

# Increases in Plasma Holotranscobalamin Can Be Used to Assess Vitamin B-12 Absorption in Individuals with Low Plasma Vitamin B-12<sup>1-3</sup>

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## Abstract

Low plasma concentrations of vitamin B-12 are common in Indians, possibly due to low dietary intakes of animal-source foods. Whether malabsorption of the vitamin contributes to this has not been investigated. A rise in the plasma holotranscobalamin (holo-TC) concentration after a standard dose of oral vitamin B-12 has been proposed as a measure of gastrointestinal absorption in people with normal plasma vitamin B-12 concentrations. We studied 313 individuals (children and parents, 109 families) in the Pune Maternal Nutrition Study. They received 3 doses of 10  $\mu\text{g}$  ( $n = 191$ ) or 2  $\mu\text{g}$  ( $n = 122$ ) of cyanocobalamin at 6-h intervals. A rise in plasma holo-TC of  $\geq 15\%$  and  $>15$  pmol/L above baseline was considered normal vitamin B-12 absorption. The baseline plasma vitamin B-12 concentration was  $<150$  pmol/L in 48% of participants; holo-TC was  $<35$  pmol/L in 98% and total homocysteine was high in 50% of participants ( $>10$   $\mu\text{mol/L}$  in children and  $>15$   $\mu\text{mol/L}$  in adults). In the 10  $\mu\text{g}$  group, the plasma holo-TC concentration increased by 4.8-fold from (mean  $\pm$  SD)  $9.3 \pm 7.0$  pmol/L to  $53.8 \pm 25.9$  pmol/L and in the 2  $\mu\text{g}$  group by 2.2-fold from  $11.1 \pm 8.5$  pmol/L to  $35.7 \pm 19.3$  pmol/L. Only 10% of the participants, mostly fathers, had an increase less than the suggested cut-points. Our results suggest that an increase in plasma holo-TC may be used to assess vitamin B-12 absorption in individuals with low vitamin B-12 status. Because malabsorption is unlikely to be a major reason for the low plasma vitamin B-12 concentrations in this population, increasing dietary vitamin B-12 should improve their status. J. Nutr. 139: 2119–2123, 2009.

Low vitamin B-12 concentrations are common in Indians (1) and contribute to hyperhomocysteinemia despite normal folate status (2,3). This is unlike the situation in Europeans (4), where low folate status is the predominant determinant of hyperhomocysteinemia. We have demonstrated an association between low maternal vitamin B-12 concentrations in pregnancy and insulin resistance (5) and reduced neurocognitive performance in the children (6). Vitamin B-12 deficiency could also contribute to the etiology of neural tube defects in Indians (7). Prevention of these conditions in India may require supplementation with vitamin B-12.

Low vitamin B-12 status among Indians is usually attributed to low dietary intake due to a mainly vegetarian diet (1,8,9), but it is not uncommon even in those who eat nonvegetarian foods (9). In our studies, even though vegetarian diet was a significant

predictor of low vitamin B-12 status, one-half to two-thirds of participants who ate nonvegetarian food more than 3 d/wk also had low plasma vitamin B-12 concentrations (C. S. Yajnik, D. S. Bhat, H. G. Lubree, C. V. Joglekar, unpublished data). We also found an inverse association of plasma vitamin B-12 concentrations with levels of education, income, and hygiene, suggesting that factors other than low dietary content could contribute to vitamin B-12 status (3). Tropical sprue and gastrointestinal infestations (10,11) and infections (including *Helicobacter pylori*) (12,13) also contribute to low vitamin B-12 concentrations, possibly due to malabsorption. This possibility is rarely investigated because of the complexity of methods for investigating vitamin B-12 absorption. For example, the Schilling (14) test requires radioactive isotope. It is essential to determine whether the low vitamin B-12 status among Indians reflects only dietary inadequacy or if malabsorption is also a contributing factor, because this will have implications for treatment.

Recently, Bor et al. (15) demonstrated an increase in plasma holotranscobalamin (holo-TC)<sup>8</sup> concentration after a small oral

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<sup>3</sup> Supplemental Figure 1 is available with the online posting of this paper at [jn.nutrition.org](http://jn.nutrition.org).

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<sup>8</sup> Abbreviations used: holo-TC, holotranscobalamin; KEM, King Edward Memorial; PMNS, Pune Maternal Nutrition Study; tHcy, total homocysteine.

dose of vitamin B-12 ( $9 \mu\text{g} \times 3$  doses) and suggested that this approach can be used to test vitamin B-12 absorption in a clinical setting. Another recent study by von Castel-Roberts et al. (16) investigated circulating concentrations of holo-TC using Bor's protocol in a sample of healthy U.S. men and women. Blood samples taken 24 h after the first dose provided the optimal results. Both these studies were performed in participants with normal vitamin B-12 concentrations. We have used the same protocol with slight modification ( $10 \mu\text{g} \times 3$  doses) as a test of vitamin B-12 absorption in a population with low vitamin B-12 status. In addition, we also investigated the response using a much lower dose ( $2 \mu\text{g} \times 3$  doses).

## Participants and Methods

The participants were families from an extended cohort of the Pune Maternal Nutrition Study (PMNS). The design and methods have been reported elsewhere (17). In short, the PMNS is a prospective community-based study to investigate the relationship of maternal nutrition to fetal growth; the participants have been followed up to assess future risk of type 2 diabetes and cardiovascular disease. The study was established in 6 villages 40–50 km from Pune city, between June 1994 and April 1996 and covered a population of >35,000. We screened all married, nonpregnant women who were <35 y and not sterilized. Of 2675 eligible women, 2466 (92%) agreed to participate. During the study period, 814 women became pregnant and participated in the main PMNS cohort. After the main study, we enrolled an additional 153 pregnant women from the same initial sample of eligible women, to study early fetal growth. Of these, 129 delivered in the study and the babies and parents have been followed up with the original cohort; 119 children remained in follow-up at 9 y of age. These children and their parents were invited to take part in the current study, which took place from May to November 2006. The women, their husbands, and the children were comparable to the main PMNS cohort in body size measurements and socioeconomic status. Children as well as adults were included in the study, because our earlier work suggested that vitamin B-12 status varies with age. The King Edward Memorial (KEM) Hospital Ethics Committee approved the study and we obtained informed written consent from the parents and assent from the children.

The participants reported to the Diabetes Unit, KEM Hospital, Pune, the evening before the study. Height was measured to the nearest 0.1 cm using a wall-mounted stadiometer (CMS Instruments) and body weight to the nearest 5 g (Conveigh, Electronic Instruments). A standard vegetarian dinner was provided between 20 and 21 h, after which participants rested overnight in the Diabetes Unit. The only food of animal origin during this period was a small amount of milk in tea. All participants were examined for gross clinical signs of protein-energy undernutrition and vitamin deficiencies (vitamins A, B complex, C, and D). In children, a FFQ was administered to record the portion size and frequency of consumption of different foods over a period of 1 y. Vitamin B-12 intakes were calculated from the FFQ data using published tables of the nutrient content of Indian foods (18). A fasting blood sample was collected in the morning (baseline) and a  $10\text{-}\mu\text{g}$  vitamin B-12 (cyanocobalamin) capsule was administered under supervision every 6 h thereafter (8, 14, and 20 h). A fasting blood sample was collected the following morning ~12 h after the last dose (postdose sample).

After completing sampling for approximately one-half of the families, it was clear from our data that absorption was satisfactory in most cases. We then reasoned that we could improve the utility of the study, including for policy makers, by investigating a smaller dose nearer to the Recommended Dietary Allowance (12) of 2.4–2.8  $\mu\text{g}$  for adults and 0.9–2.4  $\mu\text{g}$  for children. The remaining families received 2- $\mu\text{g}$  capsules rather than 10- $\mu\text{g}$  capsules. In total, 65 families received  $10 \mu\text{g} \times 3$  doses (10  $\mu\text{g}$  group) and 44 received  $2 \mu\text{g} \times 3$  doses (2  $\mu\text{g}$  group).

**Laboratory analysis.** Blood samples from the ante-cubital vein were collected in the sitting position in an EDTA vacutainer. Hemoglobin was measured on a Beckman Coulter Analyzer (A<sup>C</sup>T diff Analyzer). The remaining blood was centrifuged at  $2500 \times g$  for 15 min at 4°C within 1 h of collection and plasma was stored at  $-70^\circ\text{C}$  until further analysis. Plasma cobalamin (B-12) was measured by microbiological assay using a colistin sulfate-resistant strain of *Lactobacillus leichmanii* (19,20). Plasma holo-TC was measured using magnetic beads (microspheres) with immobilized monoclonal antibody specific for human TC (21) followed by the conventional microbiological assay developed for cobalamin estimation (22). Plasma folate was measured by microbiological assay using a chloramphenicol-resistant strain of *Lactobacillus casei* (23,24). Plasma total homocysteine (tHcy) was measured by fluorescence polarization immunoassay (Abbott) (25). In our laboratory, for vitamin B-12, holo-TC, folate, and tHcy analyses, between-day CV were <8, <9, <7, and <3%, respectively.

**Definitions.** We defined anemia as a hemoglobin concentration <120 g/L in children and mothers and <130 g/L in fathers (26). We defined microcytosis as mean corpuscular volume as <80 fL and macrocytosis as mean corpuscular volume >100 fL. Hyperhomocysteinemia was defined as plasma tHcy concentration >15  $\mu\text{mol/L}$  in adults and >10  $\mu\text{mol/L}$  in children (27). Low folate concentrations were defined as plasma folate concentration <7.0 nmol/L (28). Low vitamin B-12 concentrations were defined as plasma vitamin B-12 concentration <150 pmol/L (2). Low holo-TC concentration was defined as <35 pmol/L (29). Poor vitamin B-12 absorption was defined using the Bor et al. (16) criteria as a rise in plasma holo-TC <15% and <15 pmol/L after 3 doses of  $10 \mu\text{g}$  oral vitamin B-12.

**Statistical methods.** The data are presented as mean  $\pm$  SD. Differences between baseline and postdose measurements (vitamin B-12, tHcy, and holo-TC) were tested using paired *t* tests. Differences between groups were tested using unpaired *t* tests. Results in children and parents were analyzed separately. The results in children and parents for the 2  $\mu\text{g}$  and 10  $\mu\text{g}$  groups were combined in the multiple linear regression analysis of factors associated with rise in plasma holo-TC. SPSS version 11.0 for Windows was used for statistical analysis.

## Results

A total of 313 individuals (109 children, 96 fathers, and 108 mothers) participated in the study. None were taking vitamin supplements or drugs known to influence vitamin B-12 absorption such as proton pump inhibitors and metformin. One child from the 10  $\mu\text{g}$  group could not swallow the capsules and 1 child from the 2  $\mu\text{g}$  group vomited during the test; they were excluded from the analysis.

**TABLE 1** Anthropometric characteristics and hemoglobin concentrations of the children, fathers, and mothers<sup>1</sup>

	Children	Fathers	Mothers
<i>n</i>	109	96	108
Age, <i>y</i>	9.0 ± 0.2	36.9 ± 3.8	30.2 ± 3.0
Height, <i>cm</i>	126.5 ± 5.6	165.7 ± 7.1	153.2 ± 5.3
Weight, <i>kg</i>	21.9 ± 2.9	59.3 ± 10.0	47.7 ± 8.2
Hemoglobin, <i>g/L</i>	125.0 ± 1.0	143.0 ± 1.4	120.0 ± 1.6
Anemic, <sup>2</sup> %	24	13	46

<sup>1</sup> Values are mean ± SD, unless specified.

<sup>2</sup> Hemoglobin < 120 g/L for children and mothers and <130 g/L for fathers (26).

None of the children, fathers, or mothers had clinical signs of protein-energy undernutrition or vitamin deficiencies; some were anemic (Table 1). Sixty-four percent of anemic participants had microcytic erythrocytes and none had macrocytosis. Five participants (3 fathers and 2 mothers) had macrocytic erythrocytes but were not anemic. FFQ data were available for children only. None of them were vegan. One-third were lacto-vegetarian (milk but no other nonvegetarian foods) and two-thirds ate nonvegetarian foods (eggs, meat, and fish as well as milk). Less than one-half of the latter group (44%) consumed nonvegetarian foods more than twice a week, but the average portion size of nonvegetarian foods was small. The median calculated daily dietary intake of vitamin B-12 was <0.1 μg in the lacto-vegetarian children and 0.2 μg in the nonvegetarian children.

Baseline plasma vitamin B-12 concentrations were low, folate concentrations were normal, and tHcy concentrations were high (Table 2). Twenty-seven percent of children, 70% of fathers, and 49% of mothers had low vitamin B-12 concentrations. Ninety-eight percent of participants had low plasma holo-TC concentrations. In contrast, only 2% of children, 15% of fathers, and 9% of mothers had low folate concentrations. Hyperhomocys-

teinemia was observed in 48% of children, 74% of fathers, and 34% of mothers.

Plasma holo-TC concentrations increased after oral cyanocobalamin (Table 2; Supplemental Fig. 1). In the 10 μg group, the rise was 4.8-fold and in the 2 μg group, the rise 2.2-fold. Plasma vitamin B-12 concentrations also increased and the two were related ( $r = 0.57$ ;  $P < 0.001$ , adjusted for age and sex). The increases in holo-TC and vitamin B-12 were greater in children than in the parents ( $P < 0.001$ ) and comparable in fathers and mothers. This may have resulted from a greater dose of vitamin B-12 per kg body weight in children (10 μg group,  $1.39 \pm 0.17$  μg/kg; 2 μg group,  $0.28 \pm 0.03$  μg/kg) compared with fathers (10 μg group,  $0.53 \pm 0.09$ ; 2 μg group,  $0.10 \pm 0.02$ ) and mothers (10 μg group,  $0.65 \pm 0.10$ ; 2 μg group,  $0.13 \pm 0.02$ ).

According to the criteria listed by Bor et al. (15) (used only for the 10 μg group), 4 children (6%), 10 fathers (17%), and 5 mothers (8%) were classified as poor vitamin B-12 absorbers. The poor absorbers and normally absorbing counterparts had similar baseline plasma vitamin B-12, holo-TC, and tHcy and blood hemoglobin concentrations; none of the poor absorbers had macrocytic erythrocytes.

In a multivariate analysis, combining children and parents from both groups and including age, sex, dose/kg of cyanocobalamin, and baseline vitamin B-12 concentration, the rise in the plasma holo-TC concentration was directly proportional to the dose/kg of cyanocobalamin (standardized  $\beta = 0.446$ ;  $P < 0.001$ ) and inversely related to age (standardized  $\beta = -0.147$ ;  $P = 0.01$ ). The rise in holo-TC was higher in participants with a baseline plasma vitamin B-12 concentration >150 pmol/L at baseline than in those with concentrations <150 pmol/L [(unadjusted mean ± SEM)  $42.3 \pm 1.8$  pmol/L vs.  $30.6 \pm 1.9$  pmol/L,  $P = 0.005$ ; and  $42.0 \pm 1.6$  pmol/L vs.  $30.9 \pm 1.6$  pmol/L,  $P = 0.06$ , adjusted for age, sex, dose/kg of vitamin B-12, and body weight).

After oral cyanocobalamin, plasma folate concentrations did not change, but those of tHcy decreased from  $18.3 \pm 14.4$  to  $17.1 \pm 13.5$  μmol/L in the 10 μg group and from  $19.0 \pm 18.9$  to  $17.0 \pm 17.5$  μmol/L in the 2 μg group ( $P < 0.001$ ).

**TABLE 2** Plasma vitamin B-12, holo-TC, tHcy, and folate concentrations in Indian children and their parents before and after receiving  $3 \times 10$  μg doses or  $3 \times 2$  μg doses of cyanocobalamin<sup>1</sup>

	10 μg group			2 μg group		
	Children	Fathers	Mothers	Children	Fathers	Mothers
<i>n</i>	64	61	65	43	35	43
Plasma vitamin B-12, <i>pmol/L</i>						
Baseline	191 ± 60	136 ± 62	180 ± 176	222 ± 107	126 ± 68	161 ± 95
Postdose	302 ± 95	193 ± 74	233 ± 121	293 ± 123	158 ± 71	194 ± 103
% Change	63.1 ± 39.6*	47.7 ± 31.1*	40.1 ± 24.0*	38.9 ± 27.4*	31.3 ± 21.1*	23.5 ± 14.9*
Plasma holo-TC, <sup>2</sup> <i>pmol/L</i>						
Baseline	10.3 ± 5.6	7.7 ± 4.2	9.7 ± 9.7	11.9 ± 7.3	10.5 ± 8.1	10.7 ± 10.0
Postdose	67.4 ± 29.5	45.7 ± 22.2	47.9 ± 19.4	49.5 ± 16.3	24.9 ± 15.7	30.5 ± 17.0
% Change	655.6 ± 406.8*	577.5 ± 406.3*	498.6 ± 280.5*	399.6 ± 207.0*	150.6 ± 99.9*	229.2 ± 143.1*
Plasma tHcy, <i>μmol/L</i>						
Baseline	11.0 ± 3.7	29.2 ± 19.2	15.3 ± 8.3	10.3 ± 4.0	35.9 ± 27.5	14.1 ± 7.1
Postdose	10.0 ± 3.4	27.8 ± 18.2	14.5 ± 7.7	9.6 ± 3.9	32.9 ± 25.7	12.1 ± 6.1
% Change	-8.2 ± 12.5	-4.1 ± 11.8	-3.7 ± 21.6	-6.1 ± 11.8	-8.2 ± 11.5	-12.5 ± 16.3
Plasma folate, <i>nmol/L</i>						
Baseline	15.9 ± 5.0	10.4 ± 3.7	13.9 ± 7.0	15.7 ± 4.5	11.1 ± 4.2	12.8 ± 7.1
Postdose	16.4 ± 5.5	10.6 ± 3.6	13.0 ± 6.9	14.8 ± 4.2	10.9 ± 4.2	11.5 ± 6.9
% Change	9.8 ± 42.5	3.4 ± 17.0	-6.2 ± 20.3*	-3.0 ± 24.5	-1.1 ± 16.6	-8.6 ± 20.6*

<sup>1</sup> Values are means ± SD. \*Significant change,  $P < 0.05$ .

<sup>2</sup> Individual data are in Supplemental Figure 1.

## Discussion

We have demonstrated the usefulness of the Bor et al. (15) protocol to investigate gastrointestinal absorption of vitamin B-12 in participants with low circulating vitamin B-12 concentrations. The absolute rise in holo-TC in our participants (44 pmol/L) was comparable to that in vitamin B-12-sufficient Europeans (46 pmol/L) (15); however, the percentage rise was much higher (~550% compared with ~50%) because of lower baseline concentrations (median 7 pmol/L compared with 72 pmol/L). Our findings provide experimental evidence that vitamin B-12 absorption is adequate in the majority of Indians and argue against malabsorption as a prominent cause of their low vitamin B-12 concentrations. Furthermore, it was reassuring that a physiological dose of cyanocobalamin ( $2 \mu\text{g} \times 3$ ) was absorbed satisfactorily, suggesting that a modest increase in vitamin B-12 intake should improve their status.

Vitamin B-12 in food is protein bound. It is released in the stomach by the action of pepsin and gastric acid and then combines with salivary R-binder. This complex is broken down by pancreatic proteases in the duodenum to release free B-12, which combines with intrinsic factor secreted by gastric parietal cells (30). The intrinsic factor-vitamin B-12 complex interacts with a specific receptor in terminal ileal enterocytes and is absorbed by endocytosis (31,32). During transport across the enterocyte, vitamin B-12 is complexed with transcobalamin to form holo-TC, which is released into the blood (31). Unlike protein-bound vitamin B-12 in food (33), the free vitamin B-12 (cyanocobalamin) used in our study binds directly to R-binder, bypassing the stage of gastric degradation. From this stage onwards, vitamin B-12 from food and free B-12 follow the same absorption pathway. Thus, our study does not test gastric degradation but suggests normal handling beyond this stage. Holo-TC has a short half-life (34,35) and is therefore proposed to reflect recent vitamin B-12 absorption. However, circulating holo-TC concentrations are also influenced by hepatic and renal uptake, production, and release from the ileum and kidney, tissue requirements, and other unknown factors (36). There is a debate about whether basal holo-TC concentrations represent 'an early general cobalamin insufficiency or specifically decreased cobalamin absorption' (36). A dose-dependent rise in holo-TC during the 24 h following vitamin B-12 administration suggests that the levels are influenced by recent absorption.

The rise in holo-TC was ~28% lower ( $P = 0.005$ ) in those with low vitamin B-12 concentrations compared with that in those with normal vitamin B-12 concentration. This raises the possibility that malabsorption may contribute to low plasma vitamin B-12 concentrations in this population. It would be interesting to measure the rise in holo-TC before and after vitamin B-12 repletion to confirm whether adequate vitamin B-12 status changes vitamin B-12 absorption.

Fewer than 10% of the participants in our study had a lower than expected rise in holo-TC based on the Bor et al. protocol (15). This may be evidence of poor vitamin B-12 absorption in these individuals, but because the Bor protocol was developed in populations with normal vitamin B-12 status, whether our findings in some participants were due to malabsorption needs further investigation (37). However, the original test using a dose of  $9 \mu\text{g} \times 3$  has been validated in inherited syndromes of vitamin B-12 malabsorption (38). In our population, more parents (12%) than children (6%) had a lower than expected rise in holo-TC, which may be due to the relatively lower dose of vitamin B-12 per kilogram body weight they received. Vitamin B-12 absorption also decreases with age (39,40) and deficiency is

common in the elderly, even in Western nonvegetarian populations (41). One of the proposed mechanisms is an increasing prevalence of chronic gastritis with age, which results in hypochlorhydria and impaired synthesis of intrinsic factor and pepsin. The etiology of chronic gastritis includes autoimmune damage (pernicious anemia) and *H. pylori* infection (12,13,31). Further studies are required to identify the cause(s) of the low plasma vitamin B-12 in these participants.

Our study has many strengths. It was community based, had a large sample size, high participation rates, and low drop-out rates. We enrolled children as well as adults and included both genders. None of the participants were taking vitamin supplements and care was taken to ensure no interference from diet during the test period by requiring that vegetarian diets be consumed. Participants were admitted to our research unit to ensure that the test was conducted under strict supervision. In addition to the diagnostic dose, we also studied absorption of vitamin B-12 at a dose close to the physiological level, which may be important in guiding public health policy. Our study cannot be strictly compared with Bor et al. (15), because we used  $10 \mu\text{g} \times 3$  doses instead of  $9 \mu\text{g} \times 3$  doses because of availability. We do not think that the slightly higher dose is likely to change our conclusions, because absorption was similarly impressive for the  $2 \mu\text{g} \times 3$  doses. A definite limitation of the study is that because we used free cyanocobalamin, we could not test the gastric degradation step, which is essential for absorption of protein-bound dietary vitamin B-12 (33). However, the test would probably be able to detect the malabsorption related to tropical sprue or gastrointestinal infections, which are thought to contribute to subclinical vitamin B-12 status in tropical areas. The holo-TC test is simple, relatively inexpensive, and has the potential to be used in population-based studies, but further work regarding dosing and validation against a gold standard test would be desirable.

Thus, we have demonstrated adequate intestinal absorption of free cyanocobalamin in a community-based study in a South Asian Indian population with a high prevalence of low vitamin B-12 concentrations. The health implications of low vitamin B-12 concentrations are not fully understood, because there is poor association with macrocytosis or severe anemia, or neurological symptoms, and also because of the fact that this population has perhaps lived with such low concentrations for generations. This issue needs to be further investigated. This study is a small part of such a systematic program of research. Our results suggest that the gastro-intestinal malabsorption of the vitamin is unlikely to be a prominent cause of the high prevalence of low vitamin B-12 concentrations and the consequent hyperhomocysteinemia in children and young adults, although this could be a contributing factor in middle-aged and elderly adults. The marked increase in plasma concentrations following even low doses of vitamin B-12 suggests that fortification of food items or regular use of supplements would be useful approaches. The results of such long-term supplementation in this population are described in a separate paper.

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