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Genetic and environmental factors associated with vitamin B_{12} status in Amazonian children

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Abstract

Objective: To evaluate the prevalence of vitamin B_{12} deficiency and factors associated with vitamin B_{12} status in Amazonian children.
Design: Genetic risk score (GRS), socio-economic and nutritional status, and morbidity data were the independent variables used in multiple linear regression models to evaluate factors associated with vitamin B_{12} status in a population-based cross-sectional study. GRS was created by summing a number of known risk alleles for low serum vitamin B_{12}.
Setting: Acrelândia, western Brazilian Amazon.
Subjects: Children (*n* 988) aged <10 years.
Results: Overall prevalence of vitamin B_{12} deficiency (<150 pmol/l) was 4·2 (95 % CI 3·0, 5·6) % and was highest in children aged <24 months: 13·6 (95 % CI % 8·8, 19·7) %. For children <24 months, wealth index ($\beta = 0{\cdot}017$, $P = 0{\cdot}030$) and animal protein intake ($\beta = 0{\cdot}219$, $P = 0{\cdot}003$) were positively associated with vitamin B_{12} status. GRS ($\beta = -0{\cdot}114$, $P < 0{\cdot}001$) and serum homocysteine ($\beta = -0{\cdot}049$, $P < 0{\cdot}001$) were negatively associated. Among children aged ≥24 months, vitamin B_{12} status was positively associated with wealth index ($\beta = 0{\cdot}012$, $P < 0{\cdot}001$), height-for-age *Z*-score ($\beta = 0{\cdot}024$, $P = 0{\cdot}033$) and serum vitamin A ($\beta = 0{\cdot}089$, $P < 0{\cdot}001$). Age ≥ 60 months ($\beta = -0{\cdot}118$, $P < 0{\cdot}001$), GRS ($\beta = -0{\cdot}048$, $P < 0{\cdot}001$), maternal schooling <5 years ($\beta = -0{\cdot}083$, $P < 0{\cdot}001$), low intake of animal-derived foods ($\beta = -0{\cdot}050$, $P = 0{\cdot}030$), serum homocysteine ($\beta = -0{\cdot}053$, $P < 0{\cdot}001$), serum folate ≥23·6 nmol/l ($\beta = -0{\cdot}055$, $P = 0{\cdot}012$) and geohelminth infection ($\beta = -0{\cdot}141$, $P = 0{\cdot}017$) were negatively associated with vitamin B_{12} status.
Conclusions: GRS, poverty, low intake of animal-derived foods, geohelminth infection, vitamin A and folate status were important factors associated with vitamin B_{12} status of children in our study.

Keywords
Vitamin B_{12}
Child health
Nutritional status
Genetic polymorphism
5,10-Methylenetetrahydrofolate reductase
Fucosyltransferase 2 protein

Vitamin B_{12} plays an important role in haematopoiesis and nervous system development, and as a cofactor it participates in the conversion of methylmalonyl CoA to succinyl CoA and of homocysteine to methionine[1,2].

Children are at increased risk of vitamin B_{12} deficiency, particularly in the first 6 months of life when the lowest serum vitamin B_{12} concentrations are seen. Levels increase again from 6 months reaching a peak at 3–7 years, and thereafter the concentrations decrease gradually to those observed in adults[3]. The main cause of vitamin B_{12} deficiency in infants is low vitamin B_{12} content in the breast milk of vitamin B_{12}-deficient mothers[2]. The most common manifestations of severe deficiency in infants are failure to thrive, developmental delay[2,4], convulsions and weakness[5]. In older children, other factors may be associated with vitamin B_{12} deficiency, such as the absence of animal-derived foods or fortified foods[6], a vegetarian diet[7], low socio-economic level[8] and infection by gastrointestinal parasites[9]. Clinical presentations of vitamin B_{12} deficiency include erythrocyte deformability[10] and neurological changes, which can occur in the absence of haematological abnormality[11]. The early diagnosis and treatment of vitamin B_{12} deficiency in infants and children is important as long-term deficiency can cause developmental delay, failure to thrive, and clinical and neurological symptoms that can be irreversible[3,5].

† See Appendix for full list of members of the ACTION Study Team.

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Estimates of the global prevalence of vitamin B_{12} deficiency in childhood are scarce and vary widely according to geographic location and threshold used, ranging from 8 % to 30 % among infants or children <6 years of age[4,12,13] and from 1·6 % to 32·5 % in older children[6,8].

In addition to extrinsic factors, analysis of the contribution of polymorphisms in genes involved in B-vitamin metabolism may be helpful in providing further information about the predictors of vitamin B_{12} status early in life[14]. Recent genome-wide association studies have shown that genetic polymorphisms can influence serum vitamin B_{12} concentrations[15–17]. Hazra *et al.*[16] demonstrated that women homozygous for the rs492602 G allele of fucosyltransferase 2 (*FUT2*) had higher vitamin B_{12} concentrations. Other common *FUT2* variants such as rs602662 and rs601338 were also shown to be associated with levels of vitamin B_{12}[15,17,18]. The most commonly studied polymorphisms of enzymes involved in folate and homocysteine metabolism that are also dependent on vitamin B_{12} metabolism are mutations in the gene encoding the 5,10-methylenetetrahydrofolate reductase (MTHFR) enzyme (mutations identified are 677 C→T and 1298 A→C)[19,20]. This enzyme catalyses the biologically irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. 5-Methyltetrahydrofolate is converted by the cobalamin-dependent methionine synthase reductase (MTRR; mutation identified: 66 A→G) to tetrahydrofolate[21]. Methionine synthase and 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR; mutation identified: 2756 A→G) are required for the remethylation of homocysteine to methionine[19]. Studies have shown that the TT genotype of the *MTHFR* C677T variant and the CC genotype of the *MTHFR* A1298C variant are associated with low serum vitamin B_{12}, and also with low serum folate and high homocysteine concentrations[22,23].

The present study describes the prevalence of vitamin B_{12} deficiency and factors associated with vitamin B_{12} status in Amazonian children. To our knowledge, the study is the first to report vitamin B_{12} status, including genetic factors, in Brazilian children.

Materials and methods

Study area and population

The population-based, cross-sectional study described here was performed in 2007 in Acrelândia, a frontier town located 112 km from Rio Branco, the capital of the state of Acre, in the western Brazilian Amazon region. By 2007 Acrelândia had 11 520 inhabitants of whom 44 % resided in the urban area. Sampling strategies and field procedures were as previously reported[24]. Briefly, all households from the urban area with children up to 10 years of age (*n* 749) were identified. This resulted in 1225 children living in 734 households being enrolled in the study.

A structured questionnaire, pilot-tested previously, was administered through face-to-face interview to the mothers or guardians of 1151 children (94·0 % of those eligible). It included demographic characteristics (child's sex, age and race/ethnicity, classified as white, black, 'pardo' (brown), yellow or indigenous, according to skin colour, as used in the Brazilian census[25]), socio-economic status and environmental conditions, reproductive health variables, history of infant feeding practices, frequency of habitual food intake and morbidities.

The study protocol was approved by the institutional review board of the School of Public Health, University of São Paulo, Brazil (No. 1681/07) and it was conducted according to the guidelines laid down in the Declaration of Helsinki. Written informed consent was obtained from all parents or guardians of participating children prior to enrolment.

Anthropometric assessment

Anthropometric measurements were performed by trained research assistants following standardized procedures using calibrated equipment[26]. Among children aged <24 months, recumbent length was measured using a locally made infant measuring board; weight was measured with an electronic paediatric scale (model 1583; Tanita, Tokyo, Japan). Among children aged ≥24 months, height was measured using a stadiometer (model 208; SECA, Hamburg, Germany) and weight was measured using an electronic scale (model HS-302; Tanita, Tokyo, Japan). Each measurement was repeated and the mean value was calculated. *Z*-scores for length/height-for-age (HAZ) and BMI-for-age (BAZ) were calculated according to WHO guidelines[27]. The cut-off defined for stunting was HAZ <−2 and that for overweight was BAZ >1[28].

Dietary assessment

For children <24 months, a diet history[29] was collected by trained nutritionists. The interviewers were provided with household measures to help mothers or guardians estimate the habitual amounts of foods or beverages. The World Food Dietary Assessment System (version 2·0; University of California, USA) was used to estimate food intake. For children ≥24 months, an FFQ, based on a validation study in this area[30], was used to estimate the frequency of food consumption (fruit, green vegetables, root vegetables, dairy, beans, meat, eggs and fish) within the last month.

Biochemical measures

Approximately 5 ml of fasting venous blood was collected from 1131 children (98·3 % of those eligible) by trained phlebotomists. Serum folate and vitamin B_{12} concentrations were measured using commercial fluoroimmunoassays (Perkin Elmer, Wallac Oy, Turku, Finland). The cut-offs for vitamin B_{12} and folate deficiency were <150 pmol/l and <10 nmol/l[31], respectively. Plasma homocysteine and

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serum vitamin A concentrations were determined by HPLC (Shimadzu, Kyoto, Japan) with fluorimetric detection and isocratic elution[32]. Vitamin A concentrations <0·70 μmol/l were used to define vitamin A deficiency[33]. Anaemia, Fe deficiency and Fe-deficiency anaemia were defined according to Hb, serum ferritin and soluble transferrin receptor concentrations, respectively[24,34]. The normal range of soluble transferrin receptor concentration, as determined by the immunoassay manufacturer, was 2·9–8·3 mg/l. Fe deficiency was defined when serum ferritin concentrations were low (<12 μg/l for children aged <5 years or <15 μg/l for those ≥5 years) or when soluble transferrin receptor concentrations were high (>8·3 mg/l). Fe-deficiency anaemia was defined when Fe deficiency occurred in anaemic children; the cut-off for Hb concentration considered was 110·0 g/l for children aged 6 months to 5 years, and 115 0 g/l for children ≥5 years. Plasma C-reactive protein concentration was measured using the Immulite high-sensitivity chemiluminescent assay (DPC, Los Angeles, CA, USA). The cut-off for high C-reactive protein as an indicator of inflammation was >5 mg/l[35].

Stool samples were collected from 1016 children (97·0 % of those eligible) and analysed for eggs, cysts and larvae of parasites, according to the qualitative technique of sedimentation[36], as described elsewhere[24]. Geohelminths found in this population included *Ascaris lumbricoides*, *Trichuris trichiura* and *Strongyloides stercoralis*. Children with anaemia, nutritional deficiencies or intestinal parasitic infections received free treatment prescribed by the research clinicians.

Genotyping

SNP genotyping was performed using allele-specific PCR with the molecular beacons assay[37], under contract by Prevention Genetics (Marshfield, WI, USA). SNP included those in folate-metabolizing enzyme-encoding genes: *MTHFR* C677T (rs1801133), *MTHFR* A1298C (rs1801131), *MTR* A2756G (rs1805087), *MTRR* A66G (rs1801394) and reduced folate carrier gene (*RFC1*) G80A (rs1051266), as well as *FUT2* AG (rs492602).

The homogeneous assay used two-tailed allele-specific primers, a common reverse primer and two different fluorescently labelled universal primers in a single-well reaction. Submicrolitre PCR reactions were carried out with Array Tape instrumentation and allele calls were generated based on clustering of fluorescent signals[38]. The internal quality of genotype data was assessed by typing 10 % of blinded samples in duplicate; the resulting concordance was >99 %. Allelic and genotypes frequencies for each SNP were calculated from the Hardy–Weinberg equilibrium ($P > 0{\cdot}05$) using an available online tool.

Statistical analysis

Children were stratified into age categories (<24, ≥24–60 and ≥60 months) for the descriptive analyses in which covariates are reported as absolute frequencies and percentages or as medians and interquartile ranges.

The outcome of interest was serum vitamin B_{12} concentration (natural log-transformed). Explanatory variables comprised the above described polymorphisms, socio-economic status, maternal and child characteristics, diet, morbidities and biochemical indicators. The definitions of variables are as follows.

A genetic risk score (GRS) was developed based on polymorphisms in genes encoding the folate-metabolizing enzymes and in the *FUT2* gene. One-way ANOVA was tested for multiple comparisons of means between serum vitamin B_{12} concentrations for each genetic polymorphism. Mean differences with P value ≤0·10 for low vitamin B_{12} concentration were observed for *MTHFR* C677T, *MTHFR* A1298C and *FUT2* AG, which were selected to comprise the GRS. A code value was then assigned to each gene, ranging from 0 for the lowest-risk allele to +1 for heterozygote and +2 for the increased-risk allele, according to the present study. The GRS for each individual was created by summing these values for each SNP in the GRS. GRS was examined as a continuous variable.

Principal component analysis was used to derive a wealth index representing a proxy of household income[39], based on the presence of twelve household assets, as described elsewhere[24]. The wealth index was used as a continuous variable.

Maternal schooling was categorized as <5 years *v.* ≥5 years. Maternal age at the child's birth was categorized as ≥20 years *v.* <20 years and the child's birth weight as <2500 g *v.* ≥2500 g.

Regarding dietary information, for children <24 months of age, we quantified animal-derived protein in g/d from breast milk, cow's milk and dairy products, eggs, meat, fish and chicken. This variable was then dichotomized, according to tertiles, as low intake (first tertile, <13·5 g/d) *v.* high intake (second tertile, 13·5–30·0 g/d; and third tertile, ≥30·0 g/d). For older children (≥24 months), we created a score for animal-derived food (ADF) intake based on the FFQ, as follows. The frequencies of dairy products, meat and egg consumption were grouped and coded into three categories: 0 = low consumption (rarely/never; 1–3 times/month; 1–3 times/week; 4–6 times/week); 1 = intermediate consumption (1 time/d); and 2 = high consumption (≥2 times/d). The ADF score was created by summing the codes for each child, ranging from 0 to 6. In order to quantify whether the low consumption of ADF contributes to vitamin B_{12} variability, the ADF score was then dichotomized into below the median (<4) *v.* above the median (≥4).

Indicators of morbidities were presence of geohelminth infection and reported diarrhoea in the past 15 d; plasma C-reactive protein >5 mg/l was used as an indicator of inflammation.

Crude and multiple linear regression models were conducted separately for children aged <24 months and for those ≥24 months of age, due to the different methods

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of collecting dietary data, as stated in 'Dietary assessment'. Crude linear regression analyses were first conducted between the outcome, serum vitamin B_{12} concentration, and the covariates. The covariates were first selected for the multiple models using $P < 0{\cdot}20$, adjusted by sex and age, following a hierarchical conceptual approach[24], and were retained in the final model if they were associated with the outcome at $P < 0{\cdot}10$. Missing observations were included by creating missing-value categories. We compared results from the model with missing-value categories with those from a complete case analysis. Because the magnitudes and directions of all associations were similar, we decided to preserve all children in the multiple models. Interaction terms between GRS and biochemical measures and the outcome were tested in the models. *P* values reported are two-sided. All analyses were performed using the statistical software package Stata version 11·0.

Results

Of the 1151 participants, serum vitamin B_{12} was measured for 988 (85·8 %). Of these, the mean age was 5·2 (SD 2·8) years (range: 2·8 months to 10·4 years). Only 13·5 % of children were exclusively breast-fed until 6 months of age. Table 1 shows the characteristics of these children. The prevalence of stunting (12·0 %) and overweight (30·5 %) was higher in children aged <24 months, and this age group also saw a highest prevalence of anaemia and Fe deficiency. In addition, they presented the highest prevalence of vitamin B_{12} deficiency: 13·6 (95 % CI 8·8, 19·7) %. The overall prevalence of vitamin B_{12} deficiency was 4·2 (95 % CI 3·0, 5·6 %) % and the prevalence of vitamin B_{12} insufficiency (<221 pmol//l) was 31·7 (95 % CI 28·8, 34·6) %. Only 2·6 % of children had a low plasma folate concentration, while vitamin A deficiency was found in 14·1 % of children.

Table 1 Characteristics of urban children aged <10 years included in the study according to age group, Acrelândia, western Brazilian Amazon, 2007

	All (*n* 988)*		<24 months (*n* 169)		24–60 months (*n* 301)		60–120 months (*n* 518)	
Variable	*n* or Median	% or IQR	*n* or Median	% or IQR	*n* or Median	% or IQR	*n* or Median	% or IQR
Sociodemographic characteristics, *n* and %								
Child's sex								
Male	488	49·4	94	55·6	144	47·8	250	48·3
Race/ethnicity								
White	88	9·6	14	8·7	30	10·6	44	9·3
Black	46	5·0	10	6·3	11	3·9	25	5·3
Brown	781	85·4	136	85·0	241	85·5	404	85·4
Wealth index (quartile)								
First (low)	751	76·0	120	71·0	223	74·0	408	78·8
Others (highest)	237	24·0	49	29·0	78	26·0	110	21·2
Maternal schooling (<5 years)	373	39·1	54	33·1	101	35·0	218	43·3
Maternal age at child's birth (<20 years)	255	28·3	32	20·0	61	22·8	162	34·2
Children's characteristics, *n* and %								
Low birth weight (<2500 g)	51	5·8	9	5·6	14	5·0	28	6·4
Stunting	53	5·4	20	12·0	12	4·0	21	4·1
Overweight or obesity	147	15·0	51	30·5	49	16·4	47	9·1
Biochemical nutritional indicators								
Serum vitamin B_{12} (pmol/l)								
Median and IQR	257·5	207–319	233·0	175–296	277·0	225–360	250·0	208–307
<150 pmol/l (deficiency), *n* and %	41	4·2	23	13·6	9	3·0	9	1·7
<221 pmol/l (marginal), *n* and %	331	31·7	77	45·6	73	24·2	163	31·5
Serum vitamin A (μmol/l)								
Median and IQR	1·16	0·88–1·50	1·16	0·92–1·52	1·17	0·88–1·53	1·16	0·88–1·50
<0·70 μmol/l, *n* and %	138	14·1	23	13·9	39	13·1	76	14·8
Serum folate (nmol/l)								
Median and IQR	23·4	17·7–30·7	22·7	17·1–32·1	23·2	17·4–30·5	23·6	17·9–30·1
<10 nmol/l, *n* and %	26	2·6	7	4·1	7	2·3	12	2·3
Serum homocysteine (μmol/l)								
Median and IQR	6·65	5·88–7·80	7·92	6·31–10·86	6·25	5·54–7·30	6·69	5·98–7·62
Anaemia†, *n* and %	133	13·7	66	44·0	33	11·0	34	6·6
Fe deficiency‡, *n* and %	445	45·0	138	81·7	170	56·5	137	26·5
Fe-deficiency anaemia§, *n* and %	100	10·3	62	41·3	25	8·3	13	2·5
Morbidities, *n* and %								
C-reactive protein > 5 mg/l	94	9·9	25	15·4	28	9·8	41	8·2
Geohelminth infection	36	4·1	4	2·7	6	2·2	26	5·6
Diarrhoea in the past 15 d	227	23·2	75	44·6	74	24·7	78	15·2

IQR, interquartile range.
*Total may be less because of missing values.
†Cut-off for anaemia: Hb < 110·0 and <111·5 g/l for children 6–59 months and ≥60 months, respectively.
‡Serum ferritin concentration <12 μg/l for children <59 months or <15 μg/l for those aged ≥60 months, or serum transferrin receptor concentration >8·3 mg/l.
§Fe-deficiency anaemia was defined when Fe deficiency occurred in anaemic children.

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Mean animal-derived protein intake was 24·4 (SD 17·7) g/d in younger children. Overall, 50·3 %, 55·1 % and 35·7 % of children aged ≥24 months were observed to be in the high consumption category (≥2 times/d) for milk, meat and eggs, respectively (data not shown).

Gene allele distributions are described in Table 2. Based on the estimated risk for low serum vitamin B_{12} concentrations, the mutant allele for *MTHFR* C677T, the wild-type allele for *MTHFR* A1298C and the wild-type allele for *FUT2* were established as increased-risk alleles.

Table 3 shows the factors associated with serum vitamin B_{12} adjusted for sex and age among younger children. The final model explained 44 % of the variability in natural log-transformed B_{12} concentrations. Wealth index and animal-derived protein intake were positively associated with vitamin B_{12} status, whereas serum homocysteine level and GRS were negatively associated. Among older children (Table 4), vitamin B_{12} status was positively associated with wealth index, HAZ and serum vitamin A; age, low maternal schooling, serum homocysteine, serum folate, geohelminth infection, low ADF consumption and GRS were negatively associated with vitamin B_{12} status. A significant interaction was found in this age group between GRS and homocysteine ($P = 0·020$).

Discussion

Overall, the prevalence of vitamin B_{12} deficiency found in the present study was 4·2 %, with the highest proportion in children aged <24 months (13·6 %). This latter prevalence is higher than that observed in a national study among Mexican children aged 3 years (3·3 %)[12] and in Venezuelan children (9·7 %)[40]. Highest prevalence was found in other developing countries, such as 30 % in Guatemala[13] and 27 % in India[4], where children have low dietary intake of animal products or fruits and vegetables due to poverty or strictly vegetarian mothers, resulting in poor vitamin B_{12} concentrations in breast milk. In older children (≥24 months), the prevalence reported in our study (2·2 %) was much lower than that observed in Indian children (17·4 %)[41] or in Kenyan children (32·5 %)[6], but similar to that observed in Colombian children (1·6 %)[8].

In our study, only 2·6 % of children had folate deficiency, which suggests that mandatory folate fortification of wheat flour implemented in Brazil since 2003 is proving effective. However, the prevalence of vitamin B_{12} deficiency was higher among children <24 months of age. This might occur by the fact that infants and young children are at increased risk for vitamin B_{12} deficiency

Table 2 Gene allele distribution in urban children aged <10 years (*n* 988), Acrelândia, western Brazilian Amazon, 2007

Gene	SNP	Wild (A)	Mutant (a)	Frequency AA/Aa/aa*
MTHFR C677T	rs180133	C	T†	47·5/41·4/11·1
MTHFR A1298C	rs180131	A†	C	58·8/37·7/3·5
MTR A2756G	rs1805087	A	G	65·5/30·7/3·8
MTRR A66G	rs1801394	A	G	40·8/47·1/12·1
RFC1 G80A	rs1051266	G	A	15·8/50·7/33·5
FUT2 AG	rs492602	A†	G	36·9/48·7/14·4

*Total may be less because of missing values.
†Increased-risk allele for low serum vitamin B_{12} concentration according to the present study. Mean differences in serum vitamin B_{12} concentrations for *MTR* A2756G, *MTRR* A66G and *RFC1* G80A were not observed according to allele.

Table 3 Factors associated with vitamin B_{12} status in urban children aged <24 months, Acrelândia, western Brazilian Amazon, 2007

	Serum vitamin B_{12} concentration (*n* 114)*			
Independent variable	Adjusted β coefficient†	95 % CI	*P*	R^2‡
Polymorphisms§				0·441
GRS (continuous)	−0·114	−0·163, −0·064	<0·001	
Socio-economic status				
Wealth index (continuous)	0·017	0·001, 0·032	0·030	
Diet				
Animal protein intake (g/d)				
1st tertile (<13·5)	Ref.			
2nd tertile (13·5–30·0) + 3rd tertile (≥30·0)	0·219	0·074, 0·365	0·003	
Biochemical indicators				
Plasma homocysteine (μmol/l)	−0·049	−0·065, −0·033	<0·001	

GRS, genetic risk score; Ref., referent category.
*Dependent variable, serum vitamin B_{12}, was natural log-transformed before analysis; total may be less because of missing values.
†The model was adjusted for sex and age (continuous).
‡Final adjusted *R*-squared.
§GRS was calculated on the basis of three polymorphisms (*MTHFR* C677T, *MTHFR* A1298C and *FUT2* AG) representing increased-risk alleles.
Interaction term, GRS × homocysteine: $P = 0·053$.

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Table 4 Factors associated with vitamin B_{12} status in urban children aged ≥24 months, Acrelândia, western Brazilian Amazon, 2007

	Serum vitamin B_{12} concentration (n 747)*			
Independent variable	Sex-adjusted β coefficient	95 % CI	*P*	R^2†
Child's age (months)				0·192
24–60	Ref.			
≥60	−0·118	−0·164, −0·072	0·001	
GRS (continuous)‡	−0·048	−0·067, −0·028	<0·001	
Socio-economic status				
Wealth index (continuous)	0·012	0·006, 0·018	<0·001	
Maternal schooling (years)				
≥5	Ref.			
<5	−0·083	−0·132, −0·034	0·001	
Nutritional status				
HAZ (continuous)	0·024	0·002, 0·046	0·033	
Diet				
Score for ADF consumption				
≥4	Ref.			
<4	−0·050	−0·096, −0·004	0·030	
Morbidities				
Geohelminth infection				
Negative	Ref.			
Positive	−0·195	−0·313, −0·077	0·001	
Biochemical indicators				
Plasma homocysteine (μmol/l)	−0·053	−0·067, −0·038	<0·001	
Serum vitamin A (μmol/l)	0·089	0·047, 0·131	<0·001	
Serum folate, median (nmol/l)				
<23·6	Ref.			
≥23·6	−0·055	−0·098, −0·012	0·012	

GRS, genetic risk score; HAZ, height-for-age *Z*-score; ADF, animal-derived food; Ref., referent category.
*Dependent variable, serum vitamin B_{12}, was natural log-transformed before analysis.
†Final adjusted *R*-squared.
‡GRS was calculated on the basis of three polymorphisms (*MTHFR* C677T, *MTHFR* A1298C and *FUT2* AG) representing increased-risk alleles. Interaction term, GRS × homocysteine: $P = 0·020$.

and the most common factors that contribute to this are poor maternal nutritional status during pregnancy; this causes a lower micronutrient concentration of breast milk and influences the infant's stores of the vitamin[5,42,43]. Furthermore, exclusive breast-feeding for long periods (over 6 months of age) followed by the introduction of inadequate complementary foods lacking sufficient vitamin B_{12} can worsen the deficiency[9].

In our analysis, the GRS was negatively associated with serum vitamin B_{12} status. We observed that children with polymorphisms for the mutant *MTHFR* C677T allele and wild-type alleles in *MTHFR* A1298C and *FUT2* had the lowest mean serum vitamin B_{12}. Based on these findings, the GRS was created. The mutations in *MTHFR* (677 C→T and 1298 A→C) result in a thermolabile enzyme that impairs the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. The latter is the circulating and active form of folate[19]. Under normal conditions, 5-methyltetrahydrofolate is essential for the conversion of homocysteine to methionine, which involves the vitamin B_{12}-dependent enzyme methionine synthase reductase[19].

As previously reported, the 677T variant is associated with low plasma folate levels and hyperhomocysteinaemia[23]. In contrast, some studies have shown that low serum vitamin B_{12} was significantly associated with the TT genotype of the *MTHFR* C677T polymorphism[22] and with the CC genotype of the *MTHFR* A1298C polymorphism[23]. However, Huemer *et al.*[44] found no significant difference in vitamin B_{12} concentrations between either genotype.

Data on the prevalence and significance of the recently described *FUT2* polymorphism and its relationship to vitamin B_{12} are scarce[15–17]. In our sample, children homozygous for the G allele of rs492602 had higher vitamin B_{12} concentrations, as seen in a study conducted on women of self-reported European ancestry[16]. The secretor enzyme α-1,2-fucosyltransferase, encoded by *FUT2*, catalyses the addition of fucose to form H type 1 and H type 2 antigens[45]. A possible mechanism that has been suggested for the association between *FUT2* and low vitamin B_{12} concentration is that individuals with *FUT2* polymorphisms are more susceptible to *Helicobacter pylori* infection than those with the non-secretor status[16,18]. This could lead to reduced secretion of intrinsic factors and consequently to vitamin B_{12} malabsorption[46]. In contrast, Oussalah *et al.*[47], who evaluated the *FUT2* 461 G→A (rs601338) polymorphism in two different populations, found associations with plasma vitamin B_{12} concentration but no association with positive *H. pylori* serologic status. More recently, Chery *et al.*[48] demonstrated that individuals carrying the *FUT2* secretor variant

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who were also heterozygous for a *GIF* mutation had low vitamin B_{12} concentration independent of *H. pylori*-related gastritis. Unfortunately, in our study we could not assess the *H. pylori* infection status to better explore this relationship. More studies are necessary to elucidate the influence of *FUT2* on cobalamin concentrations.

In the present study, wealth index and maternal schooling were associated with serum vitamin B_{12} concentrations. As in other developing countries, socio-economic status is an important determinant of both deficient and marginal serum vitamin B_{12} concentrations[8,40], where the consumption of ADF is limited because of high costs and/or cultural and religious beliefs[9].

In our analyses, the lowest tertile of animal protein intake in children <24 months, as well as the lowest score of ADF in older children, were associated with vitamin B_{12} status after adjusting for other variables. The quality of diet among young Amazonian children has previously been assessed[28]. These authors found that, from an early age, this group has low intakes of fruit, vegetables and ADF, and substantial consumption of unhealthy foods (almost a third of them had already experienced cookies, sweet bread and instant noodles among other processed foods), which may partially explain the higher prevalence of vitamin B_{12} deficiency in younger children in our study.

Another factor that contributes to cobalamin deficiency because of poor absorption is intestinal parasite infection[9]. In our study, no sanitation system was available in the town[24] and cases of geohelminth infection were noted despite routine distribution of anti-helminthic medication under the Family Health Program of the municipality[24]; such infection was negatively associated with serum vitamin B_{12} concentration in children older than 24 months as this age group is at higher risk for intestinal parasite infection.

As expected, plasma homocysteine was negatively associated with serum vitamin B_{12} in the present study, which is consistent with other studies in children[49]. Impaired folate or cobalamin function in tissues leads to high plasma homocysteine levels[50]; however, because vitamin B_{12} deficiency is becoming more prevalent than folate deficiency[8], it can be said that vitamin B_{12} constitutes an important modifiable risk factor for hyperhomocysteinaemia[51].

Vitamin A deficiency prevalence was 14·1 %, which is considered a moderate public health problem by the WHO[33]. Moreover, serum vitamin A was strongly associated with serum vitamin B_{12} status in older children. Our sample consisted of low-income children who, in the presence of an inadequate diet since early childhood[28], frequent exposure to infections and insufficient basic sanitation and water treatment, have a compromised nutritional status. It is noteworthy that animal-source foods contain large amounts of retinol (preformed vitamin A)[52] as well as vitamin B_{12}, so deficiency becomes prevalent when the intake of these foods is low[1,52]. Although plasma retinol is not considered a good biomarker for dietary intake because it is tightly regulated by the mobilization of hepatic reserves[53], plasma retinol nevertheless increases rapidly when vitamin A-deficient children are fed dietary vitamin A[46] or foods fortified with vitamin A[54]. Thus, a good vitamin A nutritional status can also reflect the nutritional status of vitamin B_{12}. This may explain the positive association between vitamin A and vitamin B_{12} concentrations in our analysis.

Our study has limitations that should be considered. Because of its cross-sectional design, caution should be taken in interpreting the findings. In addition, we did not investigate methylmalonic acid levels, a sensitive marker for clinical cobalamin deficiency, or mutations in genes related to the transport of vitamin B_{12}. Despite these limitations, the study has yielded estimates of factors associated with serum vitamin B_{12}, including the joint effects of genetic polymorphisms, in a population-based study with children living in poor conditions.

Conclusion

We found a non-negligible prevalence of vitamin B_{12} deficiency in young Amazonian children. The factors associated with vitamin B_{12} status were genetic factors, poverty, low consumption of ADF, geohelminth infection, and vitamin A and folate status. Early diagnosis of vitamin B_{12} deficiency is important to prevent long-term adverse consequences. More effective public health policies to promote accessibility to and consumption of healthy foods are necessary to improve vitamin B_{12} status of young children.

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version submitted for publication. *Ethics of human subject participation:* The study was conducted according to the guidelines laid down in the Declaration of Helsinki. The institutional review board of the School of Public Health, University of São Paulo, Brazil (No. 1681/07) approved the study protocol. Written informed consent was obtained from all parents or guardians of participating children prior to enrolment.

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Appendix

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