level. Our sample size of 132 was sufficient for the analysis.

Since our earlier studies on the cognitive outcomes in children from the same cohort had showed an association between some of the maternal nutrient markers and cognitive measures (18), we reexamined the associations between trimester-specific maternal nutrient markers and childhood ERP measures. The intervention and placebo groups were combined for this analysis. Using scatter plots, we examined the associations between maternal nutrient markers [plasma vitamin B-12, total homocysteine (tHcy), and MMA concentrations, red cell folate levels, and hemoglobin] and the ERP measures of the children. Since the scatter plots showed nonlinear associations, maternal nutrient markers in each trimester were divided into tertiles and their associations with ERP outcome measures were examined using the Kruskal–Wallis test. The maternal nutrient markers that were significantly associated with ERP measures were further examined using linear regression.

TABLE 1 Demographic and baseline biochemical characteristics of women whose children underwent ERP assessment at age 72 mo in the vitamin B-12 supplementation and placebo groups

Parameter	Intervention group ($n = 62$)	Placebo group ($n = 70$)
Current maternal age, y	30.5 ± 4.4	30.3 ± 3.7
Maternal level of education		
Primary school	12 (19.4)	17 (24.3)
Middle school	25 (40.3)	23 (32.9)
High school and above	25 (40.3)	30 (42.9)
Mother employed	20 (32.4)	28 (40.0)
Monthly household income, INR	17,686 ± 10,424	19,657 ± 18,983
Only child	33 (53.2)	37 (52.9)
Child male sex	24 (38.7)	27 (38.6)
Child age, mo	72.0 ± 0.4	71.30 ± 7.7
Child IUGR	15 (24.2)	27 (38.6)
Bradley's home inventory scores	32.81 ± 7.2	33.55 ± 5.6
Baseline biochemical characteristics		
Plasma vitamin B-12, <150 pmol/L	25 (40.3)	39 (55.7)
Plasma MMA, >0.26 μmol/L	48 (77.4)	52 (74.3)
Plasma tHcy, >15.0 μmol/L	16 (25.8)	14 (20)
Erythrocyte folic acid, nmol/L	443 ± 160	432 ± 162

Values are means ± SDs, or frequencies (percentages). INR, Indian rupee; intervention, vitamin B-12 supplementation; IUGR, intrauterine growth retardation; MMA, methylmalonic acid; tHcy, total homocysteine.

The analysis was adjusted for treatment group, intrauterine growth restriction [IUGR; birth weight below the 10th centile for gestational age (30)], and home environment, since IUGR and home environment have been shown to influence childhood ERP measures in earlier studies (31, 32). The regression coefficients (β) and corresponding 95% CIs are reported. The level of significance used for interpreting the data was $P < 0.05$. Data were analyzed using SPSS for Windows, version 22.0.

Results

In the parent trial, we recruited 366 women and had birth details for 256 children, of whom 176 children were available for neurophysiological assessments at 72 mo (**Figure 1**). There were no observed differences in the sociodemographic characteristics, baseline maternal biochemical characteristics, or dropout rates between children who were available for the neurophysiological assessments and children who were unavailable (data not shown). The intervention and placebo groups were comparable on demographic and maternal biochemical characteristics (**Table 1**). A Mann–Whitney U test done to compare dietary vitamin B-12 intake between the children of mothers in the supplementation and placebo groups showed that the groups were comparable [median (quartile 1, quartile 3) for supplementation group: 0.88 (0.47,1.35); placebo group: 1.00 (0.65,1.35); $P = 0.44$].

There were no significant differences in the amplitudes or latencies of P300 data between children born to mothers who received vitamin B-12 supplementation and those who received placebo (**Table 2**). There were no significant differences in the amplitudes or latencies of MMN between children born to mothers who received vitamin B-12 supplementation and children of mothers who received placebo (Table 2).

We combined the intervention and placebo groups for the remaining analyses. We examined the associations between the various maternal nutrient markers of vitamin B-12 status across the 3 trimesters and the childhood ERP measures of P300 and MMN. The P300 amplitude of children at 72 mo was significantly different between tertiles of MMA in the third trimester (Kruskal–Wallis test, $P = 0.03$), with P 300 amplitude being significantly lower in the highest tertile of maternal MMA than the lowest tertile. (**Table 3**, **Figure 2**). None of the other ERP parameters were significantly associated with maternal vitamin B-12 status markers at other time points. In multiple variable regression analysis, after adjustment for treatment group, IUGR, and home environment, the P300 amplitude in the lowest tertile of MMA was significantly higher ($\beta = 3034.04$; 95% CI: 923.24, 5144.83) than those in the third but not the second MMA tertile ($\beta = 1612.12$; 95% CI: −258.86, 3483.10; $P = 0.005$). This association was significant after adjusting for multiple testing using Bonferroni correction ($P = 0.025$).

Discussion

In this extended follow-up of children of mothers who participated in a randomized controlled trial, we did not find any effects of maternal vitamin B-12 supplementation on neurophysiological outcomes as measured by P300 and MMN in offspring at 6 y of age. However, in the group taken together as whole, higher maternal plasma MMA concentration in the third trimester was negatively associated with P300 amplitude. While several observational studies have found an association between maternal vitamin B-12 concentrations and cognitive outcomes in children, our trial, to the best of our knowledge, is the first to examine the effects of maternal oral vitamin B-12 supplementation during pregnancy and early infancy on ERP outcomes using a randomized controlled design.

There may be several reasons for our finding of no effect of maternal vitamin B-12 supplementation on childhood ERP measures at 72 mo. These include the timing and dose of the supplement, our choice of vitamin B-12 status markers, as well as potential other effects that we were not able to measure. Specifically, in the parent trial, maternal vitamin B-12 supplementation was stopped at 6 wk postpartum, and thus, the effects of maternal supplementation on maternal and child vitamin B-12 status and related health outcomes may have

TABLE 2 Comparison of ERP measurements of children in the vitamin B-12 supplementation and placebo groups at age 72 mo[1]

ERP data	Intervention ($n = 62$)	Placebo ($n = 70$)	P value
P300			
Median (Q1, Q3) latency, ms	324 (268, 462)	352 (262, 447)	0.88
Median (Q1, Q3) amplitude, μV	5501 (3526, 8036)	5552 (2252, 8511)	0.69
MMN 1100			
Median (Q1, Q3) latency, ms	251 (198, 304)	290 (186, 326)	0.16
Median (Q1, Q3) amplitude, μV	−1558 (−3046, −985)	−2187 (−3674, −1060)	0.27

[1] MMN, mismatch negativity; P300, positive waveform ~300 ms after stimulus; Q, quartile.

TABLE 3 Comparison of P300 latency and amplitude of children based on tertiles of maternal MMA status in the 3 trimesters[1]

Trimester tertile, *n*	Maternal plasma MMA, μmol/L	P300 latency, ms	*P* value	P300 amplitude, μV	*P* value[2]
Trimester 1			0.82		0.80
1 (45)	0.24 (0.16, 0.30)	330 (266, 426)		5442 (3137, 8698)	
2 (42)	0.48 (0.41, 0.56)	326 (256, 451)		5699 (2719, 8369)	
3 (45)	0.76 (0.66, 0.94)	346 (268, 467)		5562 (2445, 8127)	
Trimester 2			0.40		0.60
1 (44)	0.18 (0.11, 0.24)	335 (263, 438)		5638 (3340, 8231)	
2 (44)	0.33 (0.30, 0.39)	323 (259, 431)		5273 (3057, 8812)	
3 (44)	0.67 (0.52, 0.82)	353 (284, 474)		5606 (2133, 7548)	
Trimester 3			0.82		0.03
1 (41)	0.14 (0.08, 0.21)	360 (248, 469)		6088 (3340, 12,160)	
2 (38)	0.35 (0.31, 0.39)	338 (277, 447)		5375 (3527, 8238)	
3 (41)	0.57 (0.48, 0.67)	307 (262, 461)		4149 (1825, 6948)	

[1] Values are medians (quartile 1, quartile 3), $n = 132$. MMA, methylmalonic acid.
[2] *P* value from Kruskal–Wallis Test.

ended with the period of supplementation. Support for this conclusion was observed in the findings of the parent study, in which higher concentrations of vitamin B-12 in breast milk were observed at 6 wk but not at later time points (17). In a recent trial from India that examined the effects of vitamin B-12 and/or folic acid supplementation on early child development, children between 6 and 30 mo of age who received both vitamin B-12 and folic acid performed better on gross motor and problem-solving functions than children who received placebo (33). In 2 Norwegian trials, young infants with symptoms of developmental regression and feeding problems or low birth weight combined with mild cobalamin deficiency were given an intramuscular injection of 400 μg hydroxycobalamin or a sham injection. Children who were injected with cobalamin had significantly improved cobalamin status and motor function (34, 35).

The lack of effect of maternal vitamin B-12 supplementation on child ERP measures in the present study may also be related to the markers of vitamin B-12 that we used. Several investigators have indicated that vitamin B-12 concentrations may not accurately reflect functional vitamin B-12 status and recommend that serum holo-transcobalamin (36) and/or a combined index of functional biomarkers be used to assess vitamin B-12 status (37). Other potential reasons behind our generally negative findings include the possibility that child genotype, diet, developmental status, or other environmental factors influenced the neurophysiologic outcomes more than maternal vitamin B-12 supplementation did, or that indeed

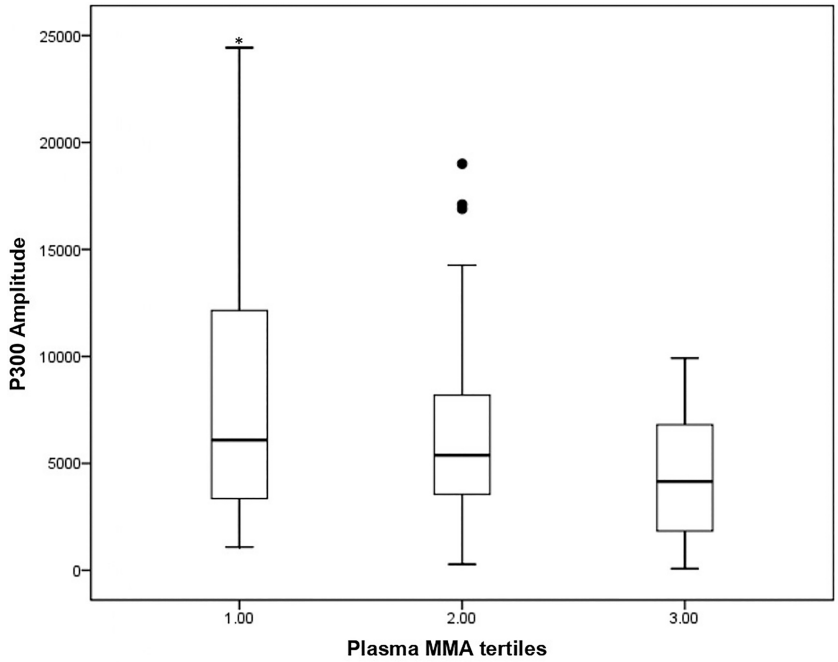

FIGURE 2 Comparison of the P300 amplitudes of children based on the tertiles of MMA of their mothers in the third trimester. Median plasma MMA concentration for tertile 1 ($n = 132$): 0.48 μmol/L; tertile 2 ($n = 132$): 0.33 μmol/L; and tertile 3 ($n = 120$: 0.35 μmol/L. $^*P < 0.05$ for the first tertile compared with the third tertile. P300, positive waveform ~300 ms after stimulus; MMA, methylmalonic acid.

maternal vitamin B-12 supplementation has no effect on child neurophysiology. Thus, in future studies it may be worthwhile to examine the effects of a longer duration of maternal supplementation as well as direct vitamin B-12 supplementation of infants on ERP outcomes, particularly in children at risk of vitamin B-12 deficiency.

Our finding of elevated maternal MMA concentrations in the third trimester as a correlate of lower P300 amplitude in children at 72 mo is a novel observation. P300 amplitude is a measure of attentional resource allocation (cognitive demands) while performing a task (38), and P300 amplitude relates to cognitive resources, compared with P300 latency, which reflects neural speed or brain efficiency (39). The third trimester of pregnancy is a period characterized by significant brain development that includes the beginning of myelination, neuronal organization, spinogenesis and synaptogenesis (40). A recent neuroimaging study using three dimensional MRI techniques in healthy human fetuses noted a significant increase in cerebral volume between 28- and 39-wk gestation, and increases in fetal white matter contributed the most to overall cerebral growth in the later periods of pregnancy (41). Low vitamin B-12 status results in impaired L-methonyl-CoA mutase function with accumulation of MMA and a decrease in succinyl CoA, a pathway that is seen as critical for maintenance of myelin integrity (42). Neuroimaging studies in adults with vitamin B-12 deficiency have shown microstructural changes in white matter (43), and thus, demyelination is the major pathological substrate in states of vitamin B-12 deficiency. Several studies have reported that elevated MMA concentrations as opposed to cobalamin concentrations are associated with poorer performance on a battery of cognitive tests in infancy and childhood (44, 45). Thus, elevated maternal MMA concentrations may serve as a marker for later adverse neurodevelopmental outcomes in children. Indeed, our previous finding that higher maternal serum total homocysteine concentrations were related to lower neurodevelopmental scores in this same cohort at earlier testing ages (46) is congruent with our neurophysiologic findings here.

The study has several limitations. Out of the 366 participants in the parent trial, only 132 participants were available for the present study, although baseline characteristics between the children who did and did not undergo ERP status were comparable. As noted above, the duration, dose, and/or route of vitamin B-12 supplementation may have been inadequate. No neuroimaging was performed in the cohort, and earlier or more extensive ERP measures were not performed. The Bradley's home inventory for early childhood used to assess home environment was not adapted or validated in the study population. Although measures of other possible confounding factors that may have influenced ERP outcomes (including child diet, developmental stimulation, and other factors) were not measured, our randomized trial design helped to mitigate these potential limitations. The strengths of the trial include its design, inclusion of women with a high rate of biochemical vitamin B-12 deficiency, long-term follow-up, and application of novel ERP measurements. In summary, maternal supplementation with 50 μg of oral vitamin B-12 during pregnancy and early lactation did not significantly influence childhood ERP measures at 72 mo. We did, however, note that elevated maternal MMA concentrations in the third trimester were associated with lower amplitude of P300, a neurophysiological marker of cognitive function. It may be worthwhile to study the impact of maternal and infant vitamin B-12 supplementation on childhood brain structure and function in longer and larger trials.

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The authors' responsibilities were as follows—KS, AVK, CD, and TAS: conceptualized the study; MJ: designed the experimental sessions; SA: conducted the neurophysiological experiment and collected the data; KS and ST: set up the study; TT: designed the statistical analyses; KS, ST, and CD: wrote the paper; and all authors: read and approved the final manuscript.

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