Total Homocysteine, Vitamin B₁₂, and Total Antioxidant Status in Vegetarians

Wolfgang Herrmann,¹ Heike Schorr,¹ Kathrin Purschwitz,² Fausi Rassoul,² and Volker Richter²

Background: Decreasing or eliminating animal products from the diet decreases the intake of some essential nutrients, such as vitamin B₁₂, which may lead to hyperhomocysteinemia. We investigated vitamin B₁₂-dependent metabolism and oxidative stress in groups with various or no intake of meat or animal products.

Methods: We investigated 44 high meat eaters, 19 low meat eaters, 34 lacto-ovo/lacto vegetarians, and 7 vegan vegetarians. Homocysteine (HCY) was assayed by HPLC, methylmalonic acid (MMA) by capillary gas chromatography–mass spectrometry, serum folate and vitamin B₁₂ with a chemiluminescence immunoassay, and total antioxidant status (TAS) by a Randox method.

Results: The mean serum HCY concentration of vegetarians was significantly increased, and in vegans the median concentration exceeded 15 µmol/L. Vegetarians had a higher serum concentration of MMA but a lower TAS. Vitamin B₁₂ and folate did not differ significantly between vegetarian and omnivorous subjects. Overall, HCY and MMA were significantly correlated. Vitamin B₁₂ correlated negatively with MMA, HCY, and folate, whereas the correlation with TAS was positive. Backward regression analysis revealed an independent influence of MMA on HCY, of HCY and vitamin B₁₂ on MMA, and of vitamin B₁₂ on TAS. The increased MMA concentration suggested a 25% frequency of functional vitamin B₁₂ deficiency in all vegetarians. Serum vitamin B₁₂ was below the lower reference limit in only five subjects.

Conclusions: Functional vitamin B₁₂ deficiency in vegetarians may contribute to hyperhomocysteinemia and decreased TAS, which may partly counteract the beneficial lifestyle of vegetarians. However, increased serum HCY is most likely not responsible for the lower TAS values in vegetarians. We recommend assaying of MMA and HCY to investigate functional vitamin B₁₂ status.

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Hyperhomocysteinemia has been recognized as an important independent cardiovascular risk factor (1). It is hypothesized that homocysteine (HCY) alters endothelial and smooth muscle cell function by generating reactive oxygen species (2–4). The resulting increase in oxidative stress diminishes antioxidative capacity, which increases the risk for atherosclerotic vessel diseases in these subjects (5, 6). Vitamin deficiencies (B₂, B₆, B₁₂, and folate), enzyme mutations with only limited loss of enzymatic activity (cystathionine-β-synthase, methionine synthase, and thermolabile methylenetetrahydrofolate reductase polymorphisms), and renal insufficiency may produce moderate hyperhomocysteinemia (15–30 µmol/L) (7–9). Dietary folate deficiency causes insufficient formation of 5-methyltetrahydrofolate, which is needed as a methyl-group donor in the remethylation of HCY to methionine (Fig. 1). However, most relevant to vegetarians is vitamin B₁₂ deficiency (dietary or inadequate absorption), which leads to impaired methyl transfer from 5-methyltetrahydrofolate to HCY during remethylation and, subsequently, increased serum HCY concentrations.

The principal difference among various vegetarian diets is the extent to which animal products are avoided. Some vegetarian diets provide less fat, less saturated fat, and fewer calories than typical omnivorous diets and have a higher content of fruits, vegetables, and whole-grain products. By total elimination of food of animal origin, vegetarians decrease their intake of some essential nutrients, including vitamin B₁₂. Vitamin B₁₂ typically is

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3 Nonstandard abbreviations: HCY, total homocysteine; MMA, methylmalonic acid; HME, high meat eater; LME, low meat eater; LVO, lacto-ovo vegetarian; LV, lacto vegetarian; TAS, total antioxidant status; and ABTS, 2,2′-azino-di-(3-ethylbenzthiazoline sulfonate).
found only in foods of animal origin. Thus, the avoidance of animal products in association with a strict vegetarian diet may lead to a deficiency of vitamin B12 (10, 11). The ultimate source of all vitamin B12 is microbial synthesis. Lacto-ovo and lacto vegetarians ingest adequate amounts of vitamin B12 from egg and dairy products (12–14). Omnivorous subjects typically ingest ~26 µg of vitamin B12 per day and excrete ~5–10 µg of vitamin B12 from their livers via bile into their intestines. When no intestinal reabsorption problems are present, the bodies of omnivorous subjects reabsorb ~3–5 µg of vitamin B12 per day. High liver stores combined with effective enterohepatic recirculation prevent healthy adult vegan vegetarians from developing vitamin B12 deficiency (15). However, people with low body storage of vitamin B12, impaired absorption or metabolism of vitamin B12, and physiological conditions with increased demands (e.g., pregnancy and breast feeding) may develop deficiency symptoms much faster. Prolonged vitamin B12 deficiency as a clinical disease usually manifests in neurologic and gastrointestinal disorders as well as anemia (16, 17).

Vitamin B12 (cobalamin) functions as an essential cofactor for only two enzymes in mammalian cells: L-methylmalonyl-CoA mutase requires adenosyl-cobalamin, and methionine synthase requires methylcobalamin (18). In vitamin B12 deficiency, increased concentrations of methylmalonyl-CoA are hydrolyzed and lead to increased amounts of methylmalonic acid (MMA). Increased serum HCY is an indicator of functional intracellular deficiency of vitamin B12 and folate, whereas increased MMA is a more specific indicator of functional vitamin B12 deficiency and is not dependent on folate status (19–22).

In the present study, we investigated omnivorous subjects and vegetarians with different dietary habits to determine the influence of vegetarian lifestyles on HCY and vitamin B12 status. The vegetarians in this study differed from omnivorous subjects not only in their dietary habits but also in their lifestyle, e.g., they consumed less alcohol, smoked less, and exercised more (Table 1). Although the actual B12 content of the different diets was not calculated, we have reason to believe that the vitamin B12 content of the food in these dietary groups was different. We tried to clarify whether MMA and HCY concentrations reflect dietary habits better than total vitamin B12 in serum. This could indicate that these metabolites are better early markers of a disturbed vitamin B12 status.

![Fig. 1. Metabolism of HCY.](image)

**Table 1. Patient characteristics.**

<table>
<thead>
<tr>
<th>Group</th>
<th>HME</th>
<th>All vegetarians</th>
<th>LME</th>
<th>LOV/LV</th>
<th>Vegans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smoke &gt;5 cigarettes/day</td>
<td>20%</td>
<td>5%</td>
<td>0%</td>
<td>9%</td>
<td>0%</td>
</tr>
<tr>
<td>Alcohol consumption&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30%</td>
<td>8%</td>
<td>16%</td>
<td>6%</td>
<td>0%</td>
</tr>
<tr>
<td>Exercise &gt;7 h/week</td>
<td>26%</td>
<td>61%</td>
<td>57%</td>
<td>62%</td>
<td>66%</td>
</tr>
<tr>
<td>BMI, kg/m&lt;sup&gt;2&lt;/sup&gt;</td>
<td>22.5 ± 3.1</td>
<td>21.6 ± 4.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>21.9 ± 2.3</td>
<td>21.4 ± 3.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>21.4 ± 5.2</td>
</tr>
<tr>
<td>Sex, F/M</td>
<td>30/14</td>
<td>36/14</td>
<td>17/2</td>
<td>24/10</td>
<td>5/2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Includes LME group.

<sup>b</sup> Less than the equivalent of 1 alcoholic drink/day.

<sup>c</sup> BMI, body mass index.

<sup>d</sup> Significant at 5% level compared with the HME group (Mann–Whitney test).
Subjects and Methods

A total of 104 apparently healthy subjects (randomly selected) living in the same region were investigated and classified into four groups based on their habitual dietary intake. All vegetarians were volunteers recruited at a conference of the German Federation of Vegetarians. High meat eaters and low meat eaters were selected from students and staff members. All participants were interviewed, and the average of three 24-h dietary recalls from the previous 3 days was used to calculate qualitative daily consumption of different nutrients. The participants had to meet the following criteria: constant dietary pattern for at least 1 year, values for basic hematologic variables within the appropriate reference intervals, no renal disease, no vitamin supplementation, no lipid-lowering drugs, no weight-loss diets, no medications or metabolic diseases that influence nutritional status, and no pregnancy. The use of oral contraceptives was not an exclusion criterion. The Human Ethics Committee at the Faculty of Medicine, University Leipzig, Germany approved the investigation. For additional subject characteristics, see Table 1. The principal difference among various vegetarian diets was the extent of which animal products were avoided; the subjects were therefore divided into the following groups:

- High meat eaters (HME; n = 44), who were the controls and consumed a typical omnivorous diet.
- Low meat eaters (LME; n = 19) usually excluded red meat and ate white meat or fish once or twice per week.
- Lacto-ovo vegetarians did not consume meat, poultry, or fish, but had no restrictions as to egg or dairy product consumption. Lacto vegetarians also excluded eggs. These subjects were combined into one group (LOV/LV; n = 34).
- Vegans (n = 7) excluded all foods of animal origin.

Laboratory Tests

All tests, with the exception of total antioxidant status (TAS), were performed on serum, which was collected after an overnight fast. The blood was allowed to clot on ice, and serum was obtained by centrifugation (4 °C) within 45 min after venipuncture and stored at ~70 °C.

HCY was measured by HPLC with fluorescence detection according to the method of Araki and Sako (23) (between-day CV, 4.5%). We found no significant difference between the HCY concentrations in plasma and serum. The HCY results for serum were ~5% higher than results for optimally prepared plasma. MMA was assayed by a modified capillary gas chromatography–mass spectrometry method according to the method described by Allen et al. (24) (capillary gas chromatograph Model 6890 with a Model 5973 mass-selective detector; Hewlett-Packard). We used a serum pool prepared in-house for quality control (within-day CV, 2.9%; between-day CV, 6.3%). Serum folate and vitamin B12 were measured with a chemiluminescence immunoassay (Bayer) on an ACS Centaur (Bayer). Control sera were obtained from the same company (between-day CVs, 9% for serum folate and 2.7% for vitamin B12).

Plasma TAS was measured on a Hitachi Analyzer with a Randox reagent set (Randox). Control samples were obtained from the same company (between-day CV, <5%). The latter determination is based on the reaction of 2,2'-azinodi-(3-ethylbenzthiazoline sulfonate) (ABTS®) with a peroxidase (metmyoglobin) and H2O2 to produce the radical cation ABTS+•:

$$\text{HX-Fe}^{3+} + \text{H}_2\text{O}_2 \rightarrow X-[\text{Fe}^{4+} = \text{O}] + \text{H}_2\text{O}$$

$$\text{ABTS} + X-[\text{Fe}^{4+} = \text{O}] \rightarrow \text{ABTS}^{+} + \text{HX-Fe}^{3+}$$

where HX-Fe4+ is metmyoglobin. The radical cation has a relatively stable blue-green color, which is measured at

Table 2. Medians (5th/95th percentiles) of metabolites, vitamins, and TAS in vegetarians.

<table>
<thead>
<tr>
<th>Group</th>
<th>HME (n = 44)</th>
<th>All vegetarians* (n = 60)</th>
<th>LME (n = 19)</th>
<th>LOV/LV (n = 34)</th>
<th>Vegans (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>23 (21/53)</td>
<td>22 (19/56)</td>
<td>24 (17/61)</td>
<td>22 (19.5/49.3)b</td>
<td>22 (19/30)</td>
</tr>
<tr>
<td>HCY, μmol/L</td>
<td>9.8 (5.9/16.7)</td>
<td>11.6 (6.3/19.3)c</td>
<td>11.8 (6.1/17.0)b</td>
<td>11.0 (5.7/20.8)</td>
<td>15.2 (9.3/18.5)c</td>
</tr>
<tr>
<td>TAS, mmol/L</td>
<td>1.21 (1.05/1.43)</td>
<td>1.17 (0.98/1.34)c</td>
<td>1.10 (0.95/1.24)c</td>
<td>1.21 (0.97/1.40)</td>
<td>1.14 (1.02/1.21)</td>
</tr>
<tr>
<td>MMA, nmol/L</td>
<td>169 (111/280)</td>
<td>209 (111/522)b</td>
<td>210 (95/257)</td>
<td>205 (110/572)</td>
<td>246 (153/292)b</td>
</tr>
<tr>
<td>Vitamin B12, pmol/L</td>
<td>276 (172/406)</td>
<td>243 (148/386)</td>
<td>240 (118/331)b</td>
<td>253 (153/376)</td>
<td>217 (153/438)</td>
</tr>
<tr>
<td>Folate, nmol/L</td>
<td>17.3 (7/36.5)</td>
<td>17.7 (9.1/33.8)</td>
<td>19.1 (10/26.1)</td>
<td>17.3 (7.9/44)</td>
<td>15.4 (10.4/24.5)</td>
</tr>
</tbody>
</table>

* Includes LME group.

c Significant compared with the HME group (Mann–Whitney test): c significant within 1% level; c significant within 5% level.
600 nm. Antioxidants contained in the serum sample suppress the formation of this color.

**Statistical Analysis**

Median values and 5th and 95th percentiles were calculated, and the Mann–Whitney test, correlation analysis by the Spearman $r$, and backward regression analyses were performed with the software package SPSS (Ver. 9.0 for Windows; SPSS).

**Results**

All results were median values because the test results showed skewed distribution, with the exception of TAS and vitamin B12. Compared with age- and sex-matched members of the HME group, the serum HCY concentrations of vegetarians were significantly increased (Table 2). Vegetarians also had higher serum MMA but slightly lower TAS. The serum concentrations of vitamin B12 and folate did not differ significantly between both groups. Only vegans showed a median HCY $>15$ mol/L. Compared with the HME group, the HCY median value was increased in the other subgroups, but did not reach the pathologic range of $>15$ mol/L. Similarly, only the serum MMA concentration of the vegans was significantly increased compared with the HME group, whereas in the other subgroups, the MMA concentration was only slightly increased. Compared with the HME group, all subgroups of vegetarians showed lower vitamin B12 serum concentrations, but the median serum vitamin B12 was significantly lower only in the LME group. For folate, we found rather uniform concentrations in all subgroups. The TAS of all vegetarian subgroups was lower than that of the HME group, reaching statistical significance only in the LME group. Additionally, vegetarians differed from the omnivorous HME group not only in their dietary habits but also in their lifestyle, e.g., they consumed less alcohol, smoked less, and exercised more (Table 1).

Correlation analysis revealed a highly significant correlation of MMA with HCY (Table 3). Vitamin B12 correlated with the other investigated variables (MMA, HCY, folate, TAS) at a 5% significance level. From backward regression analysis, it followed that the HCY concentration was significantly and independently influenced by MMA, age, and sex (Table 4). MMA was independently modulated by age, vitamin B12, and HCY. The TAS was influenced by vitamin B12 and sex only.

We found a high frequency of subjects with pathologically increased metabolite concentrations (HCY and/or MMA), whereas serum vitamin B12 and folate were pathologically decreased in only five cases (Table 5).

The scatter plots of MMA vs vitamin B12, HCY vs vitamin B12, and MMA vs HCY are depicted in Fig. 2. From Fig. 2A it follows that increased MMA was found only in subjects with serum vitamin B12 concentrations up to 360 mol/L. This concentration is approximately twice as high as the upper reference limit for vitamin B12.
Decreased serum vitamin B12 was linked with increased as well as normal MMA concentrations at almost the same frequency. Fig. 2B shows that, in our subjects, decreased serum vitamin B12 concentrations were detected only at HCY serum concentrations >8 µmol/L. Additionally, we found that increased MMA already occurred at HCY serum concentrations >8 µmol/L (Fig. 2C). The plots in Fig. 2 clearly indicate that MMA showed the highest discriminative power between the dietary groups in our study. Therefore, MMA represents the most sensitive test for early vitamin B12 deficiency. The odds ratio for all vegetarians, compared with the HME group, to have an increased MMA was 7 (95% confidence interval, 1.51–32.46) and to have an increased HCY was 3.42 (95% confidence interval, 0.90–12.95). An odds ratio for decreased vitamin B12 was not calculable.

**Discussion**

Our investigation clearly demonstrates that vegetarians have higher serum concentrations of HCY than omnivorous controls. The median value for all vegetarians was 11.6 µmol/L, compared with 9.8 µmol/L in omnivorous controls. The HCY concentration increased as the vegetarian diet became more restrictive and peaked in the group of vegans. Twenty percent of all vegetarians (inclusive the LME group) had moderate hyperhomocysteinemia (>15 µmol/L). To date, there has been no unanimous definition of hyperhomocysteinemia. In an European Concerted Action Program, hyperhomocysteinemia had been defined as HCY >12 µmol/L (25). Stampfer et al. (26) reported 90th and 95th percentiles for HCY of 14.1 and 15.8 µmol/L, respectively, for men free of a diagnosed vascular disease. They also found a 3.4-fold increased risk for myocardial infarction among men with a HCY concentration >15.8 µmol/L. In the Framingham Heart Study (27), it was shown that the risk for vascular disease was increased at concentrations ≥11.4 µmol/L. It has been suggested that HCY concentrations should be lowered to 9–10 µmol/L and that HCY values <10 µmol/L may be considered desirable (28, 29). HCY values <12 µmol/L are considered as optimal, the range 12–15 µmol/L as borderline, and values >15 to 30 µmol/L are defined as moderate hyperhomocysteinemia (3). The median age of our vegetarian group was 22 years and differed in this respect from the studies mentioned above. However, an age-related risk definition for HCY values is not suggested. Therefore, it can be assumed that a greater proportion of our vegetarians have HCY values in an unfavorable range.

The correlation analysis indicated a significant correlation between MMA and HCY and inversely between vitamin B12 and HCY, which is in agreement with results obtained by other investigators (30, 31). Compared with the occurrence of decreased vitamin B12 in serum, the vegetarians showed a higher frequency of increased MMA, which has also been reported in elderly subjects (30, 31). Additionally, from backward regression analysis it follows that the HCY is significantly and independently modulated by the MMA concentration but not by serum vitamin B12. This analysis also indicated that MMA is significantly influenced by HCY and serum vitamin B12. It should be mentioned that smoking, alcohol consumption, and physical exercise were not included in the multiple regression model. The results from backward regression analysis were confirmed by a study on elderly subjects who also have a high frequency of vitamin B12 deficiency.

![Fig. 2. Scatter plots illustrating the relationship between MMA and vitamin B12 (A), HCY and vitamin B12 (B), and MMA and HCY (C).](image-url)
The increased HCY concentration in a greater portion of vegetarians may possibly contribute to an increased atherosclerotic risk in these subjects (13, 35). In general, antioxidants play a significant role in the pathogenesis of atherosclerotic and age-related diseases (6). Epidemiologic data strongly support the hypothesis that high consumption of fruits and vegetables that are rich in monounsaturated and polyunsaturated fatty acids, minerals, fiber, complex carbohydrates, antioxidant vitamins, flavonoids, and nutrients together with a otherwise healthy lifestyle protects against degenerative diseases (36–40). A recent publication (41) reports that 1 week after a change to a vegan diet-based lifestyle, HCY was significantly reduced (~13%). The authors concluded that because of the short duration of this lifestyle change, factors other than B vitamins are involved in lowering HCY. However, it can be supposed that the generally healthier lifestyle of vegetarians could be partly reversed by increases in HCY as a consequence of vitamin B12 deficiency. Mezzano et al. (35) reported that increased platelet function and HCY may counteract the known cardiovascular health benefits of a vegetarian diet.

We were able to show that vegetarians, especially the LME and vegan groups, had a reduced TAS, whereas the TAS of LOV/LV was not different from that of the HME group. The TAS decreased with increasing avoidance of vitamin B12-containing animal products, whereas HCY increased in the same order. The total antioxidant concentration correlated highly significantly with the vitamin B12 concentration in serum but not with HCY. In addition, the correlation as well as backward regression analysis demonstrated that serum vitamin B12, but not MMA, as a marker for a functional B12 status is the variable that influences the TAS. Therefore, from our study we cannot totally exclude that components other than vitamin B12 in cobalamin-rich food could possibly improve TAS. The missing significant correlation between HCY and TAS might be attributable to the fact that of total HCY, 98% is oxidized HCY and only ~2% is reduced (free) HCY, which can be oxidized and in this way modulate TAS. Rauma and Mykkanen (6) reported that measurements of antioxidant status in vegetarians showed that a vegetarian diet maintains a high antioxidant vitamin status (vitamins C and E, β-carotene) but a variable antioxidant trace...
element status compared with omnivorous diet. They therefore recommended evaluation of the total antioxidant capacity rather than the status of a single antioxidant nutrient. Our results underscore this statement and add that in subjects on restrictive vegetarian diets, insufficient vitamin B₁₂ intake is a very important factor that influences the TAS. Therefore, sufficient vitamin B₁₂ supplementation for persons on restrictive vegetarian diets is of great importance.

Additional studies confirming our results are needed. These studies should focus on determining the diagnostic value of vitamin B₁₂ markers, such as transcobalamin II and MMA, compared with vitamin B₁₂. Studies on well-characterized vegetarian groups having quantitative dietary protocols could investigate the influence of different vegetarian diets on HCY metabolism, taking special consideration of the content of vitamin B₁₂ and other vitamins. A possible influence of confounders, such as renal function, alcohol and coffee consumption, smoking habits, intake of supplements, sex hormones, physical exercise, duration of dietary habit, and other factors should be taken into account and, if possible, excluded or minimized. Last but not least, the importance of supplements, especially vitamin B₁₂, to compensate for the adverse effects of certain vegetarian diets should be considered.

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References

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