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We report that serum holotranscobalamin (holoTC) compares favorably with serum vitamin B12 for identifying vegans likely to have vitamin B12 deficiency as judged by measurements of the metabolites methylmalonic acid (MMA) and homocysteine (tHcy). We also report that measurement of holoTC may possibly replace combined testing with serum vitamin B12 (B12), MMA, and tHcy in this population.

Recently, two new markers for vitamin B12 deficiency, holoTC (TC saturated with vitamin B12) and the related TC saturation (the fraction of total TC present as holoTC), have been introduced (1–3). Approximately 30% of circulating B12 is attached to TC, whereas the major part of B12 is attached to another protein, haptocorrin. Because only B12 attached to TC (holoTC) is able to enter all the cells of the body, holoTC may be a more useful marker than total B12 in serum.

We compared holoTC with the tests currently used for diagnosis of vitamin B12 deficiency, i.e., B12, MMA, and tHcy, in vegan men, whose diets are devoid of food of animal origin (and thus low in vitamin B12) and who thus are susceptible to developing B12 deficiency.

The vegan men were recruited from two sources: 100 (of a subset of 233) vegan men enrolled in the Oxford cohort of the EPIC study (European Prospective Investigation into Cancer and Nutrition) between 1993 and 1997 for whom measurements of B12 had been performed at enrollment [mean (range) B12 at enrollment, 170 (67–397) ng/L; mean (range) age at recruitment, 49 (27–78) years]; and 72 men (of a total of 96 respondents) who were members of the London Vegan Society between 2001 and 2002 [mean (range) age, 38 (18–70) years]. The vegan men had consumed no food of animal origin for a mean (range) of 24 (2–76) years. The reported analyses are on blood samples obtained from the vegan men between February 2001 and February 2002. A reference group of 23 men consuming a mixed diet (omnivores) was recruited among the staff and student population of King’s College London [mean (range) age, 41 (23–61) years]. Individuals with a history of major gastrointestinal or liver disease, current medication for psychiatric disorders, injections of vitamin B12, or diabetes mellitus were excluded. The protocol of the study was approved by the Research Ethics Committee of King’s College London, and all participants gave informed written consent.

Blood samples were obtained by venipuncture in the morning after an overnight fast and collected in Vacutainer Tubes containing no anticoagulant (B12, folate, total and holoTC) and in chilled lithium-heparin Vacutainer Tubes (MMA and tHcy; plasma was separated within 5 min of collection). Samples were stored at −70 °C until shipped on dry ice or thawed for analysis.

Serum total TC and holoTC were measured by an ELISA (2) modified for use on an automated analyzer (BEP-2000; Dade Behring) at 37 °C. The reference intervals for 161 blood donors (age interval, 21–65 years) were 700–1500 pmol/L for total TC, ≥50 pmol/L for holoTC, and ≥0.05 for TC saturation. The assay imprecision (CV) was 4% for holoTC, 6% for total TC, 8% for MMA and folate, and 5% for tHcy and B12, as judged by internal quality-control systems. Analysts performing the holoTC assays were unaware of the other results and vice versa.

MMA (reference interval, 0–0.28 μmol/L) and tHcy (reference interval, 0–12 μmol/L) were measured by gas chromatography–mass spectrometry (4). Serum folate (reference interval, 3–15 μg/L) and B12 [reference interval ≥180 ng/L (≥135 pmol/L)] were measured on an Immuno 1 analyzer (Bayer Diagnostics). Two vegans and one omnivorous individual had serum folate concentrations below the reference interval. Parietal cell and intrinsic factor antibodies were analyzed in all individuals with B12 <120 ng/L, but none was positive.

Statistical analyses were conducted using Prism 4 (GraphPad Software): between-group comparisons were made using a Mann–Whitney test; associations were tested using Pearson’s correlation coefficient; and ROC curves and areas (with 95% CI) were used to estimate diagnostic accuracy for holoTC and B12.

HoloTC (median and range) was significantly lower (P <0.000001) in vegan men [41 (8–240) pmol/L] than in omnivores [95 (62–210) pmol/L], as was the TC saturation in the vegans [0.045 (0.008–0.37)] compared with the omnivores [0.099 (0.061–0.19)]. No significant difference was observed for total TC [vegans, 980 (760–1400) pmol/L; omnivores, 920 (550–2000) pmol/L], the medians for vegans and omnivores were 210 and 380 pmol/L, respectively, for B12; 0.27 and 0.14 μmol/L for MMA; and 13 and 9.8 μmol/L for tHcy. The fractions of vegan men showing test results outside the reference intervals were 0.58 for holoTC, 0.49 for TC saturation, 0.45 for B12, 0.48 for MMA, and 0.55 for tHcy. Although there have been reports of inadequate vitamin B12 status in vegetarians (5,6) and vegans (7), the severity was more marked in the present study, possibly because the individuals had followed their diets for longer.

HoloTC correlated strongly to TC saturation (r = 0.96; P <0.0001). For this reason, only holoTC was included in further statistical analysis. Both holoTC and B12 showed similar negative correlations with MMA and tHcy (r = −0.70 and −0.66, respectively, for correlation with MMA; r = −0.69 and −0.67, respectively, for correlation with tHcy; see the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol49/issue12/). B12 was strongly correlated with holoTC (r = 0.75), as were MMA and tHcy (r = 0.72). The strong correlation between...
holoTC and B12 is in agreement with recently published results (8). The correlations between MMA and both holoTC and B12 are in agreement with a previous study on vegetarians and vegans (7), but they are considerably stronger than observed for other target groups (9,10). A likely explanation for this difference is that the vegetarians/vegans suffer from no competing condition, whereas this may very well be the case in other population groups with suspected vitamin B12 deficiency. ROC curves were used to compare the ability of holoTC and B12 to predict the men likely to have metabolic vitamin B12 deficiency as indicated by MMA >0.75 μmol/L and tHcy >15 μmol/L (n = 36) compared with the others (n = 159) in a combined group of vegans and omnivores. The areas under the ROC curves were similar for holoTC [0.87; 95% confidence interval (CI), 0.81–0.93] and B12 (0.86; 95% CI, 0.81–0.92; Fig. 1A). A comparison was made of holoTC and B12 in omnivores and in vegan men grouped according to likelihood of suffering from vitamin B12 deficiency.

### Table 1. Biochemical markers of vitamin B12 deficiency in omnivores and in vegan men grouped according to likelihood of suffering from vitamin B12 deficiency.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Reference limits</th>
<th>Omnivores (n = 23)</th>
<th>Vegans (n = 172)</th>
<th>Comparisons</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Unlikely to be B12 deficient&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Uncertain to be B12 deficient&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>B12, ng/L</td>
<td>≥180</td>
<td>380 (340–500)</td>
<td>350 (270–490)</td>
<td>175&lt;sup&gt;d&lt;/sup&gt; (109–270)</td>
</tr>
<tr>
<td>HoloTC, pmol/L</td>
<td>≥50</td>
<td>95 (76–130)</td>
<td>71&lt;sup&gt;e&lt;/sup&gt; (56–110)</td>
<td>35&lt;sup&gt;e&lt;/sup&gt; (26–52)</td>
</tr>
<tr>
<td>MMA, μmol/L</td>
<td>≤0.28</td>
<td>0.14 (0.11–0.18)</td>
<td>0.15 (0.12–0.20)</td>
<td>0.27&lt;sup&gt;f&lt;/sup&gt; (0.21–0.41)</td>
</tr>
<tr>
<td>tHcy, μmol/L</td>
<td>≤12</td>
<td>9.4 (8.2–12)</td>
<td>9.4 (8.4–11)</td>
<td>14&lt;sup&gt;f&lt;/sup&gt; (12–16)</td>
</tr>
<tr>
<td>HoloTC, pmol/L</td>
<td>≥50</td>
<td>95 (76–130)</td>
<td>73&lt;sup&gt;f&lt;/sup&gt; (60–110)</td>
<td>41&lt;sup&gt;f&lt;/sup&gt; (31–55)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Unlikely: MMA ≤0.28 μmol/L and tHcy ≤12 μmol/L.
<sup>b</sup> Uncertain: at least one value outside the limits indicated in "unlikely", but less than two values outside the limits indicated in "likely".
<sup>c</sup> Likely: MMA >0.75 μmol/L and tHcy >15 μmol/L.
<sup>d</sup> P <0.0001;  <sup>e</sup> P <0.01;  <sup>f</sup> P <0.02.
<sup>e</sup> Compared with omnivores:  <sup>e</sup> P <0.0001;  <sup>f</sup> P <0.001;  <sup>f</sup> P <0.02.
<sup>a</sup> Unlikely: MMA ≤0.28 μmol/L, tHcy ≤12 μmol/L, and B12 ≥180 ng/L.
<sup>b</sup> Likely: at least two values outside MMA >0.75 μmol/L, tHcy >15 μmol/L, and B12 <120 ng/L.
men classified as unlikely to be, uncertain to be, or likely to be vitamin B12 deficient (Table 1A). HoloTC was significantly ($P <0.01$) lower in the group of vegan men unlikely to have vitamin B12 deficiency compared with omnivores, whereas this was not the case for B12 ($P = 0.30$; Table 1A).

Finally we examined whether holoTC might replace combined testing with B12, MMA, and tHcy. In this analysis, individuals were classified as likely to have vitamin B12 deficiency if at least two of three tests were outside the cutoff limits ($B12 <120 \text{ ng/L, MMA } >75 \text{ mmol/L, tHcy } >15 \text{ mmol/L; } n = 56$) or not ($n = 139$; all others). The diagnostic accuracy of holoTC was then assessed by ROC curve analysis (Fig. 1B). The areas under the ROC curves were 0.91 (95% CI, 0.87–0.95) for all individuals, 0.88 (95% CI, 0.82–0.95) for vegan men recruited from the London Vegan Society, and 0.92 (95% CI, 0.85–0.99) for the men recruited from the London Vegan Society.

We also compared holoTC and the other three markers in omnivores and in vegan men classified as unlikely to be, uncertain to be, or likely to be vitamin B12 deficient based on test results for B12, MMA, and tHcy (Table 1B). HoloTC was significantly lower ($P = 0.017$) in the vegan men who were unlikely to be vitamin B12 deficient compared with the omnivores, whereas no significant difference was observed for the other three measures: $B12 (P = 0.49), \text{MMA (P = 0.37), and tHcy (P = 0.47)}$.

Each of the laboratory tests used for diagnosis of vitamin B12 deficiency has its weaknesses. The metabolites MMA and tHcy are influenced by kidney function (10, 11), and tHcy also depends on the folate status of the patient. B12 is influenced by the concentration of the binding proteins of vitamin B12, and is increased in patients with myeloproliferative diseases (12).

Our data are consistent with an early decrease of holoTC in vitamin B12 deficiency in vegans and further suggest that holoTC might replace combined testing with B12, MMA, and tHcy in this population group. If holoTC is used as the primary screening test, we suggest no further testing for patients with holoTC $>50 \text{ pmol/L}$ (unlikely to suffer from vitamin B12 deficiency) and holoTC $<25 \text{ pmol/L}$ (likely to suffer from vitamin B12 deficiency). For patients with holoTC between 25 and 50 pmol/L, we would suggest further testing with one of the metabolic markers.

Further studies are needed to evaluate the validity of holoTC in other patient groups, especially those with conditions likely to influence markers of vitamin B12 deficiency. Measurement of holoTC may be of particular value in identifying vitamin B12 deficiency in patients with kidney malfunction, in whom the metabolites may show falsely increased values (10, 11), and in patients with myeloproliferative diseases, in whom the concentration of B12 may be falsely increased (12). We conclude that holoTC is a promising indicator of vitamin B12 deficiency.

This work was supported by the Internationale Stiftung für Ernährungsforschung und für Ernährungsaufklärung, EUREKA (CT-T2006) and by EU Biomed (QLK3-CT-2002-01775). The assistance of Anna-Lisa Christensen, Roy Sherwood, and Jette Fisker Pedersen is warmly acknowledged. None of the authors has a conflict of interest to declare.

References


DOI: 10.1373/clinchem.2003.020743

UDP-glucuronosyltransferase 1A1 (UGT1A1) is the key enzyme for bilirubin conjugation. Defects in this enzyme can cause a nonhemolytic unconjugated hyperbilirubinemia, such as Crigler–Najjar syndrome type 1 (CN1)