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Identification of vitamin B_{12} deficiency in vegetarian Indians

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Abstract

The prevalence of a sub-clinical vitamin B_{12} deficiency in the vegetarians is high. Total serum vitamin B_{12} concentration alone does not reliably reflect vitamin B_{12} status. Holotranscobalamin (holo-TC) II is a bioactive B_{12} fraction promoting specific uptake of B_{12} by cells and the circulating concentration reflects the intake of B_{12}, whereas total homocysteine (tHcy) indicates the metabolic ability. In this study, we investigated the diagnostic value of circulating holo-TC, B_{12}, folate and homocysteine in vegetarians who were at risk of B_{12} deficiency. B_{12}-related biomarkers were measured in 119 young, healthy graduate vegetarians. None was folate deficient. As per reported definition, half were B_{12} deficient; 70 % of males and 50 % of females had low plasma holo-TC concentrations; and 92 % of males and half of females had hyperhomocysteinaemia. None had any clinical signs of B_{12} deficiency. Receiver operating characteristic curve analysis demonstrated similar AUC at the B_{12} concentration of 100 and 150 pmol/l when holo-TC (0·777 and 0·784) and homocysteine (0·924 and 0·928) were used as variables. Cut-off value of 100 pmol/l resulted in the highest sensitivity of 77·78 % and specificity of 71·05 % with a predictive value of 19·6 pmol/l for holo-TC and a sensitivity of 82·72 % and specificity of 89·7 % with a predictive value of 21·7 μmol/l for homocysteine. The combination of B_{12}, holo-TC and tHcy improves the diagnostic accuracy at these cut-offs, and we suggest that for the young Indian vegetarians the cut-off for plasma B_{12} and holotrancobalamin is 100 pmol/l and 19·6 pmol/l, respectively, and for homocysteine it is 17·6 (females) and 27 μmol/l (males) for identifying B_{12} deficiency.

Key words: Vitamin B_{12}: Holotranscobalamin: Folate: Homocysteine

Vitamin B_{12} is essential for 1-C metabolism and cell division. Foods derived from animals are the main sources of vitamin B_{12}. Strict vegetarians have limited sources of vitamin B_{12} in their diet and therefore likely to have vitamin B_{12} deficiency[1–3]. Reduced consumption of cobalamin from food or impaired intestinal absorption leads to severe deficiency when tissue stores of the vitamin are depleted. The clinical consequence of vitamin B_{12} deficiency includes megaloblastic anaemia and progressive neurologic disease of the central and peripheral nervous system[4] and hyperhomocysteinaemia, a risk factor for CVD[5]. Early diagnosis of vitamin B_{12} deficiency is useful to prevent irreversible neurological damage by cobalamin supplementation[6–8]. It has been observed that asymptomatic Indian lacto-vegetarians, who make up for more than half of the Indian population, had distinctly lower vitamin B_{12} concentrations than non-vegetarians[9] and was confirmed by studies from different geographic regions of India. However, total plasma vitamin B_{12} concentration may not reliably reflect vitamin B_{12} status. To obtain more specificity and sensitivity in diagnosing vitamin B_{12} deficiency, the concept of measuring holotranscobalamin (holo-TC) II, a transport protein, has aroused great interest. holo-TC is a biologically active vitamin B_{12} fraction that promotes the aspecific uptake of vitamin B_{12} by cells[10].

Vitamin B_{12} deficiency has been divided into four stages[11]. In stages I and II, indicated by a low plasma level of holo-TC, the plasma and cell stores become depleted. Stage III is characterised by increased plasma levels of total homocysteine (tHcy) and methylmalonic acid (MMA) in addition to lowered holo-TC. In stage IV, clinical signs become recognisable such as macroovalocytosis, elevated mean corpuscular volume (MCV) or lower Hb levels. Stage III of vitamin B_{12} deficiency has been found in over 60 % of vegetarians[12]. Thus, it is important to monitor vitamin B_{12} status in this dietary group. Measurement of plasma vitamin B_{12}, holo-TC, tHcy and MMA has been suggested for optimal monitoring of vitamin B_{12} status in vegetarians[13]. In this study, we investigated the diagnostic cut-off values of plasma vitamin B_{12}, holo-TC and functional marker, tHcy, of vitamin B_{12} metabolism in Indian vegetarians.

Methods

The study was conducted at Deenanath Mangeshkar Hospital and Research Centre. Young, healthy, postgraduates and staff members of the hospital and their relatives were invited to participate in the study. Self-explained information regarding the participants was captured by an interview, which included

Abbreviations: Hcy, homocysteine; holo-TC, holotranscobalamin; MMA, methylmalonic acid; tHcy, total homocysteine.

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age, vitamin supplementation, food habits and routine lifestyle. Study subjects were enrolled after the purpose and requirements of the study were explained. They were clinically examined for gross clinical signs of protein-energy under-nutrition and vitamin deficiencies (vitamin A, B-complex, C and D). Non-vegetarians and pregnant women were not included. Subjects with diabetes, cancer and those taking drugs known to influence vitamin B_{12} absorption were also excluded. A total of 119 eligible participants (forty-six male and seventy-three female) were enrolled for the study. The study protocol was approved by the Hospital Ethical Committee and all the participants gave written informed consent (2015_APR/SN/169).

Experimental procedure

A volume of 10 ml of fasting blood sample was collected in EDTA vacutainers. Haematological parameters were measured on a five-part differential cell counter (Sysmex) and the remaining blood was centrifuged at 1500 ***g*** for 20 min. Separated plasma was stored for biochemical investigations at −20°C. Plasma holo-TC was measured using microparticle enzyme immunoassay. Microparticle enzyme intrinsic factor assay was used for the quantitative determination of plasma vitamin B_{12}. Plasma folate and tHcy were measured by the fluorescence polarisation immunoassay technique. All four biomarkers were analysed on an AxSYM immunoassay analyzer (Abbott Laboratories)[17]. Plasma creatinine was measured using the alkaline picrate method with a Daytona analyser (Randox)[14].

Dietary intake data

A 24-h dietary recall questionnaire was administered to capture detailed information about all food, beverages and dietary supplements consumed in the past 24 h from midnight to midnight the previous day by an experienced nutritionist. Energy, protein, fat and vitamin B_{12} intake was calculated using Dietsoft[15,16].

Definitions. Folate and vitamin B_{12} deficiency was defined as concentrations <2 ng/ml and <148 pmol/l, respectively; hyperhomocysteinaemia as plasma tHcy concentrations <15 μmol/l[17]; low holo-TC concentration as <35 pmol/l; anaemia as Hb concentration <120 g/l in females and <130 g/l in males; and macrocytosis as MCV > 100 fl[18].

Statistical analysis

The data are presented as medians and 25th and 75th percentiles. Between-group comparisons were calculated using Mann–Whitney *U* test and associations were tested using Pearson's correlation coefficient.

Sample size justification. Prevalence of vitamin B_{12} deficiency in vegetarian Indians is found to be 70 %[9]. Sample size was calculated using Buderer's method considering prevalence rate[19]. We selected sensitivity and specificity to be 80 and 90 %, respectively. We chose clinically acceptable width of 95 % CI for sensitivity and specificity to be no more than 10 %, significance ($a = 0{\cdot}05$). The sample size calculated was 88 for the expected sensitivity of 80 % and 116 for the expected sensitivity of 90 %. Taking maximum of both, we arrived at the estimate of 116.

We enrolled 119 subjects. Receiver operating characteristic (ROC) curves and AUC (with 95 % CI) were used to measure the diagnostic accuracy of (cut-offs) vitamin B_{12}, holo-TC and tHcy. ROC decision plots depicting sensitivity and specificity for 100 and 150 pmol/l concentration of vitamin B_{12} using holo-TC (<35 pmol/l) and tHcy (>15 μmol/l) variables were determined to identify the cut-off values.

Results

The participants had no clinical symptoms of vitamin B_{12} deficiency (neurological symptoms such as paresthesia, weakness, gait abnormality, tingling of hands and feet or anaemia symptoms such as skin pallor, fatigue, shortness of breath and so on) and were not taking vitamin B_{12} supplementation or any drugs known to influence vitamin B_{12} absorption.

All subjects had normal plasma folate and creatinine concentrations (5·9, 6·6 ng/ml and 1·2, 0·95 mg/dl in males and females, respectively). Median plasma vitamin B_{12}, holo-TC and tHcy concentrations were 146·5 and 164 pmol/l, 22·3 and 26·5 pmol/l, and 22·6 and 14·2 μmol/l in males and females, respectively (Table 1). In all, 39 and 30 % had very low concentration (<100 pmol/l), 11 and 20 % had low concentration (100–148 pmol/l), and 50 and 52 % had normal plasma vitamin B_{12} concentration (>148 pmol/l) in males and females, respectively. holo-TC was lower in three-fourth of the participants (22 and 24 pmol/l). A significant sex difference was found in plasma tHcy concentrations (22·6 μmol/l in males and 14·2 μmol/l in females, $P = 0{\cdot}0001$), with no sex difference in holo-TC and vitamin B_{12} concentrations (Table 1). Group-wise distribution of vitamin B_{12} (<100, 100–150 pmol/l) was strongly related to plasma holo-TC and inversely to tHcy concentrations in both males and females (Table 2, $P < 0{\cdot}0001$). At normal level of vitamin B_{12} (>148 pmol/l), the median concentrations of plasma holo-TC concentrations were 27·7 and 29·8 pmol/l in vegetarian males and females, which are lower than those found in non-vegetarians (Enexo). Between plasma vitamin B_{12} concentrations of 113 and 122 pmol/l, nineteen participants had normal holo-TC (34–52 pmol/l) with higher tHcy (34 μmol/l) concentrations, which is unexplainable with existing cut-offs (Table 3). ROC analysis demonstrated similar AUC at the vitamin B_{12} concentration of 100 and 150 pmol/l when holo-TC (0·784 and 0·777, respectively) and homocysteine (Hcy) (0·928 and 0·924, respectively) were used as variables. Cut-off value of 100 pmol/l resulted in the highest sensitivity (77·78 %) with acceptable specificity (71·05 %) with a predictive value of 19·6 pmol/l for holo-TC and a sensitivity of 82·72 % and specificity of 89·47 % with a predictive value of 21·2 μmol/l for tHcy (Fig. 1 and 2).

Discussion

Our data support the concept that the measurement of plasma holo-TC and tHcy along with vitamin B_{12} provides a better index of cobalamin status than the measurement of vitamin B_{12} alone[20]. Plasma holo-TC and tHcy are both sensitive markers

Table 1. Baseline characteristics and biochemistry of the participants (male and female) (Medians and 25th, 75th percentiles)

	Male (*n* 46)		Female (*n* 73)		
	Median	25th, 75th percentiles	Median	25th, 75th percentiles	*P**
Age (years)	29·0	25·5, 33·0	28·2	27, 32·5	NS
Energy intake (kJ/d)	10006	9372, 10334	9455	8786, 10446	0·042
Protein intake (g/d)	68	60, 74	62	58, 70	NS
Fat intake (g/d)	48	40, 55	47	40, 57	NS
Folate intake (μg/d)	268	220, 310	275	230, 300	NS
Vitamin B_{12} intake (μg/d)	1·65	1·3, 1·90	1·80	1·4, 2·0	0·04
Hb (g/l)	140	134, 148	124	117, 132	0·000
Anaemia (%)	4·0		26		
Mean corpuscular volume (fl)	85·8	82·0, 89·5	82·5	78·0, 85·0	0·001
Macrocytic (%)	12		15		
Creatinine (mg/dl)	1·2	0·9, 1·3	0·95	0·8, 1·15	0·001
Vitamin B_{12} (pmol/l)	146	84, 244	164	100, 288	NS
Vitamin B_{12} deficiency (%)	77		50		
holo-TC (pmol/l)	22·3	12·3, 31·4	24·4	15·5, 41·6	NS
Low holo-TC (%)	78		75		
Folate (ng/ml)	5·9	4·2, 14·9	6·6	4·0, 15·2	NS
Folate deficiency (%)	0		0		
tHcy (μmol/l)	22·6	13·9, 52·0	14·2	9·9, 24·4	0·0001
Hyperhomocysteinaemia (%)	92		50		

holo-TC, holotranscobalamin; tHcy, total homocysteine.
* Difference between male and female.

Table 2. Sex difference in total homocysteine (tHcy) at different vitamin B_{12} concentrations (Medians and 25th–75th percentiles)

			tHcy (μmol/l)				
Plasma vitamin B_{12} (pmol/l)	Sex	*n*	Median	25th, 75th percentiles	*P* (sex difference)	holo-TC (pmol/l)	
≤100	Male	18	50	46, 55	0·0001	13·2	NS
	Female	20	26·4	21·3, 40·4		14·6	
100–150	Male	5	23·8	21·4, 27	0·366	21·4	NS
	Female	15	20·9	15·6, 25·8		23·4	
>150	Male	23	13·9	12·0, 18·2	0·0068	31·3	NS
	Female	38	10·1	8·9, 12·6		35·3	

holo-TC, holotranscobalamin.

Table 3. Plasma vitamin B_{12} concentrations and corresponding concentrations of plasma holotranscobalamin (holo-TC) and total homocysteine (tHcy)

Median			Participants			
Vitamin B_{12} (pmol/l)	holo-TC (pmol/l)	tHcy (μmol/l)	Male (*n* 46)	Female (*n* 73)	Total (*n* 119)	Vitamin B_{12} status
314	46·75	9·64	4	14	18	Normal (B_{12} > 148 pmol/l)
266	36·3	11·2	5	9	14	
175	43·0	11·4	2	5	7	
174	24·9	12·49	3	5	8	
170	20·7	17·7	4	5	9	
122	41·65	34·06	2	5	7	Metabolic deficiency
113	52·7	34·06	3	10	13	tHcy >15 μmol/l, holo-TC > 35 pmol/l)
102	15·6	25·7	14	9	23	Deficiency
50	10·5	52·0	9	11	20	B_{12} <200 pg/ml, tHcy > 15 μmol/l, holo-TC > 35 pmol/l

holo-TC, holotranscobalamin; tHcy, total homocysteine.

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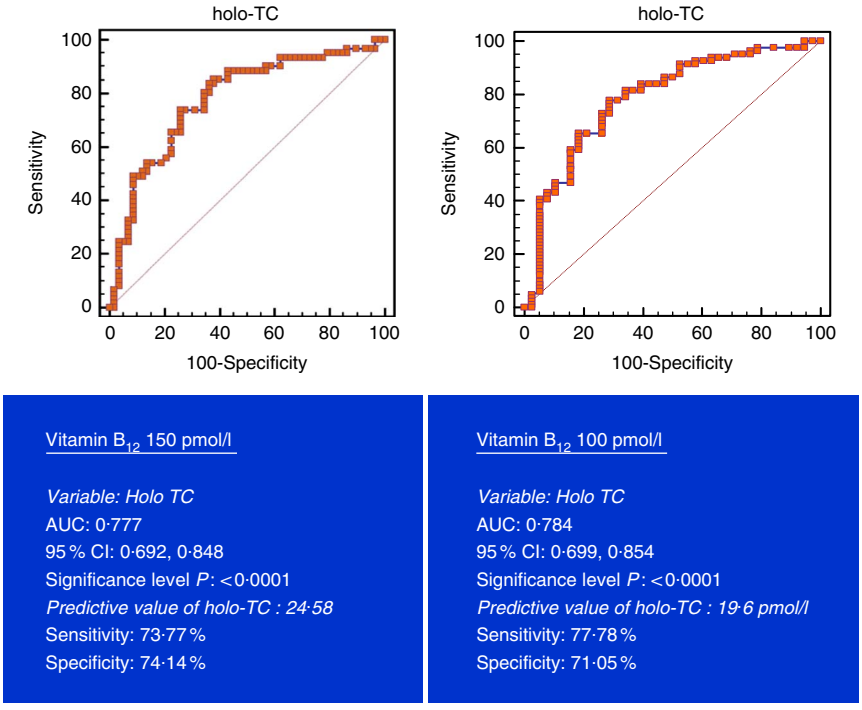


ROC analysis using holo-TC as a variable (>35 pmol/l); see Fig. 1

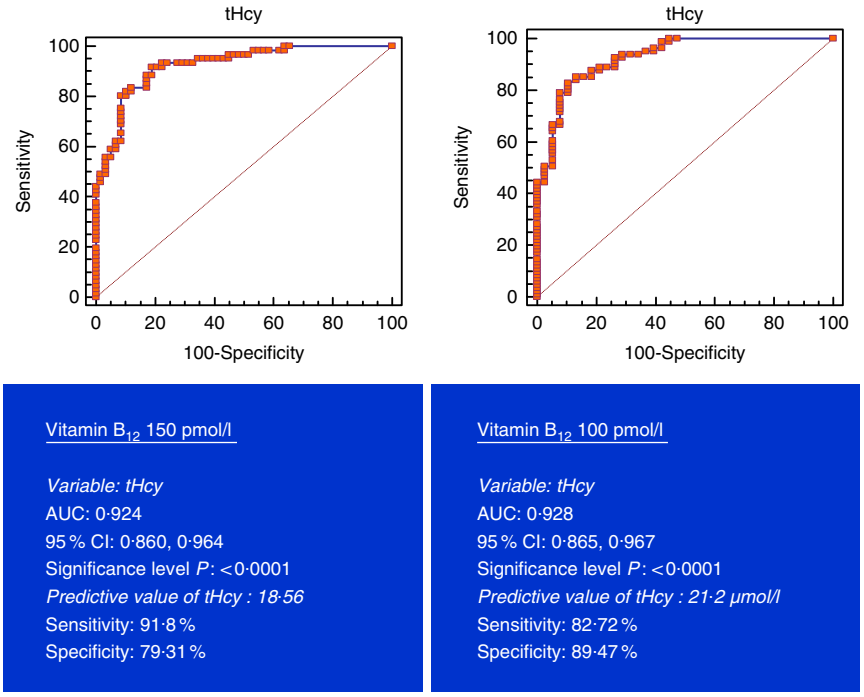
Vitamin B_{12}cut-off (pmol/l)	AUC	Sensitivity (%)	Specificity (%)	Predictive value (holo-TC, pmol/l)
100	0·784 (95 % CI: 0·699, 0·854) *P*<0·0001	77·78	71·05	19·6
150	0·772 (95 % CI: 0·691, 0·848) *P*<0·0001	73·77	74·14	24·58

Fig. 1. Plasma vitamin B_{12} at concentrations of 150 and 100 pmol/l were used for analysis. Metabolic deficiency was defined as plasma holotranscobalamin (holo-TC) < 35 pmol/l in 119 vegetarian Indians. Predictive values of plasma holo-TC were 24·58 and 19·6 μmol/l, respectively, with similar sensitivity and specificity at both 150 and 100 pmol/l of vitamin B_{12}. ROC, receiver operating characteristic.

for cobalamin status[21]. The lowest median levels of holo-TC were observed in the low vitamin B_{12} concentration group, followed by the 100–150 pmol/l group and then by the normal group (>148 pmol/l): 13·2, 21·4 and 31·3 pmol/l, respectively ($P = 0·0001$). At similar plasma cobalamin concentrations, men had different metabolic effects compared with women. The use of holo-TC and tHcy results enables the differentiation between storage, depletion and functional vitamin B_{12} deficiency.

Fedosov[22] reported that a combination of vitamin B_{12}, holo-TC, MMA and Hcy (cB_{12}) biomarkers is a reliable diagnostic tool. Fedosov *et al.*[23] derived equations that combined two, three or four biomarkers into one diagnostic indicator and provided a guidance for treatment. They suggested that adults having plasma vitamin B_{12} levels between 116 and 119 pmol/l, holo-TC between 8·4 and 20 pmol/l, and Hcy between 19·2 and 51 μmol/l be grouped as low vitamin B_{12} status and adults with B_{12} between 119 and 186 pmol/l, holo-TC between 20 and 37 pmol/l, and Hcy between 13·6 and 19·2 μmol/l as transitional vitamin B_{12} deficiency. A Brito *et al.*[24] stated that cB_{12} could only detect improved neurophysiological function in asymptomatic Chilean elderly with poor vitamin B_{12} status and also suggested to identify functional indicators of sub-clinical vitamin B_{12} deficiency.

This is the first study to investigate the diagnostic value of circulating plasma vitamin B_{12}, holo-TC and tHcy (a functional marker) concentrations associated with 1-C metabolism in Indian vegetarians. We found that at similar circulating concentrations of plasma vitamin B_{12} and holo-TC, there is a sex difference in plasma tHcy concentrations, with men having higher tHcy concentrations than women ($P < 0·0001$). The marked sex difference[17] in plasma tHcy concentrations is confirmed in this study. The difference suggests a higher threshold for supplementation of vitamin B_{12} to improve reproductive and cardiovascular outcomes[25]. The sex difference is not significant in participants whose plasma vitamin B_{12} concentrations are between 105 and 148 pmol/l. The strong inverse relation between plasma vitamin B_{12} and tHcy and direct relation with holo-TC[17] concentrations are confirmed in this study. Although age and sex reference intervals of tHcy have been established[25,26], they are usually ignored in reporting tHcy levels, because of dual biochemical origin (B_{12} and folate). Elevated Hcy in plasma has been very often used as



ROC analysis using tHcy as a variable (>15 µmol/l); see Fig. 2

Vitamin B_{12} cut-off (pmol/l)	AUC	Sensitivity (%)	Specificity (%)	Predictive value (tHcy, µmol/l)
100	0·928 (95 % CI: 0·865, 0·967) $P<0·0001$	82·72	89·47	21·2
150	0·924 (95 % CI: 0·860, 0·964) $P<0·0001$	91·8	79·31	18·56

Fig. 2. Plasma vitamin B_{12} at 150 and 100 pmol/l were used as variables. Metabolic deficiency was defined as total homocysteine (tHcy) >15 µmol/l in 119 vegetarian Indians. Predictive values of plasma tHcy were 18·56 and 21·2 µmol/l, respectively, with better specificity (89·47 %) at 100 pmol/l of vitamin B_{12}. ROC, receiver operating characteristic.

a biomarker, but its relationship to the molecular mechanisms of disease has not been established[27].

In this study, we aimed to meet two criteria: (a) vitamin B_{12} absorption capacity in Indian vegetarians and (b) optimum plasma vitamin B_{12} concentration required for methylation of Hcy. At normal plasma vitamin B_{12} levels (>150 pmol/l), median holo-TC concentrations were 31·3 and 35·3 pmol/l in men and women, respectively, thereby attaining normal tHcy concentrations (13·9 and 10·17 µmol/l, respectively).

A cohort of 100 known patients with CVD and sixty-three normal healthy subjects (median age 44 years) were examined for their vitamin B_{12} status in a case–control study in Pune. Median plasma vitamin B_{12}, holo-TC and tHcy concentrations were 160 pmol/l, 24 pmol/l and 19·7 µmol/l, respectively, in normal subjects. They stated that hyperhomocysteinaemia and elevated MMA were due to vegetarianism[18]. Similarly, another report from Pune, wherein subjects with low plasma vitamin B_{12} levels were studied, showed very low plasma holo-TC concentrations (7·7 (SD 4·2) and 9·8 (SD 8·7) pmol/l in men and women, respectively), with tHcy concentrations of 29·2 (SD 19·2) and 15·3 (SD 8·3) µmol/l[28]. Low vitamin B_{12} concentration (median 110 pmol/l) and hyperhomocysteinaemia (>15 µmol/l) have been reported to be common in Indian men, particularly in vegetarians and urban middle-class residents. Most of the participants from these studies were vegetarians. In the study by Naik *et al.*[17], young Indian vegetarian subjects with low plasma vitamin B_{12} status (<148 pmol/l) were found to have holo-TC and tHcy concentrations of 14·4 pmol/l and 31·9 µmol/l, respectively, and the subjects with normal status (>200 pmol/l) were found to have holo-TC and tHcy concentrations of 27·7 pmol/l and 11·9 µmol/l, respectively. Most of the Indian studies did not use plasma holo-TC measurements. A Dutch study reported 49 (8–388) pmol/l of plasma holo-TC concentrations in healthy subjects with corresponding vitamin B_{12} concentrations of 217 (119–1210) pmol/l[29]. A study from USA reported plasma holo-TC concentration of 85 (SD 48) pmol/l for 495 (SD 119) pmol/l of vitamin B_{12} concentrations in healthy volunteers[30]. In both these studies the participants were non-vegetarians with high plasma holo-TC concentrations and the referred cut-offs may not be appropriate for vegetarians.

In healthy individuals, all four biomarkers (plasma vitamin B_{12}, holo-TC, tHcy and MMA) had a strong relation to vitamin B_{12} intake, with steady-state concentrations at a daily intake of 4–7 µg vitamin B_{12}[31]. These studies suggest that all four markers may be useful for monitoring a population's vitamin B_{12} status over time.

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Carmel[32] categorised available biomarkers as those that directly measured plasma vitamin B_{12} and those that measured metabolites that accumulated with inadequate amounts of vitamin B_{12}. Plasma holo-TC and vitamin B_{12} measured circulating vitamin B_{12} concentrations. These two therefore reflected the broad vitamin B_{12} status from high risk of severe deficiency to adequacy. Miller *et al.*[33] have stated that holo-TC and total vitamin B_{12} have equal diagnostic accuracy in screening for metabolic vitamin B_{12} deficiency. Measurement of both holo-TC and total vitamin B_{12} provided better screen for vitamin B_{12} deficiency than either assay alone. According to Green[34], low vitamin B_{12} status was indicated by values lower than the reference range (for vitamin B_{12} < 148 pmol/l; for holo-TC <35 pmol/l), whereas for indirect measures of metabolites (MMA or tHcy) low vitamin B_{12} status measures were indicated by a level above the upper limit of the reference range (for MMA >260 nmol/l; for tHcy >12 µmol/l). However, Valente *et al.*[35] suggested a diagnostic strategy using holo-TC as the front-line test. The cut-offs for deficiency were defined as 20 pmol/l for holo-TC and 123 pmol/l for serum vitamin B_{12} after studying employees and medical students of a local hospital at Dundee, UK.

Conclusions

holo-TC levels may prove most useful if the aim is to monitor a population with a borderline sub-optimal vitamin B_{12} supply. In contrast, total vitamin B_{12} may be superior if the goal is to monitor a possible surplus load of vitamin. We advocate a diagnostic cut-off level of plasma vitamin B_{12} (105 pmol/l), holo-TC (22·6 pmol/l) and Hcy (17·6 µmol/l for females and 27·0 µmol/l for males) in vegetarian Indian population. In addition to this, we recommend that vegetarians do take a supplement of vitamin B_{12} to ensure adequate supply of the micronutrient. This would be of particular importance for females in reproductive age, to prevent the risks associated with maternal–fetal vitamin B_{12} deficiency.

The limitation of the study is that plasma MMA has not been measured. However, if none of the participants is folate deficient, the measurement of Hcy shall indicate vitamin B_{12} status.

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S. N. designed the study. V. B. and N. M. prepared the manuscript draft. S. N. prepared the final draft.

The authors declare that there are no conflicts of interest.

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