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Vitamin B-12 status, particularly holotranscobalamin II and methylmalonic acid concentrations, and hyperhomocysteinemia in vegetarians[1–3]

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ABSTRACT

Background: Vegetarians have a lower intake of vitamin B-12 than do omnivores. Early and reliable diagnosis of vitamin B-12 deficiency is very important.

Objective: The objective was to investigate vitamin B-12 status in vegetarians and nonvegetarians.

Design: The study cohort included 66 lactovegetarians or lactoovovegetarians (LV-LOV group), 29 vegans, and 79 omnivores. Total vitamin B-12, methylmalonic acid, holotranscobalamin II, and total homocysteine concentrations were assayed in serum.

Results: Of the 3 groups, the vegans had the lowest vitamin B-12 status. In subjects who did not consume vitamins, low holotranscobalamin II (< 35 pmol/L) was found in 11% of the omnivores, 77% of the LV-LOV group, and 92% of the vegans. Elevated methylmalonic acid (> 271 nmol/L) was found in 5% of the omnivores, 68% of the LV-LOV group, and 83% of the vegans. Hyperhomocysteinemia (> 12 μmol/L) was present in 16% of the omnivores, 38% of the LV-LOV group, and 67% of the vegans. The correlation between holotranscobalamin II and vitamin B-12 was weak in the low serum vitamin B-12 range ($r = 0.403$) and strong in the high serum vitamin B-12 range ($r = 0.769$). Holotranscobalamin II concentration was the main determinant of total homocysteine concentration in the vegetarians ($\beta = -0.237$, $P < 0.001$). Vitamin B-12 deficiency led to hyperhomocysteinemia that was not probable in the upper folate range (> 42.0 nmol/L).

Conclusions: Vegan subjects and, to a lesser degree, subjects in the LV-LOV group had metabolic features indicating vitamin B-12 deficiency that led to a substantial increase in total homocysteine concentrations. Vitamin B-12 status should be monitored in vegetarians. Health aspects of vegetarianism should be considered in the light of possible damaging effects arising from vitamin B-12 deficiency and hyperhomocysteinemia. *Am J Clin Nutr* 2003;78:131–6.

KEY WORDS Vitamin B-12, homocysteine, methylmalonic acid, holotranscobalamin, vegetarians

INTRODUCTION

Consumption of a vegetarian diet is associated with a favorable lipid profile and high antioxidant consumption, which afford potential protections against cardiovascular disease (1–3). Studies confirmed the potential of vegetarianism as a protection against coronary disease (4, 5). However, these findings were not confirmed by other investigators (6). The harmful effects of vegetarianism that result from the deprivation of some essential micronutrients have been addressed repeatedly (7, 8).

Natural sources of vitamin B-12 (cobalamin) in the human diet are restricted to foods of animal origin (9), and persons who adopt a plant-based diet are known to be at risk of cobalamin deficiency (8, 10–12). Vitamin B-12 is an essential micronutrient that plays a fundamental role in cell division and in one-carbon metabolism (9–12). Chronic vitamin B-12 depletion (ie, prolonged low intake or intestinal malabsorption) results in a state of negative vitamin balance. The depletion process may take years to become clinically evident. Early diagnosis of vitamin B-12 deficiency is crucial, owing to the latent nature of this disorder and the resulting possible irreversible neurologic damage (13). A single reliable diagnostic approach for ruling out vitamin B-12 deficiency is not available (8, 14–16). The total serum vitamin B-12 concentration, the first variable often determined, does not reliably rule out a functional cobalamin deficiency (8, 12, 17). On the contrary, elevated methylmalonic acid (MMA) and total homocysteine (tHcy) concentrations are sensitive metabolic markers for vitamin B-12 deficiency (11, 17). However, renal insufficiency may also cause elevation of MMA and tHcy (16–18), and elevated tHcy may reflect folate deficiency as well as vitamin B-6 deficiency (17, 18).

The assay for holotranscobalamin II was introduced to achieve more sensitivity and specificity in the diagnosis of vitamin B-12 deficiency (12, 14, 19). Holotranscobalamin II is composed of vitamin B-12 attached to transcobalamin, and it represents the biologically active fraction that can be delivered into all DNA-synthesizing cells.

The observations of Herbert et al (12) suggested that vitamin B-12 deficiency is developed through 4 stages of negative balance. In stages I and II, plasma and cell stores become depleted and the concentration of holotranscobalamin II is reduced. Stage III is characterized by functional imbalances indicated by elevated tHcy

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[2] The reagents used for the holotranscobalamin II assay were a gift from Axis-Shield (Oslo).

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and MMA concentrations in plasma. In stage IV, clinical signs may become recognizable.

In an earlier investigation, we studied the status of tHcy, MMA, and total vitamin B-12 in a group of vegetarians (8). The present study was designed to shed more light on vitamin B-12 status in subjects who have adopted different types of diets. Vitamin B-12 status was investigated by measuring total vitamin B-12, tHcy, MMA, and holotranscobalamin II concentrations. The interpretation of these variables and the involvement of cobalamin deficiency in inducing hyperhomocysteinemia were also addressed in the present study.

SUBJECTS AND METHODS

Subjects

A total of 174 apparently healthy subjects living in Germany and the Netherlands were recruited. The vegetarians [66 lactovegetarian or lactoovovegetarian (LV-LOV) subjects and 29 vegans] were volunteers recruited either at a conference of the German Federation of Vegetarians or at a Vegan Society summer camp in the Netherlands. The study was approved by the German Federation of Vegetarians and the Vegan Society of the Netherlands, and the participants gave informed consent. Omnivorous control subjects (n = 79) were students at the University of Saarland and staff members in the central laboratory of the University of Saarland. A constant dietary pattern, maintained for ≥ 1 y, was the main inclusion criterion. Exclusion criteria included renal disease, current consumption of weight-loss diets, pregnancy, and medication or metabolic diseases known to influence nutritional status. All subjects were interviewed and asked to complete a preliminary questionnaire about lifestyle factors, the degree of animal-products restriction they followed, and vitamin consumption. Seventeen vegans (59%) and 13 LV-LOV subjects (20%) supplemented their diet with B vitamins. Not all vitamin users provided details about the dose and the frequency of vitamins used. The participants in the current study were classified into groups on the basis of their habitual dietary intake: control subjects (n = 79) consumed an omnivorous diet; lactoovovegetarians excluded meat, poultry, and fish, and lactovegetarians further excluded eggs (LV-LOV group, n = 66); and vegans (n = 29) excluded all foods of animal origin.

Methods

Twelve-hour fasting blood samples were collected, placed directly on ice for no more than 45 mim, and centrifuged for 15 min at 2000 × g and 4 °C; then serum was separated and stored at −70 °C. MMA, tHcy, and cystathionine were assayed in serum by gas chromatography-mass spectrometry (20, 21). Vitamin B-12 and folate were measured with the use of a chemiluminescence immunoassay (ADVIA Centaur system; Bayer, Leverkusen, Germany). Serum vitamin B-6 was analyzed by HPLC with fluorescence detection using reagents from Immundiagnostik (Bensheim, Germany). Holotranscobalamin II concentrations were measured in serum, using a radioimmunoassay-based reagent set (Axis-Shield, Oslo) as recently described (14).

Statistical analysis

Statistical analyses were performed with SPSS software, version 9.0 (SPSS Inc, Chicago). We performed one-way analysis of variance and followed that with a post hoc Tamhane test for between-group comparisons. Logarithmic transformation was applied to correct for skewness of distribution of data. Correlation between variables was evaluated with the use of Spearman's rank-order coefficient correlation. Data are presented as medians and 5th and 95th percentiles. All tests were two-tailed and were considered significant when $P < 0.05$.

RESULTS

Population characteristics and biochemical markers according to the type of diet and vitamin consumption

Omnivorous control subjects had higher vitamin B-12 and holotranscobalamin II concentrations and lower tHcy and MMA concentrations than did vegans and the LV-LOV subjects. However, not all differences were significant when the LV-LOV subjects taking vitamins (n = 13) were compared with omnivorous control subjects (**Table 1**).

When we compared vitamin users and nonusers, holotranscobalamin II values were significantly different in vegans, and vitamin B-12, MMA, and cystathionine concentrations were different in LV-LOV subjects. When we compared vegans and LV-LOV subjects, we found significant differences in age, vitamin B-12, holotranscobalamin II, MMA, and cystathionine concentrations irrespective of vitamin consumption.

Irrespective of vitamin usage, low vitamin B-12 concentrations (< 156 pmol/L) were found in 1% of the omnivores compared with 26% of the LV-LOV subjects and 52% of the vegans. Reduced holotranscobalamin II concentrations (< 35 pmol/L) were present in 11% of the omnivores, 73% of the LV-LOV subjects, and 90% of the vegans. An elevated MMA concentration (> 271 nmol/L) was found in 5% of the omnivores, 61% of the LV-LOV subjects, and 86% of the vegans. As expected, LV-LOV vitamin users had pathologically abnormal concentrations of the markers that were intermediate to those in omnivorous control subjects and vegans. To eliminate any possible interference with the results, vitamin takers were not included in any of the other statistical analyses.

Vitamin B-12 status as indicated by serum concentrations of holotranscobalamin II and MMA

Study participants were stratified into groups according to their vitamin B-12 status, as previously suggested by Herbert et al (12). The intergroup distribution of subjects according to the type of diet is presented in **Table 2**. Stage I or II of vitamin B-12 deficiency was characterized by an isolated decrease in the holotranscobalamin II concentration. Stage III of vitamin B-12 deficiency consists of increased MMA and decreased holotranscobalamin II concentrations. Vegetarians had a distribution pattern that tended toward stage III, whereas omnivores were mostly in the normal vitamin B-12 group. Creatinine did not differ significantly between the subjects in the different stages. Only 6 persons had an elevated MMA together with a normal holotranscobalamin II concentration.

Correlation between different vitamin B-12 status indexes

The correlation between serum vitamin B-12 and holotranscobalamin II in 2 vitamin B-12 deficiency states (above and below the cutoff of 156 pmol/L) is shown in **Figure 1**. A strong

TABLE 1
Main characteristics and vitamin B-12 status in the study population according to the type of the diet and vitamin consumption[1]

	Omnivorous control subjects (n = 79)	LV-LOV subjects			Vegan subjects		
		Vitamin user (n = 13)	Vitamin nonuser (n = 53)	All (n = 66)	Vitamin user (n = 17)	Vitamin nonuser (n = 12)	All (n = 29)
Age (y)	51 (23, 68)[2]	50 (31, 67)	46 (22, 76)	48 (24, 75)	36 (14, 59)	40 (19, 60)	37 (15, 64)[3]
Women (%)	56	54	55	55	47	67	55
Vitamin B-12 (pmol/L)	287 (190, 471)	303 (146, 771)	179 (124, 330)[4,5]	192 (127, 450)[4]	192 (125, 299)[4]	126 (92, 267)[4]	148 (99, 314)[3,4]
Low vitamin B-12, <156 pmol/L (%)	1	8	32	26	29	83	52
HoloTC (pmol/L)	54 (16, 122)	26 (3, 235)	23 (4, 84)[4]	23 (3, 155)[4]	14 (3, 53)[4]	4 (2, 35)[4,5]	10 (2, 78)[3,4]
Low holoTC, <35 pmol/L (%)	11	62	77	73	88	92	90
MMA (nmol/L)	161 (95, 357)	230 (120, 1344)[4]	368 (141, 2000)[4,5]	355 (138, 1948)[4]	708 (163, 2651)[4]	779 (222, 3480)[4]	708 (193, 3470)[3,4]
MMA >271 nmol/L (%)	5	31	68	61	88	83	86
tHCY (μmol/L)	8.8 (5.5, 16.1)	9.6 (5.5, 19.4)	10.9 (6.8, 28.2)[4]	10.6 (6.4, 27.7)[4]	11.1 (5.3, 25.9)[4]	14.3 (6.5, 52.1)[4]	12.8 (5.9, 57.1)[4]
tHCY >12 μmol/L (%)	16	15	38	33	47	67	55
Folate (nmol/L)	21.8 (14.5, 51.5)	30 (14.8, 119)	27.7 (16, 76.9)[4]	28.8 (16.1, 77)[4]	29.5 (18.8, 71.8)[4]	34.3 (20.7, 72.7)[4]	31.8 (19.7, 78.1)[4]
Creatinine (μmol/L)	71 (53, 93)	71 (62, 85)	71 (50, 91)	71 (53, 88)	71 (44, 81)	71 (53, 86)	71 (49, 88)
Vitamin B-6 (nmol/L)	12.9 (6.1, 31.8)	13.7 (3.6, 120)	12.3 (4.9, 25.7)	12.4 (4.6, 66.9)	10.2 (5.6, 31.2)	12.2 (2.4, 26.3)	10.5 (3.5, 31.4)
Cystathionine (nmol/L)	228 (127, 383)	195 (109, 374)	226 (146, 656)[5]	214 (131, 596)	188 (138, 227)[4]	202 (134, 310)[4]	189 (136, 306)[3]

[1] LV-LOV, lactovegetarian or lactoovovegetarian; holoTC, holotranscobalamin II; MMA, methylmalonic acid; tHcy, total homocysteine.
[2] Median; 5th and 95th percentiles in parentheses.
[3] Significantly different from LV-LOV subjects, $P < 0.05$ (ANOVA and Tamhane test).
[4] Significantly different from omnivorous control subjects, $P < 0.05$ (ANOVA and Tamhane test).
[5] Significantly different from vitamin users (within the group), $P < 0.05$ (ANOVA and Tamhane test).

TABLE 2
Metabolites and B vitamins in cobalamin deficiency stages among subjects not taking vitamins[1]

	Cobalamin status group[2]		
	Normal vitamin B-12 status (n = 69)	Stage I or II (n = 20)	Stage III (n = 44)
Women [n (%)]	42 (61)	10 (50)	21 (48)
Omnivorous control subjects (n)	62	8	2
LV-LOV subjects (n)	6	11	32
Vegan subjects (n)	1	1	10
Age (y)	51 (23, 70)[3]	35 (13, 54)	45 (24, 75)[4]
Vitamin B-12 (pmol/L)	291 (194, 476)	206 (128, 266)[5]	152 (108, 267)[4,5]
HoloTC (pmol/L)	58 (39, 12)	26 (8, 35)[5]	11 (2, 33)[4,5]
MMA (nmol/L)	161 (100, 254)	172 (97, 253)	763 (341, 2835)[4,5]
tHCY (μmol/L)	8.6 (5.4, 13.8)	8.5 (5.9, 54.0)	13.1 (7.2, 47.4)[4,5]
Folate (nmol/L)	24.7 (13.8, 51.6)	27.8 (14.5, 72.3)	28.1 (15.5, 73.1)
Creatinine (μmol/L)	70.7 (53.0, 90.7)	70.7 (44.6, 96.8)	70.7 (53.0, 88.4)

[1] n = 79, 53, and 12 among the omnivorous control subjects, the lactovegetarian and lactoovovegetarian (LV-LOV) subjects, and the vegan subjects, respectively. Six subjects (2 omnivorous control subjects and 4 LV-LOV subjects) had elevated methylmalonic acid (MMA) and normal holotranscobalamin II (holoTC) levels (data not shown in the table), and MMA data were missing for 1 omnivorous control subject.

[2] Normal, MMA ≤ 271 nmol/L and holoTC ≥ 35 pmol/L; stage I or II, MMA ≤ 271 nmol/L and holoTC < 35 pmol/L; stage III, MMA > 271 nmol/L and holoTC < 35 pmol/L.

[3] Median; 5th and 95th percentiles in parentheses.

[4] Significantly different from stage I or II, $P < 0.05$ (ANOVA and Tamhane test).

[5] Significantly different from subjects with normal vitamin B-12 status, $P < 0.05$ (ANOVA and Tamhane test).

direct correlation was found between the 2 values in the group with high serum vitamin B-12 (panel B: $r = 0.769$, $P < 0.001$), but the correlation in the group with low serum vitamin B-12 was much weaker (panel A: $r = 0.403$, $P = 0.034$). As shown in **Figure 2**, holotranscobalamin II and vitamin B-12 are plotted against MMA concentrations. MMA was elevated in 27 subjects (19%), although vitamin B-12 concentrations were > 156 pmol/L (Figure 2, left, field II). A higher concordance was found between MMA and holotranscobalamin II concentrations ($r = -0.686$, $P < 0.001$) (Figure 2, right, field II) than was found between holotranscobalamin II and vitamin B-12 concentrations. A direct correlation was found between MMA and tHcy ($r = 0.521$, $P < 0.001$) (data not shown).

The "folate trap" phenomenon in vegetarians

The correlation between tHcy and serum folate in the omnivorous control subjects and the LV-LOV and vegan subjects is shown in **Figure 3**. We found tHcy concentrations < 12 μmol/L in omnivorous control subjects with serum folate concentrations as low as

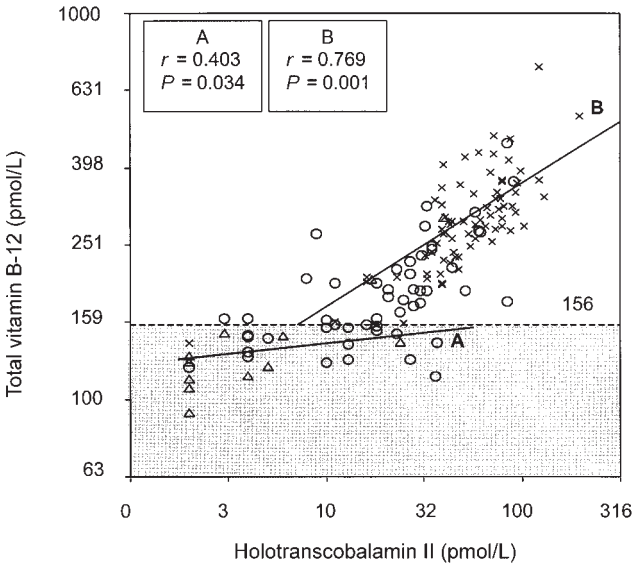

FIGURE 1. Scatter plots of holotranscobalamin II and total vitamin B-12 concentrations in the subjects not taking vitamins: ×, omnivorous control subjects (n = 79); ○, lactovegetarians and lactoovovegetarians (n = 53); and △, vegans (n = 12). The correlation coefficients are calculated for 2 vitamin B-12 ranges (A, < 156 pmol/L; B, ≥ 156 pmol/L). The numbers on the axes are anti-log.

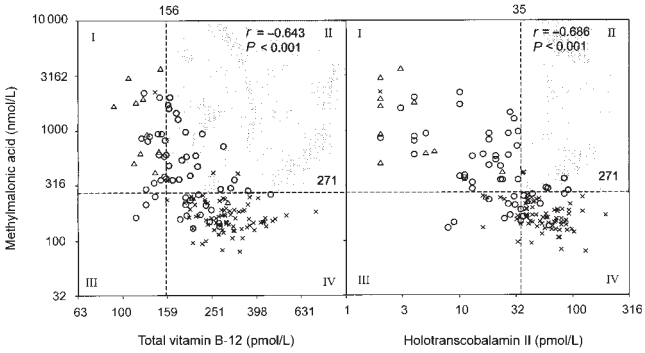

FIGURE 2. Scatter plots of methylmalonic acid and total vitamin B-12 (left) and of methylmalonic acid and holotranscobalamin II (right) in the subjects not taking vitamins: ×, omnivorous control subjects (n = 79); ○, lactovegetarians and lactoovovegetarians (n = 53); and △, vegans (n = 12). The dashed lines indicate the upper limit of the normal range for methylmalonic acid (271 nmol/L) and the lower limit of the normal range for vitamin B-12 (156 pmol/L) and holotranscobalamin II (35 pmol/L). The numbers on the axes are anti-log.

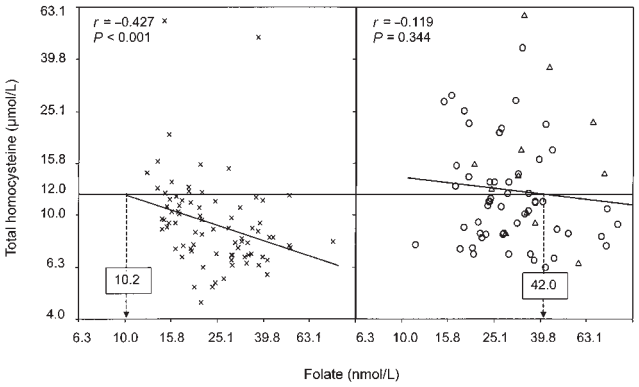

FIGURE 3. Expected serum folate concentration required in omnivorous control subjects (×, $n = 79$), lactovegetarians and lactoovovegetarians (○, $n = 53$), and vegans (△, $n = 12$) to reach a total homocysteine concentration < 12 μmol/L. Correlation coefficients are according to Spearman's rank-order coefficient correlation. The numbers on the axes are anti-log.

10.2 nmol/L (normal: > 7 nmol/L). In contrast, hyperhomocysteinemia occurred in the LV-LOV subjects and vegans when serum folate was as high as 42.0 nmol/L.

Stepwise multiple regression analyses performed in the vegetarians (LV-LOV and vegan subjects) showed that holotranscobalamin II was the strongest predictor of tHcy concentrations ($\beta = -0.237$, $P < 0.001$) and that female sex was the next strongest ($\beta = -0.149$, $P < 0.001$). Neither creatinine ($P = 0.236$) nor serum folate ($P = 0.405$) concentrations significantly affected tHcy concentrations in the vegetarians (total $n = 65$) ($R^2 = 0.52$).

DISCUSSION

In this study, which included subjects who had adopted different types of diets, omnivorous control subjects had lower tHcy concentrations and better cobalamin status than did both the LV-LOV subjects and the vegans. The same tendency was found when comparing omnivores with LV-LOV subjects who took vitamins, but the differences were not statistically significant, except the difference for MMA. The incidence of pathologically abnormal indexes of vitamin B-12 status was clearly related to the type of diet, because it was considerably higher in the vegans than in the other 2 groups. It was not clear in the current study whether the slightly better vitamin B-12 status in the LV-LOV subjects taking vitamins was attributable to vitamin B supplements or to the weak statistical power resulting from the small number of subjects in this group (Table 1). Therefore, subjects taking vitamins were excluded from further analysis.

The weak correlation between holotranscobalamin II and vitamin B-12 at the lower concentrations of vitamin B-12, which is the most significant range for establishing a diagnosis, may indicate the limitation of total vitamin B-12 assay in this regard (Figure 1) (8, 12, 17). In contrast to this weak correlation, a stronger link between MMA and holotranscobalamin II was evident (Figure 2). All but 6 subjects (discussed later) with elevated MMA also had a low holotranscobalamin II concentration.

Isolated reduced holotranscobalamin II with normal MMA and tHcy (stage I or II) may indicate a depletion of plasma and cell stores of vitamin B-12 (Table 2). When the negative balance progresses (stage III), depleted vitamin stores and functional disturbances lead to elevated concentrations of MMA and tHcy, which indicate a more advanced stage of deficiency. It is important to note that a normal MMA concentration may not rule out stage I or II of vitamin B-12 deficiency and that low holotranscobalamin II may not distinguish between stage I or II and stage III of negative balance.

The combination of normal holotranscobalamin II and an elevated MMA concentration found in 6 subjects was not consistent with vitamin B-12 deficiency. Similar findings have been reported in persons with chronic renal failure (16, 22). However, disturbed renal function seems unlikely in these cases because creatinine concentrations were normal in all 6 of those subjects. Elevated serum concentrations of MMA have also been reported in the presence of intestinal bacterial overgrowth (23). A study of Asian Indians found that only 5% of subjects with relatively high vitamin B-12 concentrations had reduced holotranscobalamin II, whereas ≈40% had elevated MMA (> 260 nmol/L) (24). The higher proportion of Asian Indian subjects with normal holotranscobalamin II and an elevated MMA concentration (24) and the greater prevalence of gastrointestinal infections in Asia than in Europe may suggest an artificial increase in MMA concentrations (23).

Overt folate deficiency (serum folate: < 7 nmol/L) was not found in any subject in this study, and, as might be expected, vitamin B-12 deficiency was the strongest determinant of tHcy concentration in all of the vegetarians, both vegan and LV-LOV subjects (β coefficient for holotranscobalamin II = −0.237, $P < 0.001$). Similarly, holotranscobalamin II was the main determinant for tHcy concentrations in cobalamin-deficient Asian Indians (24). Folate, age, creatinine, and vitamin B-12 status are well established as determinants of tHcy concentrations within the general population (25). The observation that folate was not a significant determinant of tHcy in our vegetarians may be explained by the relatively high baseline folate status in our subjects (Table 1). Furthermore, the homocysteine concentration increases with advancing age, whereas vitamin B-12 status declines (17). Our omnivorous control subjects were slightly older than were the LV-LOV subjects ($P = 0.092$) and the vegans ($P = 0.126$), which may have influenced the current findings.

5-Methyltetrahydrofolate is the methyl group donor in the remethylation of homocysteine to methionine, mediated by methionine synthase that requires vitamin B-12 as cofactor. Thus, in vegetarians with vitamin B-12 deficiency, homocysteine may not be efficiently remethylated and hence accumulates in association with the trapping of reduced folate in the form of 5-methyltetrahydrofolate, the so-called folate trap (26). Our data support this concept (Figure 3), because vitamin B-12 deficiency in vegetarians was associated with a relative shortage of folate, and hyperhomocysteinemia occurred unless the folate concentration was > 42.0 nmol/L. The same phenomenon was evident in our recent investigations in nonvegetarian Syrian subjects who had a high prevalence of vitamin B-12 deficiency (27). It is worthy of mention that, in severe vitamin B-12 deficiency, normal to high-normal serum folate concentrations might be expected, but they do not necessarily indicate subcellular folate sufficiency (28).

Megaloplastic anemia was not a common finding in either the vegan and LV-LOV subjects in this study (7% displayed mean cell volume > 95 fL) (29) or in those reported by others (24). It should

be noted, however, that macrocytic anemia may be masked in vegetarians by excess folate intake (30) or by concomitant iron deficiency (29, 30). There is compelling evidence for elevated tHcy as a risk factor for cardiovascular disease (31). In addition, DNA hypomethylation and disturbed formation of neurotransmitters were reported in vitamin B-12–deficient subjects (32). In the absence of a classic hematologic picture and with consideration of the latent nature of vitamin B-12 deficiency, it is particularly important to establish an early diagnosis for this disturbance in suspected cases.

Taken together, the vegetarians investigated in this study had different degrees of vitamin B-12 deficiency, which were related to the degree of animal product restriction. Hyperhomocysteinemia and relative folate shortage were linked to vitamin B-12 deficiency. According to our data, the assessment of holotranscobalamin II, accompanied by that of metabolic markers such as tHcy and MMA, may offer sensitive and reliable tools for early diagnosis and hence proper intervention in persons who are prone to vitamin B-12 deficiency. More emphasis should be placed on effective vitamin B-12 supplementation and monitoring of vitamin B-12 status in persons who have chosen lifelong adherence to a vegetarian diet.

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WH had the original idea for the study and wrote the manuscript; HS planned the study and participated in sample collection, the analysis of samples, and the statistical analysis and wrote part of the manuscript; RO participated in sample collection, the analysis of samples, the analysis and interpretation of the data, and the revision of the manuscript; and JG participated in sample collection and supervised the study. None of the authors had any personal or financial interest in any organization sponsoring this research or any conflict of interest related to their participation in this study.

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