Anaemia and Haemoglobinuria

Holotranscobalamin – An Early Marker for Laboratory Diagnosis of Vitamin B₁₂ Deficiency

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Abstract

Vitamin B₁₂ deficiency is widespread. Among the population groups at risk are older people, vegetarians, pregnant women and patients with renal or intestinal diseases. The neurological symptoms of vitamin B₁₂ deficiency are unspecific and can be irreversible. Early detection is therefore important. This article reviews the diagnostic performance of the different laboratory markers for vitamin B₁₂ status. Total serum vitamin B₁₂ is a relatively insensitive and unspecific biomarker of deficiency that does not reflect recent variations in cobalamin status. Holotranscobalamin (holoTC), the metabolically active portion of vitamin B₁₂, is the earliest laboratory parameter that becomes decreased in case of a vitamin B₁₂ negative balance. Concentration of methylmalonic acid (MMA) is a functional vitamin B₁₂ marker that will increase when the vitamin B₁₂ stores are depleted. Isolated lowering of holoTC shows vitamin B₁₂ depletion (negative balance), while lowered holoTC plus elevated MMA (and homocysteine) indicates a metabolically manifested vitamin B₁₂ deficiency, although there still may be no clinical symptoms. The diagnostic use of holoTC allows the initiation of therapeutic measures before irreversible neurological damage develops. Because the clinical manifestations of vitamin B₁₂ deficiency are unspecific, people at risk should be identified and should regularly test their holoTC with or without MMA.

Keywords

Cobalamin, holotranscobalamin, diagnosis, deficiency

Pernicious anaemia, which causes severe vitamin B₁₂ (cobalamin) deficiency, used to be a fatal, non-curable disease. However, in 1926 George Minot and William Murphy discovered that pernicious anaemia can be treated by including large amounts of liver in patients’ food. Vitamin B₁₂ is synthesised exclusively in microorganisms. Animals obtain vitamin B₁₂ from foods contaminated with vitamin B₁₂-synthesising bacteria, and thus foods of animal origin represent the only source of vitamin B₁₂ in the human diet.

Together with 5-methyltetrahydrofolate, vitamin B₁₂ is an essential co-factor in methyl group transfer, cell division and catabolism of homocysteine (Hcy). In addition, vitamin B₁₂ is required by all cells for one-carbon metabolism and DNA synthesis and maintenance. Only two vitamin B₁₂-dependent enzymes are known in humans: methionine synthase and L-methylmalonyl-CoA mutase. Methionine synthase mediates the formation of methionine from Hcy, and requires methylcobalamin as a co-factor. L-methylmalonyl-CoA mutase requires adenosylcobalamin, and catalyses the formation of succinyl-CoA from methylmalonyl-CoA. The latter compound can be converted into methylmalonic acid (MMA). Serum concentrations of MMA and Hcy are therefore considered metabolic indicators of vitamin B₁₂ status.

Vitamin B₁₂ is conserved in humans through the enterohepatic circulation and via active re-absorption in the proximal tubule. The estimated stores of vitamin B₁₂ in the human liver are relatively high. Therefore, in the case of intact absorption, the depletion of the vitamin takes years to be expressed as a metabolic or clinical dysfunction. Acquired vitamin B₁₂ deficiency can be related to prolonged insufficient intake, disturbed absorption, increased requirements or an accelerated loss of the vitamin.

Subtle Vitamin B₁₂ Deficiency and Recommended Daily Amount

Subtle vitamin B₁₂ deficiency is common in the general population and it is more widespread than has been assumed so far. Chronic insufficient intake or disrupted absorption of vitamin B₁₂ are the most common causes of cobalamin deficiency.

According to the recommended daily amount (RDA) guidelines from the National Research Council of the US National Academy of Sciences, adults should ingest 2.4µg daily (pregnant women up to 6µg), which can be met by a typical western diet. The calculation of the required amount is based on the calculation of the amount of vitamin B₁₂ necessary to sustain a normal haematological status (normal haemoglobin and mean corpuscular volume of erythrocytes [MCV]) and to maintain remission in pernicious anaemia. At the time when the RDA was set, functional metabolic markers of vitamin B₁₂ had not been sufficiently investigated in clinical studies. Neurological manifestations are found at a relatively late stage of...
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Recent investigations have suggested that this daily intake is too low to ensure normal blood cobalamin markers. Recent data have shown that the plasma concentration of MMA and Hcy falls when vitamin B12 is ingested, whereas the concentration of holotranscobalamin (holoTC) as metabolically active B12 fraction rises. At present, a daily intake between 4 and 6µg vitamin B12 is thought to be necessary to maintain the optimal plasma concentration of cobalamin biomarkers. It is concluded that the recommended daily intake of B12 seems too low and should be newly determined, especially for older people.

Early Diagnosis of Vitamin B12 Deficiency

Since vitamin B12 deficiency can lead to irreversible neurological damage, early diagnosis is essential. Early diagnosis of this disease is crucial for prevention of further complications. Moreover, vitamin B12 deficiency can cause hyperhomocysteinaemia, which has been related to the risk of vascular and cerebral diseases. Studies have confirmed that serum concentrations of total vitamin B12 are insensitive in detecting early depletion of the vitamin. Furthermore, people who express a benign low serum concentration of vitamin B12 related to low haptocorrin but who are not deficient are far from rare. This implies that a low concentration of vitamin B12 in serum does not necessarily indicate a deficiency and indicates the need for a more specific and early laboratory marker. In addition, low serum concentrations of vitamin B12 are uncommon in patients with renal insufficiency. However, this disagrees with the finding that renal patients show a marked MMA elevation that can be corrected by vitamin B12 supplementation, indicating a pre-treatment deficiency.

In recent years, new and sensitive laboratory markers to determine vitamin B12 status have become available. Cobalamin-saturated transcobalamin, also called holoTC, constitutes between 6 and 20% of total plasma vitamin B12. This portion is the only one that can be delivered into all DNA-synthesising cells. Holohtocorrin constitutes approximately 80% of total serum cobalamin and has no known function. Serum concentration of holoTC has been suggested as a sensitive marker for early changes (depletion or repletion) of vitamin B12.

A study by our group shows a clear difference in serum holoTC concentrations according to diet. The holoTC values found in vegans were notably shifted towards the lower end of the distribution. On the other hand, the distribution of holoTC concentrations in lacto vegetarians/lacto-ovo vegetarians showed an intermediate pattern between that found in the vegans and the omnivores (see Figure 1). A low concentration of holoTC (<35pmol/l) was detected in 8% of the omnivores, 61% of the lacto/lacto-ovo vegetarians and 76% of the vegans. A combined two abnormal results (holoTC and MMA) were detected in 43 and 64% of the lacto/lacto-ovo vegetarians and vegan subjects, respectively. More importantly, about 45% of subjects with low holoTC and elevated MMA had normal serum vitamin B12, again indicating the insensitivity of serum vitamin B12 (see Figure 2).

Moreover, the distribution of holoTC in elderly subjects (see Figure 1) was only slightly shifted towards lower concentrations compared with healthy younger people. However, this did not explain higher serum concentrations of MMA compared with younger adults. Approximately 20% of elderly subjects showed an elevated concentration of MMA but normal holoTC. This group had a significantly higher median serum concentration of creatinine compared with the group with normal MMA.

Vitamin B12 deficiency and are not unique. In addition, haematological signs are expressed by only a subpopulation. Therefore, the criteria used the define the RDA are too insensitive to detect cobalamin-deficient cases.
and normal holoTC. A combination of a low concentration of holoTC and an elevated MMA was found in approximately 16% of the elderly subjects; this represents subjects with a metabolic sign suggesting cobalamin deficiency.

Taken together, available functional biomarkers such as MMA and holoTC facilitate the laboratory diagnosis of cobalamin deficiency. However, as before, there is no single "gold standard" marker that can be applied for all clinical conditions.

Risk Groups
The prevalence of subclinical functional vitamin B₁₂ deficiency is higher than expected when sensitive and relatively specific markers are used such as MMA, holoTC and Hcy. Risk groups for vitamin B₁₂ deficiency (see Table 1) include: patients with unexplained anaemia; patients with unexplained neuropsychiatric symptoms; patients with gastrointestinal manifestations, including stomatitis, anorexia and diarrhoea; elderly people; vegetarians; patients with gastrointestinal disorders, such as Crohn’s disease or H. pylori infection; and patients with stomach resection. To date, the rate of people in the at-risk population who will develop clinical symptoms because of vitamin B₁₂ deficiency has not been studied systematically.

In the general population, the prevalence of vitamin B₁₂ deficiency in younger people is 5–7%. Functional vitamin B₁₂ deficiency – i.e. raised MMA and lowered holoTC – is common in old age and has been diagnosed in 10–30% of patients over 65 years of age. A high prevalence of a slightly abnormal vitamin B₁₂ status has been reported in elderly people despite intake of the recommended daily dose (>2.4µg/day). This deficiency is not presumed to be associated with dietary causes but rather with malabsorption. Fifty-three percent of elderly patients from Strasbourg who had vitamin B₁₂ deficiency had malabsorption problems and 33% had pernicious anaemia; in only 2% was vitamin B₁₂ deficiency related to insufficient dietary intake, and in 11% the aetiology of the vitamin B₁₂ deficiency remained unexplained. However, because the currently recommended dietary intake for vitamin B₁₂ in elderly people is low, dietary deficiencies are underdiagnosed. Using synthetic vitamin B₁₂ preparations can protect elderly persons from symptoms of deficiency. However, dietary intake of vitamin B₁₂ does not provide any information on vitamin B₁₂ status because malabsorption is a common and important factor. Furthermore, elderly persons often have atrophic gastritis, pernicious anaemia or achlorhydria. Disorders that affect the gastrointestinal pH can also result in malabsorption and thus vitamin B₁₂ deficiency. The incidence of H. pylori is high in elderly people and can lead to atrophic gastritis, and in turn to B₁₂ malabsorption, owing to disrupted production of hydrochloric acid. H. pylori was found in 56% of patients with vitamin B₁₂ deficiency. In 40% of patients, serum concentrations of B₁₂ rose after treatment for H. pylori infection. According to recent reports, longer-term treatment of H. pylori (one year) resulted in a significant rise in mean vitamin B₁₂ (from 146 to 271pmol/l) and a fall in mean Hcy concentrations (from 41 to 13µmol/l). Vitamin B₁₂ malabsorption owing to H. pylori infection can thus lead to vitamin B₁₂ deficiency and hyperhomocysteinemia.

Vegetarians are at high risk of developing vitamin B₁₂ deficiency because animal products are the main sources of vitamin B₁₂. A functional B₁₂ deficiency (lowered holoTC, raised MMA and Hcy) is common in vegetarians and depends on the strictness of the diet and the amount of time for which the vegetarian diet has been followed. Persons with an increased vitamin requirement – such as pregnant and breastfeeding women, patients with autoimmune disorders or persons with HIV infection – are a further risk group for vitamin B₁₂ deficiency. Persons who regularly take proton pump inhibitors can also develop vitamin B₁₂ deficiency.

Vitamin B₁₂ deficiency is also widespread in patients with renal disorders. In spite of normal plasma concentrations of vitamin B₁₂ or holoTC, these patients often have raised serum concentrations of MMA and Hcy. The likely cause is disrupted cellular absorption of holoTC, which results in intracellular vitamin B₁₂ deficiency and raised metabolites. Studies have shown that patients with renal disorders may have higher concentrations of holoTC, which seems to contradict vitamin B₁₂ deficiency. This can be explained by the role of the kidney in transcobalamin filtration and resultant secondary accumulation of holoTC. The plasma concentration of holoTC in such patients therefore does not correctly reflect the functional vitamin B₁₂ status.

Utility of Holotranscobalamin as an Early Diagnostic Marker
Recent studies have emphasised the need for testing the clinical utility of holoTC compared with vitamin B₁₂ and MMA. The pros and cons of the different laboratory tests of cobalamin status have

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Table 1: Population Groups at Risk of Vitamin B₁₂ Deficiency

<table>
<thead>
<tr>
<th>Group</th>
<th>Causes/Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vegetarians, vegans and macrobiotic diet</td>
<td>Low intake of vitamin B₁₂</td>
</tr>
<tr>
<td>Neonates from vegetarians or vitamin B₁₂-deficient mothers</td>
<td>Low vitamin B₁₂ available during foetal life and later low intake via human milk from deficient mothers</td>
</tr>
<tr>
<td>Elderly</td>
<td>Pernicious anaemia, achlorhydia, gastrointestinal diseases or drugs that alter pH, ileum resection, Helicobacter pylori</td>
</tr>
<tr>
<td>Neurodegenerative and psychiatric diseases</td>
<td>Either causally related to the clinical symptoms or secondary to the disease because of low intake</td>
</tr>
<tr>
<td>Chronic atrophic gastritis</td>
<td>Malabsorption of vitamin B₁₂</td>
</tr>
<tr>
<td>Diseases of the terminal ileum</td>
<td>Crohn’s disease, ileum resection, bacterial overgrowth</td>
</tr>
<tr>
<td>Macrocytic anaemia</td>
<td>Low B₁₂ intake or pernicious anemia (antibodies against intrinsic factor)</td>
</tr>
<tr>
<td>Chronic alcoholism</td>
<td>Low intake, disturbed absorption of the vitamin</td>
</tr>
<tr>
<td>Medications</td>
<td>Proton pump inhibitors, H₂-receptor antagonists, nitric oxide exposure</td>
</tr>
<tr>
<td>AID5-associated myelopathy</td>
<td>Abnormal vitamin B₁₂ utilisation</td>
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The development of automated methods for evaluating holoTC has made large-scale screening studies possible. With regard to the cost–benefit effect of early detection of vitamin B12 deficiency by using holoTC, it is anticipated that holoTC will soon replace vitamin B12 as a first-line laboratory parameter to screen for vitamin B12 deficiency.

Studies on vegetarians with different dietary habits have suggested that a lowered serum holoTC concentration is the earliest marker of vitamin B12 deficiency, and indicates that the body does not have sufficient available vitamin B12 and that vitamin B12 stores are emptying as a result of the negative balance of the vitamin. At this stage, clinical or haematological symptoms may not yet be present. Lowered holoTC combined with raised MMA and Hcy levels is indicative of metabolically manifest vitamin B12 deficiency. Clinical signs may already be present, but can still be missing. At this stage, people may be clinically inconspicuous.

The use of total vitamin B12 assay as a first-line parameter to screen for cobalamin deficiency has been disappointing, despite the relatively low costs of the assay. The limitations of the vitamin B12 assay are especially important in the lower range of serum vitamin B12. A significant positive correlation was found between the two parameters in the group with serum vitamin B12 concentrations >300pmol/l (r=0.46; p<0.001) (see Figure 3). However, the correlation line in the group with low serum vitamin B12 concentrations (<300pmol/l) was quite different from that seen in those with higher vitamin B12 concentrations; the slope of that curve was significantly steeper, indicating that the part of holoTC that contributes to total vitamin B12 is significantly decreased in the lower vitamin B12 concentration range to below 10%. Therefore, in the lower vitamin B12 concentration range (<300pmol/l), total vitamin B12 overestimates the cobalamin status.

In a recent study on over 1,000 samples from patients referred for testing total serum vitamin B12, we studied the distribution of serum concentrations of total vitamin B12 in subjects defined as cobalamin-deficient (holoTC ≤35pmol/l and MMA ≥300nmol/l) and those defined as non-deficient (holoTC >35pmol/l and MMA <300nmol/l) (see Figure 4). Only patients with normal renal function were included. The majority of patients with high MMA and low holoTC (cobalamin-deficient) had vitamin B12 concentrations between 156 and 400pmol/l and would be classified (utilising total vitamin B12 as a diagnostic tool) as false-normal. Few subjects had low concentrations of vitamin B12 and normal MMA and holoTC (false-positive).

The receiver operating characteristic (ROC) curves testing the performance of holoTC and vitamin B12 for detecting
concentrations of MMA above 300nmol/l in individuals with normal renal function showed a larger area under the curve (AUC) for holoTC compared with vitamin B12 (0.71 versus 0.60). This supports a better diagnostic specificity and sensitivity for holoTC compared with vitamin B12. A 72% sensitivity could be expected by using a cut-off of 35pmol/l for holoTC and 243pmol/l for vitamin B12.

Renal insufficiency constitutes a common and important exceptional condition for the interpretation of cobalamin markers. The artificial increase of serum concentrations of MMA and tHcy in some clinical settings is a major limitation of these parameters. Both parameters correlate to serum concentration of creatinine and increase even in mild degrees of renal insufficiency. In general, results of the metabolites should be interpreted with caution because it is difficult to determine the extent to which the impaired kidney function may participate in MMA and Hcy elevation. Cobalamin deficiency is common in patients with renal dysfunction. Moreover, concentrations of holoTC in people with renal insufficiency are markedly elevated and they are not consistent with what we have learned about vitamin B12 deficiency. This one important exception is relatively common and may raise some uncertainty about using holoTC as a marker of cobalamin status in renal patients and in elderly people even with subclinical degrees of renal insufficiency. A laboratory diagnosis of cobalamin deficiency in renal patients is an important challenge that remains unresolved because of the simultaneous increase of both serum MMA and holoTC. Our observations of patients with renal dysfunction or elderly people with mild renal complications suggest that cobalamin deficiency in these patients can be ruled out only after cobalamin treatment. Whenever cobalamin deficiency is suspected in renal patients, cobalamin treatment should be started. A significant reduction of serum MMA (by approximately 250nmol/l) after cobalamin treatment indicates a pre-treatment deficiency. A residual increment of MMA is then related to renal dysfunction. We have suggested an algorithm for laboratory diagnosis of vitamin B12 deficiency (see Figure 6). This algorithm takes into account renal dysfunction as a common cause for elevated both holoTC and MMA.

**Screening**

At present there is no consensus regarding screening for vitamin B12 deficiency. Screening makes sense when the first signs of vitamin B12 deficiency can be detected before neurological or haematological anomalies develop. HoloTC and MMA are suitable screening tools; however, renal dysfunction should be kept in mind.

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