Altered Vitamin B\textsubscript{12} Status in Recreational Endurance Athletes

Markus Herrmann, Rima Obeid, Juergen Scharhag, Wilfried Kindermann, and Wolfgang Herrmann

This study aimed to compare the vitamin B\textsubscript{12} and folate status of recreational endurance athletes and inactive controls by modern biomarkers. In 72 athletes (38 ± 7 y) and 46 inactive controls (38 ± 9 y) serum levels of vitamin B\textsubscript{12}, methylmalonic acid (MMA), holotranscobalamin II (holoTC), folate, and homocysteine (Hcy) were measured. Vitamin B\textsubscript{12} and folate levels of both groups were comparable, but athletes had higher median (25.–75. percentile) MMA [242 (196 to 324) versus 175 (141 to 266) nmol/L] and holoTC concentrations [67 (52 to 93) versus 55 (45 to 70) pmol/L] than controls. Hcy was slightly lower in athletes [9.2 (7.2 to 12.6) versus 10.8 (8.9 to 12.9) nmol/L]. In controls, we found the following correlations: vitamin B\textsubscript{12} and MMA (\(r = -0.38\)), vitamin B\textsubscript{12} and holoTC (\(r = 0.51\)), MMA and holoTC (\(r = -0.36\)). In athletes, MMA did not correlate with vitamin B\textsubscript{12} and holoTC. Our data suggests an altered vitamin B\textsubscript{12} metabolism in recreational athletes that needs further investigation.

Key Words: vitamin B\textsubscript{12}, methylmalonic acid, holotranscobalamin, folate, homocysteine

Recreational as well as elite athletes with a normal Western diet frequently use vitamin supplements (19, 23). Most of these preparations contain vitamin B\textsubscript{12} and folate. Since there are only a few studies investigating the vitamin B\textsubscript{12} and folate status in athletes, an evidence-based recommendation for vitamin B\textsubscript{12} and folate supplementation is actually not justified (8, 9, 29). A previous study by Singh et al. indicates, however, that vitamin B\textsubscript{12} metabolism might be altered in endurance athletes (26).

Vitamin B\textsubscript{12} and folate are important co-factors of the methionine pathway, the most important source of methyl groups in the human organism (10, 25). Many substrates centrally involved in physical exercise, such as catecholamines, creatine, and DNA, obtain their methyl groups from this pathway (3, 4, 28). All previous studies investigating vitamin B\textsubscript{12} in athletes measured vitamin B\textsubscript{12} directly. Compared to modern markers of vitamin B\textsubscript{12} status, however, such as holotranscobalamin II (holoTC) and methylmalonic acid (MMA), the sensitivity and specificity...
of common vitamin B₁₂ measurement is limited (5, 13, 14, 21). Especially for vitamin B₁₂ levels < 300 pmol/L, the clinical performance of holoTC and MMA is considerably better than direct measurement of vitamin B₁₂ (14). The limitations of common vitamin B₁₂ detection are mainly due to the fact that only 6 to 20% of circulating vitamin B₁₂ is metabolically active (12). Moreover, it has been shown that the extracellular vitamin B₁₂ level is not always correlated with the intracellular vitamin B₁₂ supply (13, 14).

Hcy is the final product of the methionine pathway and can be cleared by the transsulfuration or the re-methylation pathway (10, 25). The vitamin B₁₂ dependent re-methylation recycles Hcy by the transfer of a methyl group from 5-methyltetrahydrofolate, which results in the formation of methionine. The transsulfuration couples Hcy with serine to form cystathionine, which is then cleaved into cysteine and α-ketobutyrate. Methylmalonic acid is a metabolite in the degradation cascade of α-ketobutyrate. The conversion of methylmalonyl-CoA into succinyl-CoA, catalyzed by the L-methylmalonyl-CoA-mutase, is an exclusively vitamin B₁₂ dependent step during the degradation of α-ketobutyrate (27). It has been shown that vitamin B₁₂ deficiency can result in an accumulation of MMA prior to the enzyme (2). Therefore, MMA is used as a marker of intracellular vitamin B₁₂ deficiencies.

Serum vitamin B₁₂ is bound to three different transport proteins, transcobalamin I, II, and III. Only the complex of vitamin B₁₂ and transcobalamin II, named holo-transcobalamin II (holoTC), can be utilized by all cells. Therefore, holoTC represents the metabolically active fraction of circulating vitamin B₁₂ (24). Since MMA, holoTC, and Hcy have never been used to estimate vitamin B₁₂ and folate status in athletes, we studied the supply and utilization of these 2 vitamins in recreational endurance athletes and inactive controls using the above-mentioned modern biomarkers.

Materials and Methods

Subjects and Design

In the present study, we investigated serum levels of vitamin B₁₂, MMA, holoTC, Hcy, and folate in 72 healthy recreational endurance athletes (64 men and 8 women) and 46 nonexercising healthy controls (34 men and 12 women), who did not use vitamin supplements. The study design was approved by the institutional review board. All subjects gave written informed consent.

Blood Samples and Assays

Nonfasting venous blood samples were taken at rest. In athletes, blood sampling was done at least 8 h after the last training. After 20 min of coagulation, serum was immediately separated from blood cells by centrifugation and transported on ice to our laboratory, where samples were stored at –20 °C until analysis.

Vitamin B₁₂ and folate were measured with commercial, competitive chemiluminescence immunooassays (Bayer Diagnostics, Fernwald, Germany) on an ACS Centaur automated analyzer (Bayer Diagnostics). The folate assay detects 5-methyltetrahydrofolate. Inter- and intraassay CVs were < 4.0 and < 4.4% for vitamin B₁₂ and < 5.5 and < 5.3% for folic acid, respectively. Hcy was detected by a commercial HPLC assay (Immundiagnostik, Bensheim, Germany) with fluorescence detection.
MMA was assayed by a modified capillary gas chromatography-mass spectrometry method (capillary gas chromatograph, model 6890 with a Hewlett-Packard model 5973 mass-selective detector) according to the method described by Allen et al. (1). Inter- and intraassay CVs were ≤ 7.2% for Hcy and ≤ 3.3% for MMA. HoloTC measurement was done with a commercial radioimmunassay (Axis-Shield, Oslo, Norway). Inter- and intraassay CVs were < 8%.

Statistical Analysis

A Kolmogorov-Smirnov test was used to test for normal distribution. Vitamin B₁₂, MMA, holoTC, folate, and Hcy did not show a normal distribution. Hence, all laboratory variables are provided as median (25.-75. percentile). Anthropometrical data are given as mean ± standard deviation. Group comparison was done with a Mann-Whitney U-test. Furthermore, we performed a Spearman’s correlation analysis with all biochemical markers.

Results

Athletes (runners and cyclists) had a mean age of 38 ± 7 y, a mean height of 178 ± 7 cm, and a mean weight of 72 ± 9 kg. The weekly training volume ranged between 5 to 10 h and regular training had continued over the last 7 ± 6 y. Training was not standardized. Controls had a mean age of 38 ± 9 y, a mean height of 176 ± 9 cm, and a mean weight of 73 ± 12 kg. All subjects had an omnivorous diet and did not use vitamin supplements. The descriptive statistics of all laboratory parameters are presented in Table 1. Median vitamin B₁₂ and folate levels were comparable in athletes and controls, and ranged within the reference intervals of the applied assays (lower reference limit of folate: 5.38 ng/mL; reference interval of vitamin B₁₂: 211 to 911 pg/mL). These results indicate a comparable and sufficient micronutrient status in both groups. MMA and holoTC were, however, significantly higher in athletes than in controls. The median MMA level in athletes was close to the upper reference limit of 270 nmol/L.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Controls (n = 46)</th>
<th>Athletes (n = 72)</th>
<th>P–value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin B₁₂ (pg/mL)</td>
<td>350 (255–428)</td>
<td>343 (285–431)</td>
<td>0.83</td>
</tr>
<tr>
<td>Holotranscobalamin II (pmol/L)</td>
<td>55 (45–70)</td>
<td>67 (52–93)</td>
<td>0.004*</td>
</tr>
<tr>
<td>Methylmalonic acid (nmol/L)</td>
<td>175 (141–266)</td>
<td>242 (196–324)</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>Folate (ng/mL)</td>
<td>8.6 (6.1–15.1)</td>
<td>8.3 (6.1–10.9)</td>
<td>0.52</td>
</tr>
<tr>
<td>Homocystein (μmol/L)</td>
<td>10.8 (8.9–12.9)</td>
<td>9.2 (7.2–12.6)</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Note. Results are given as median (25.–75. percentile); *P < 0.05.
Figure 1 depicts the individual vitamin B₁₂, holoTC, and MMA levels of all subjects investigated. Four athletes exhibited vitamin B₁₂ serum levels below the lower reference limit and 4 athletes had lowered holoTC concentrations (lower reference limit: 35 pmol/L). One athlete with lowered holoTC had also a lowered vitamin B₁₂. Based on holoTC and vitamin B₁₂ results, 7 athletes (10%) would have been classified as vitamin B₁₂ deficient. Both markers, however, represent only the extracellular vitamin B₁₂ status. By contrast, MMA, a marker of the functional intracellular vitamin B₁₂ supply, was increased in 25 athletes (35%). Only one of those 25 athletes had a lowered vitamin B₁₂ serum level. In controls, we found 2 subjects with lowered vitamin B₁₂ and five with lowered holoTC levels. Altogether, we would have classified 7 controls (15%) as deficient for circulating vitamin B₁₂. Only 9 of 47 controls (19%) had elevated MMA levels, which is significantly less than in athletes. Considering folate levels, we found a comparable frequency of folate deficiencies (15%) in athletes (n = 10) and controls (n = 7). Furthermore, median Hcy, a cumulative marker of the methionine metabolism, was clearly below the upper reference limit in both groups (upper reference limit < 12 μmol/L), and tended to be somewhat lower in athletes than in controls (about 15%; \( P = 0.06 \)).

As expected, in controls there were significant correlations between vitamin B₁₂, and MMA \( (r = -0.38, P = 0.008) \), vitamin B₁₂, and holoTC \( (r = 0.51, \)
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P = 0.000), MMA, and holoTC (r = –0.36, P = 0.016) as well as Hcy and folate (r = –0.404, P = 0.006). Furthermore, Hcy correlated with vitamin B12 and holoTC on a 10% level (Hcy vs. vitamin B12: r = –0.27, P = 0.075; Hcy vs. holoTC: r = 0.28, P = 0.067).

In athletes, vitamin B12 correlated significantly with holoTC (r = 0.58, P = 0.000). Hcy correlated with folate (r = –0.43, P = 0.000), vitamin B12 (r = –0.33, P = 0.006), and holoTC (r = –0.29, P = 0.017). In contrast to controls, the correlations of vitamin B12 with MMA (r = –0.01, P = 0.913) and MMA with holoTC (r = 0.01, P = 0.931) were not present in athletes.

In a next step, we divided athletes and controls in tertiles of vitamin B12 and holoTC, and calculated the corresponding medians of MMA (Figure 2). In controls MMA levels decreased from the first to the third tertile independently if classification was done for vitamin B12 or holoTC. Athletes exhibited no such decrease.

Figure 2 — Analysis of median MMA concentrations in tertiles of vitamin B12 and holoTC. The numbers beside each data point represent the median (25.-75. percentile) vitamin B12 or holoTC concentration in the corresponding tertile. The dashed lines display the upper reference limit of MMA.
Athletes and controls had similar vitamin B\textsubscript{12} and folate serum levels (within the reference intervals) indicating a comparable and sufficient status of these micronutrients. Analysis of Hcy, MMA, and holoTC suggest an altered vitamin B\textsubscript{12} metabolism, however, but a normal folate status in athletes.

Median holoTC was significantly higher in athletes than in controls (Table 1). This finding was somewhat surprising, since serum vitamin B\textsubscript{12} was comparable in both groups. Vitamin B\textsubscript{12} measurement detects a group of chemically related molecules consisting of four pyrrol rings with a central cobalt atom. Only holoTC, however, which accounts for 6 to 20\% of circulating vitamin B\textsubscript{12}, can be utilized by all cells (12). Recent studies have demonstrated that measurement of holoTC represents better circulating vitamin B\textsubscript{12} than common vitamin B\textsubscript{12} assays (5, 13, 14, 21). Since athletes had higher holoTC levels, we expected a better cellular vitamin B\textsubscript{12} supply, expressed by lower MMA levels. But we found significantly higher MMA levels in athletes than in controls. Median MMA in athletes was close to the upper reference limit, suggesting a critical intracellular vitamin B\textsubscript{12} supply. Correlation analysis revealed a significant association between vitamin B\textsubscript{12} and holoTC, which was comparable in both groups. In addition, in controls we found significant correlations between MMA, vitamin B\textsubscript{12}, and holoTC. These results are in accordance with previous results by others and ourselves (11, 15, 16). In athletes, however, the correlations of vitamin B\textsubscript{12} and holoTC (markers of extracellular vitamin B\textsubscript{12} supply) with MMA (marker of intracellular vitamin B\textsubscript{12} supply) were not present, suggesting a disturbed cellular vitamin B\textsubscript{12} metabolism.

What might be the reason for the discrepancy between MMA and holoTC in athletes? The main difference between athletes and controls was the regular endurance training. Endurance exercise is known to cause changes in plasma volume, which might affect the serum concentration of all molecules which can not freely pass the vessel wall (7). MMA, however, represents a very small molecule that is not concentrated or diluted by changes in plasma volume. Consequently, hemoconcentration as the underlying cause for higher MMA levels in athletes can be ruled out. An exercise-induced stimulation of β-oxidation in athletes might be another hypothetical explanation for the discrepancy between MMA and holoTC. Propionic acid, a metabolite in the β-oxidation cascade, is transformed into propionyl-CoA, representing the direct precursor of MMA. Therefore, a stimulated β-oxidation in athletes might result in an increased formation of MMA (independent of vitamin B\textsubscript{12}). We have demonstrated, however, that endurance exercise causes only a brief (reversible within a few hours) transient MMA increase (unpublished data). In the present study, blood sampling was performed at rest (≥ 8 h after the last training session) and 1 to 12 h before a long-distance race (marathon running or a 110 km mountain bike race). Accordingly, the last training session before blood sampling was short and of low intensity. That is why a stimulated β-oxidation as an explanation for higher MMA levels in athletes seems improbable. Elevated MMA levels in the presence of normal vitamin B\textsubscript{12} and holoTC levels can also be due to intestinal bacterial overgrowth (20). It has been shown that intestinal bacteria are able to synthesize MMA, which appears in the circulation. A high prevalence of bacterial overgrowth in athletes has never been described, however, and would probably have been recognized by clinical symptoms.
Compared to inactive controls, athletes are exposed to oxidative stress, inducing multiple modifications of cell physiology (6, 17, 18). Two previous studies on vegetarians and neurological patients have shown that oxidative stress could be related to a disturbed vitamin B$_{12}$ metabolism (16, 22). McCaddon et al. reported that in patients with Alzheimer’s disease oxidative stress might hamper the intracellular reduction of vitamin B$_{12}$ to its metabolically active form (22). Herrmann et al. found significantly higher MMA levels and a reduced antioxidative status in vegetarians than in omnivorous controls (16). Moreover, in vegetarians, the antioxidative status and MMA were significantly correlated. Based on these findings, we hypothesize that oxidative stress might be a relevant causative factor for the altered vitamin B$_{12}$ metabolism in athletes. We did not, however, quantify oxidative stress and, therefore, this hypothesis is highly speculative and remains to be proven.

Given that vitamin B$_{12}$ deficiencies are involved in the pathogenesis of various diseases, such as anemia and neurodegenerative disorders (11), it would be interesting to know whether vitamin B$_{12}$ supplementation can improve intracellular vitamin B$_{12}$ status in athletes. Since our subjects did not use supplements, we cannot answer this question. A hint, however, can be obtained by the analysis of MMA when subjects are divided in tertiles of vitamin B$_{12}$ or holoTC. As expected, controls exhibited a significant decline of MMA from the first to the third tertile. In contrast, athletes showed no decline of MMA. Even in the third tertile (vitamin B$_{12}$: 501 pg/mL; holoTC: 115 pmol/L) MMA did not fall below 250 nmol/L, which indicates a critical intracellular B$_{12}$ status. Because of the possibly disturbed cellular vitamin B$_{12}$ metabolism it is questionable whether athletes would benefit from an oral vitamin B$_{12}$ substitution. Only an intervention trial, however, can clarify this question.

The second vitamin investigated was folate. Most folate assays detect 5-methyltetrahydrofolate, the major form in human serum, which correlates well with the intracellular folate supply. The methyl group of 5-methyltetrahydrofolate is intracellularly transferred onto Hcy to form methionine (remethylation). Folate deficiency leads to an inhibition of this reaction with a subsequent accumulation of Hcy (14). Athletes and controls exhibited comparable folate levels within the reference interval. Moreover, athletes had slightly lower Hcy levels than controls, indicating a sufficient intracellular folate status. The correlation between Hcy and folate was similar in athletes and controls. These results demonstrate that folate metabolism is comparable in the 2 groups.

Lastly, our data suggest a disturbed cellular vitamin B$_{12}$ utilization among recreational endurances athletes. The underlying mechanism of the disturbed vitamin B$_{12}$ metabolism is not understood and needs clarification. In contrast, folate status is normal in athletes and controls. Therefore, a general recommendation to substitute folate in athletes is not justified.

References
