Nonradioactive Vitamin $B_{12}$ Absorption Test Evaluated in Controls and in Patients with Inherited Malabsorption of Vitamin $B_{12}$

Mustafa Vakur Bor,1,2* Mualla Çetin,3 Selin Aytaç,3 Çiğdem Altay,3 and Ebba Nexo1

**Background:** Current tests for evaluation of vitamin $B_{12}$ absorption are problematic because they involve the use of radioactively labeled vitamin $B_{12}$. We describe a vitamin $B_{12}$ absorption test that circumvents this problem.

**Methods:** We measured cobalamin or transcobalamin saturated with cobalamin (holo-TC) 24 h after three 9-μg doses of vitamin $B_{12}$ given orally at 6-h intervals. We studied 17 patients with inherited malabsorption of vitamin $B_{12}$ attributable to Imerslund–Grasbeck syndrome ($n = 13$) or intrinsic factor deficiency ($n = 4$), their obligate heterozygous biological parents ($n = 19$), and healthy controls ($n = 44$).

**Results:** In the patients, the median (range) change of holo-TC after the $B_{12}$ load was not significant [1 (–42 to 5) pmol/L], nor was the change of cobalamin [3 (–32 to 22) pmol/L], consistent with a lack of measurable active or passive absorption. In controls, however, the median (range) increases of holo-TC and cobalamin were 26 (–6 to 63) pmol/L and 41 (–37 to 109) pmol/L, respectively. Similarly, the parents showed increases of 23 (–2 to 47) pmol/L and 27 (–15 to 94) pmol/L. The mean areas under the ROC curves (95% confidence intervals) were 0.97 (0.93–1.0) for holo-TC and 0.87 (0.79–0.94) for cobalamin, distinguishing patients from controls. At a cutoff of 6 pmol/L for holo-TC, the diagnostic sensitivity (95% confidence interval) was 100 (81–100)% and the diagnostic specificity was 92 (82–97)%.

**Conclusion:** Measurement of holo-TC after administration of vitamin $B_{12}$ is a promising approach for evaluating vitamin $B_{12}$ absorption.

© 2005 American Association for Clinical Chemistry

---

1 Department of Clinical Biochemistry, NBG, AS, and 2 Department of Clinical Biochemistry, Aalborg Hospital, Aarhus University Hospital, Aarhus, Denmark.

Address correspondence to this author at: Department of Clinical Biochemistry, NBG, AS, Aarhus University Hospital, Norrebrogade 44, DK-8000 Aarhus C, Denmark. Fax 45-89493060; e-mail vakurbor@hotmail.com.

Received June 5, 2005; accepted August 23, 2005.

Previously published online at DOI: 10.1373/clinchem.2005.055509

---

Evaluation of the intestinal absorption of vitamin $B_{12}$ is a necessary step in determining the etiology of vitamin $B_{12}$ deficiencies. All available tests, including the classic Schilling test, rely on measurement of radioactively labeled vitamin $B_{12}$ after an oral dose of the vitamin (1). The use of a radioactively labeled compound has made the tests increasingly unacceptable (2). An alternative approach is therefore needed for estimation of the absorption of vitamin $B_{12}$. Measurement of vitamin $B_{12}$-saturated transcobalamin (holo-TC)4 or total cobalamin after oral ingestion of vitamin $B_{12}$ may represent such a test.

We recently demonstrated that a consistent and significant increase in holo-TC occurs in healthy individuals after oral intake of three 9-μg doses of vitamin $B_{12}$ given at 6-h intervals, indicating that this analyte reflects active vitamin $B_{12}$ absorption (3). In the present study, we evaluated the use of this new approach as a vitamin $B_{12}$ absorption test in patients with inherited malabsorption of vitamin $B_{12}$ attributable to Imerslund–Grasbeck syndrome (IGS) or lack of intrinsic factor (IF), their obligate heterozygous parents, and healthy controls.

**Materials and Methods**

The participants, all Turkish, were divided into 3 groups: patients [$n = 17$ (8 females and 9 males); median (range) age, 17 (8–32) years; 13 with IGS and 4 with hereditary IF deficiency]; their biological parents [$n = 19$; 9 females and 10 males; median age, 40 (30–57) years], and volunteers assumed to be healthy [$n = 44$; 22 females and 22 males; median age, 25 (9–58) years]. Patient characteristics are shown in Table 1.

All 17 patients have been described previously (4–8) and had been given vitamin $B_{12}$ from initial diagnosis. The treatment schedule was 1000 μg of oral vitamin $B_{12}$ given at 2-week intervals for the last 5 years (5). The Schilling tests indicating no ability to absorb vitamin $B_{12}$
had been performed in 13 of the patients around the time of diagnosis (Table 1).

IGS and hereditary IF deficiency are characterized by recessive inheritance. Thus, the biological parents (father and mother) of these patients should be heterozygous, but genetic analyses were not available for any of the parents. One of the parents of 3 patients and both parents of 8 patients participated in the study.

The control individuals were recruited from the outpatient clinic of the Institute of Child Health at the Department of Pediatrics, Hacettepe University, and from medical staff working in the same department. Inclusion criteria for the study included no known disorders related to vitamin B12 deficiency; no chronic systemic disease; no medical treatment, including vitamin tablets, within the past week; and written informed consent (for adult participants).

Written informed consent was obtained from all participants and the parents of patients who were under the age of 18. The Research Ethics Committee of Hacettepe University Hospital approved the study protocol. The study was carried out from November 2003 to October 2004.

The nonlabeled oral vitamin B12 absorption test is based on measurement of serum holo-TC before and after oral intake of 3 oral 9-μg doses of vitamin B12 given at 6-h intervals (3). Blood samples were taken at 0800 on the day before the start of the study (day 0) and on day 1. After the blood sample was taken on day 0, oral 9-μg doses of vitamin B12 (Natur Drogeriet A/S) were administered with a glass of water 3 times (0800, 1400, and 2000); time points were allowed to deviate ±45 min. The participants were allowed to have a light breakfast, not including meat or any diary products, 30–60 min before blood sampling but were otherwise allowed to eat their typical diet. For those patients taking vitamin B12 as a treatment for disease, the test was performed 2 weeks after the last oral intake of vitamin B12.

The blood samples were centrifuged within 1 h and stored at −20 °C until further processing.

Serum holo-TC was measured by ELISA (9), and vitamin B12 was measured on the Advia Centaur Analyzer (Bayer Diagnostics) by competitive immunoassay using direct chemiluminescent technology, in which vitamin B12 from the patient sample competes with labeled vitamin B12 for a limited amount of purified intrinsic factor.

Spearman correlation coefficients were used to describe the correlation between continuous variables. P values <5% were regarded as statistically significant. ROC curves and areas [with 95% confidence intervals (CIs)] were used to estimate diagnostic accuracy for an

| Table 1. Genetic analyses, Schilling test results, baseline concentrations of holo-TC and vitamin B12, and holo-TC and vitamin B12 increments after vitamin B12 load in patients with inherited vitamin B12 malabsorption.  

<table>
<thead>
<tr>
<th>Patients</th>
<th>Sex</th>
<th>Age, years</th>
<th>Siblings</th>
<th>Mutated gene</th>
<th>Schilling test I (58Co),%</th>
<th>Holo-TC, pmol/L</th>
<th>Cobalamin, pmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>32</td>
<td>A</td>
<td>AMN</td>
<td>0.2</td>
<td>221</td>
<td>−8</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>27</td>
<td>A</td>
<td>AMN</td>
<td>1.4</td>
<td>164</td>
<td>−20</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>9</td>
<td>B</td>
<td>GIF</td>
<td>72</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>8</td>
<td>B</td>
<td>GIF</td>
<td>5</td>
<td>135</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>13</td>
<td></td>
<td>CUBN</td>
<td>15</td>
<td>135</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>10</td>
<td></td>
<td>GIF</td>
<td>29</td>
<td>122</td>
<td>22</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>18</td>
<td></td>
<td>AMN</td>
<td>30</td>
<td>384</td>
<td>−18</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>15</td>
<td>C</td>
<td>NA</td>
<td>18</td>
<td>184</td>
<td>−32</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>17</td>
<td>C</td>
<td>NA</td>
<td>17</td>
<td>135</td>
<td>−17</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>17</td>
<td></td>
<td>CUBN</td>
<td>16</td>
<td>167</td>
<td>−13</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>15</td>
<td>D</td>
<td>NA</td>
<td>7</td>
<td>96</td>
<td>14</td>
</tr>
<tr>
<td>12</td>
<td>M</td>
<td>9</td>
<td>D</td>
<td>NA</td>
<td>13</td>
<td>113</td>
<td>5</td>
</tr>
<tr>
<td>13</td>
<td>F</td>
<td>18</td>
<td>E</td>
<td>AMN</td>
<td>22</td>
<td>187</td>
<td>−12</td>
</tr>
<tr>
<td>14</td>
<td>F</td>
<td>17</td>
<td>E</td>
<td>AMN</td>
<td>15</td>
<td>201</td>
<td>1</td>
</tr>
<tr>
<td>15</td>
<td>F</td>
<td>15</td>
<td></td>
<td>NA</td>
<td>6</td>
<td>149</td>
<td>15</td>
</tr>
<tr>
<td>16</td>
<td>M</td>
<td>17</td>
<td></td>
<td>AMN</td>
<td>12</td>
<td>155</td>
<td>−3</td>
</tr>
<tr>
<td>17</td>
<td>M</td>
<td>23</td>
<td></td>
<td>GIF</td>
<td>6</td>
<td>122</td>
<td>−28</td>
</tr>
</tbody>
</table>

a A genetic diagnosis was available for 12 patients, as described in Tanner et al. (7) for patients 1, 2, 5, 7, 10, 13, and 14 and in Tanner et al. (8) for patients 3, 4, and 6. The genetic diagnoses for patients 16 and 17 have been determined but not yet published. Five patients (?) are still under evaluation, but they are believed to have IGS.
b Patients who are siblings are indicated by the same letter.
c Reference interval for Schilling test (58Co): 11%–28%.
d Reference interval >50 pmol/L.
e Reference interval 200–600 pmol/L.
f AMN, amnionless; GIF, gastric IF; NA, not available; CUBN, cubillin.
g Genetic analysis has not been completed.
increase in holo-TC and cobalamin as a diagnostic marker for diagnosing vitamin B$_{12}$ malabsorption. Data were analyzed with Prism 4 (GraphPad) software.

**Results**

We evaluated an oral nonradioactive vitamin B$_{12}$ absorption test in 3 groups of individuals. One group consisted of 17 patients unable to actively absorb the vitamin because of inherited lack of IF (n = 4) or defects in the IF receptors cubillin or amnionless (n = 13; Table 1). The second group was 19 parents of patients, all expected to be heterozygous for nonactive absorption of vitamin B$_{12}$. The last group consisted of 44 healthy controls.

The patient group showed no significant change in either holo-TC or cobalamin as measured in blood samples collected after the intake of the test dose of vitamin B$_{12}$ (Fig. 1). The median (range) changes were 1 (−42 to 5) pmol/L for holo-TC and −3 (−32 to 22) pmol/L for cobalamin. There was no difference between findings in the patients with IGS and those with hereditary IF deficiency.

The control group showed a highly significant increase in holo-TC and cobalamin (P < 0.001 for both) 1 day after an oral dose of vitamin B$_{12}$. There was no relationship between sex and the observed increases in cobalamin and holo-TC, whereas we found a significant negative relationship between age and the increase in holo-TC (Spearman $r = 0.51$; $P = 0.0004$). We also found a significant negative relationship between the starting concentrations of holo-TC (Spearman $r = 0.40$; $P = 0.007$) and cobalamin (Spearman $r = -0.34$; $P = 0.02$) and the increments in these analytes after the oral dose of vitamin B$_{12}$.

In the parents, concentrations of both holo-TC and cobalamin increased significantly (P < 0.001 and P < 0.01, respectively; Fig. 1) after the test dose of vitamin B$_{12}$. The absolute increases in holo-TC and cobalamin concentrations did not differ significantly between the parents [median (range) increase, 23 (−2 to 47) and 27 (−15 to 94) pmol/L, respectively] and the controls [26 (−6 to 63) and 41 (−37 to 109) pmol/L, respectively].

We used ROC curves to compare the diagnostic accuracy of holo-TC and cobalamin as an oral vitamin B$_{12}$ absorption test (Fig. 2). For this purpose, we used changes in holo-TC and cobalamin concentrations after the absorption test observed in patients (n = 17) and in controls (n = 44). The areas under the ROC curves were 0.97 (95% CI, 0.93–1.0) for holo-TC and 0.87 (0.79–0.94) for cobalamin. A cutoff limit of 6 pmol/L for holo-TC gives diagnostic sensitivity of 100% (95% CI, 81%–100%) and diagnostic specificity of 92% (82%–97%).

**Discussion**

The results of this study indicate that measurement of holo-TC after an oral dose of vitamin B$_{12}$ can identify patients with hereditary disorders of absorption of vita-
min B₁₂. The patient group was not able to actively absorb vitamin B₁₂; therefore, any absorption of the vitamin during the proposed test would suggest the occurrence of passive absorption. Passive absorption did not take place with the vitamin B₁₂ dose used in our study: the patient group showed no significant change in either holo-TC or cobalamin measured in blood samples after the vitamin B₁₂ load. This result is an important prerequisite for the use of the proposed test. Passive absorption of vitamin B₁₂ is believed to account for ~1% of the administered dose of the vitamin; we were therefore concerned whether the high physiologic dose used in our new test would lead to passive absorption, mimicking active uptake. Our results strongly support the conclusion that significant passive absorption does not occur when a challenge involving three 9-μg doses of vitamin B₁₂ is used, at least not in patients with the 2 hereditary disorders of absorption of vitamin B₁₂ studied here.

In both the parents and the healthy controls, holo-TC and cobalamin concentrations increased highly significantly after the vitamin B₁₂ load. These results strongly suggest that those heterozygous for nonactive vitamin B₁₂ absorption retain a normal absorptive capacity. In accord with this observation, an increased occurrence of vitamin B₁₂ deficiency in persons heterozygous for nonactive absorption of vitamin B₁₂ has not been reported.

A higher basal concentration of holo-TC was followed by a smaller increment in holo-TC after ingestion of vitamin B₁₂. Regulation of vitamin B₁₂ uptake according to need was observed many years ago in mice, in which increased absorption of vitamin B₁₂ was reported during pregnancy (10).

We also showed in controls 9 to 58 years of age an inverse relationship between age and holo-TC increase, suggesting that the cobalamin absorptive capacity decreases from childhood through adult life. A decrease in absorptive capacity with age has been suggested previously by studies in adults (11).

Although holo-TC seems to be a better marker than total cobalamin for studying the uptake of vitamin B₁₂, we found that the increase in cobalamin reflects the absorption better than reported previously (6, 12, 13). The difference in previous designs and the design used in this study relates to the timing of the doses of vitamin B₁₂. We took advantage of the fact that a new dose of vitamin B₁₂ may be absorbed after a few hours, and as a consequence, we administered three 9-μg doses of vitamin B₁₂ at 6-h intervals. In contrast, most previous studies used a single dose of vitamin B₁₂ ranging from 100 to 1000 μg (6, 12, 13).

Vitamin B₁₂ absorption has been studied by various methods, including the urinary excretion of orally administered radioactively labeled vitamin B₁₂ alone (Shilling test I) or in combination with IF (Shilling test II) (1). Performance of these tests has been increasingly difficult because of the limited availability of radioactively labeled vitamin B₁₂ and decreasing acceptance of a radioactively labeled vitamin in a diagnostic test (2). Moreover, the IF of human origin used in these tests has been removed from the market in most countries. Our new test may represent a suitable alternative to the Shilling test and have the advantage of requiring neither labeled vitamin B₁₂ nor the collection of a 24-h urine sample. In the present study, we tested only the absorption of free vitamin B₁₂. Recombinant human IF is now available commercially (14, 15), and as soon as it becomes available for human use, our design can be used to test whether IF can correct negative absorption of free vitamin B₁₂. In our patient group, this would help distinguish those with vitamin B₁₂ malabsorption attributable to a defective receptor from those with inherited lack of IF. Only in the latter group would one expect to be able to correct vitamin B₁₂ absorption by addition of IF.

In conclusion, measurement of holo-TC before and after oral intake of vitamin B₁₂ is a promising approach to evaluate vitamin B₁₂ absorption and may constitute a substitute for the Shilling test I.

We warmly acknowledge the excellent technical assistance of Anna-Lisa Christensen and Jette Fisker Pedersen. This work was supported in part by a European Union demonstration project on the diagnostic utility of holo-TC (QLK3-CT-2002-01775). Ebba Nexo is a cofounder and a member of the board of Cobento Biotech A/S. This company produces recombinant human IF in plants. The company has patents pending for the use of this protein.
that also cover its use in the vitamin B12 absorption test described in this report, which is named as CobaSorb.

References


