Novel and Established Markers of Cobalamin Deficiency: Complementary or Exclusive Diagnostic Strategies

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ABSTRACT

New developments in diagnostic markers and a better understanding of the limitations of traditional diagnostic strategies have allowed diagnosis of earlier stages and atypical forms of cobalamin deficiency. Still, there are no generally accepted guidelines for the definition, diagnosis, treatment, and follow-up of cobalamin deficiency. The new trend toward defining cobalamin deficiency purely on the basis of biochemical test outcomes in the absence of overt clinical signs and symptoms could, however, be problematic and may result in overdiagnosis and overtreatment. Use of metabolic markers for the assessment of cobalamin deficiency allows the demonstration of tissue deficiency, but the establishment of the cause of deficiency should also be part of the diagnostic approach. Four groups of diagnostic tests are currently available and these include total cobalamin and cobalamin fractions (such as holo-transcobalamin), tests of gastrointestinal dysfunction, tests of metabolic function, and different gene tests. Among the available tests, only homocysteine, methylmalonic acid, holo-transcobalamin, and possibly methylcitric acid are considered to be useful in clinical practice to add to cobalamin. Gastrointestinal function tests may identify the cause of cobalamin deficiency, whereas the diagnostic usefulness of genetic testing needs to be evaluated. This article provides an overview of recent developments and a reappraisal of novel and established diagnostic markers for cobalamin deficiency.

KEYWORDS: Diagnostic markers, cobalamin deficiency, holoTC, homocysteine, methylmalonic acid

Educational Objectives: Upon completion of this article, the reader should be able to (1) understand the metabolic function of cobalamin; (2) recognize clinical symptoms and findings in cobalamin deficiency; (3) identify causes of cobalamin deficiency; (4) recognize diagnostic approach and strategies; and (5) appreciate intercomparison of diagnostic markers of cobalamin deficiency.

Vitamin B_{12} or cobalamin (Cbl) deficiency is considered a frequent disorder, especially among elderly patients, but is often unrecognized because the initial clinical manifestations are subtle and nonspecific or are attributed to the normal aging process.[1] Because of the potential seriousness and irreversibility of the symptoms (particularly neurological), detection and treatment of early stages of cobalamin deficiency is important.[2]

Since the development of microbiological assays for detection of total cobalamin in blood in the early

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1940s, several new diagnostic markers have become available.[3] The development of more specific and sensitive diagnostic tests has made it possible to diagnose cobalamin deficiency at an earlier stage—sometimes even before the occurrence of clinical symptoms—and to identify atypical forms of deficiency.[4]

However, with the advent of new, more sensitive diagnostic tests, it became a widespread practice to define cobalamin deficiency purely on the basis of biochemical test outcomes, without clinical signs and symptoms or clinical response to treatment.[3] It has been questioned whether this practice is justified, and there certainly is a balance between the risks of underrecognition and those of potential overdiagnosis and overtreatment of the disease.[3]

The aim of this article is to provide an overview of the novel and established diagnostic tests for cobalamin deficiency and to compare and summarize the test characteristics and diagnostic utilities of these markers.

METABOLISM AND FUNCTION

In human metabolism, cobalamin serves as a cofactor in only two enzyme reactions, in methionine synthase, responsible for the methylation of homocysteine into methionine, and in methylmalonyl-CoA mutase, transforming methylmalonyl-CoA into succinyl-CoA (Fig. 1). Deficiency of the cofactor results, first, in an accumulation of the substrates of these reactions and, second, in an increase of blood concentrations of homocysteine and methylmalonic acid.[5]

For efficient absorption and retention of vitamin B_{12}, several tissues, receptors, and transport systems are involved, which include the gastric mucosa, pancreas, distal ileum, liver and biliary system, and kidney (Fig. 1).

The transport of vitamin B_{12} in blood and cellular uptake involves several specific binding proteins and receptors, including transcobalamin II (TC II), the transcobalamin II-receptor (TC II-R), R-binders, intrinsic factor, and mucosal cells carrying cubilin and amnionless as subunits of a receptor complex that recognizes intrinsic factor-Cbl-complexes, and which interacts with TC II-R (Fig. 1).

CLINICAL SYMPTOMS

The clinical symptoms of "classical" cobalamin deficiency, that is, of severe megaloblastic anemia combined with neuropsychiatric symptoms, "megaloblastic madness," are rarely seen today.[6] Cobalamin deficiency is a slowly progressive process (Fig. 2) that can take many years to develop.[7] Nowadays, most cases are detected at an earlier stage, at which clinical manifestations are often subtle and highly variable, and neuropsychiatric symptoms may occur in the absence of hematological signs.[8]

Thus, in clinical practice, many patients may present with diffuse, nonspecific symptoms, and vitamin cobalamin deficiency is only one of many differential diagnoses. As a consequence, the diagnostic value of most of these symptoms and signs is low.[9]

In principle, to prevent irreversible neurological damage, prophylactic cobalamin treatment merely on the basis of early biochemical signs may be justifiable. This approach poses particular demands on the specificity and sensitivity of diagnostic tests.[10]

Typical clinical signs of cobalamin deficiency have been related to the biochemical function of the vitamin; that is, DNA synthesis, methylation reactions, and energy production.[5] Major symptoms are related to the hematological and nervous system but may also affect other tissues (Table 1).

At an early stage, impaired DNA synthesis was recognized as being responsible for the development of megaloblastic anemia,[11] whereas methylation defects are postulated to be the likely cause of myelin damage and disturbed neurotransmitter metabolism.[12] The pathogenesis of neurological damage and relevance to cognitive dysfunction is, however, still uncertain.[13,14]

DEFINING COBALAMIN DEFICIENCY

The definition of cobalamin deficiency is problematic, as there is neither an independent gold standard nor a reference method for unequivocal characterization of the disease.[15] Moreover, there is no agreement on how to treat (with regard to formulation, dosage, cobalamin preparation, intensity, and duration of treatment), nor on when and how to monitor effects of treatment.[16]

In principle, it is important to distinguish between the diagnosis of the disease and identifying the cause of cobalamin deficiency. The first issue raises the problem of definition in the absence of generally accepted criteria. Another dilemma emerges if the finding of a plausible (known) cause is required for definition and diagnosis of cobalamin deficiency. There may be other, unknown or undiagnosed causes of cobalamin deficiency. For example, normal gastroscopic findings or absence of biochemical signs of gastritis do not exclude cobalamin deficiency, as there may be additional causes of cobalamin malfunction (Fig. 1).[15,17]

The clinical definition of cobalamin deficiency is usually based on a combination of clinical signs and symptoms and biochemical parameters, which may vary considerably between the different medical specialties.[16] Moreover, the picture of cobalamin deficiency in clinical practice is generally not at all similar to what is described in textbooks, which further distracts from a uniform diagnostic approach.[18]

Gastroenterologists may seek endoscopic evidence of typical changes in the gastric or duodenal mucosa—or at least positive surrogate markers of gastric

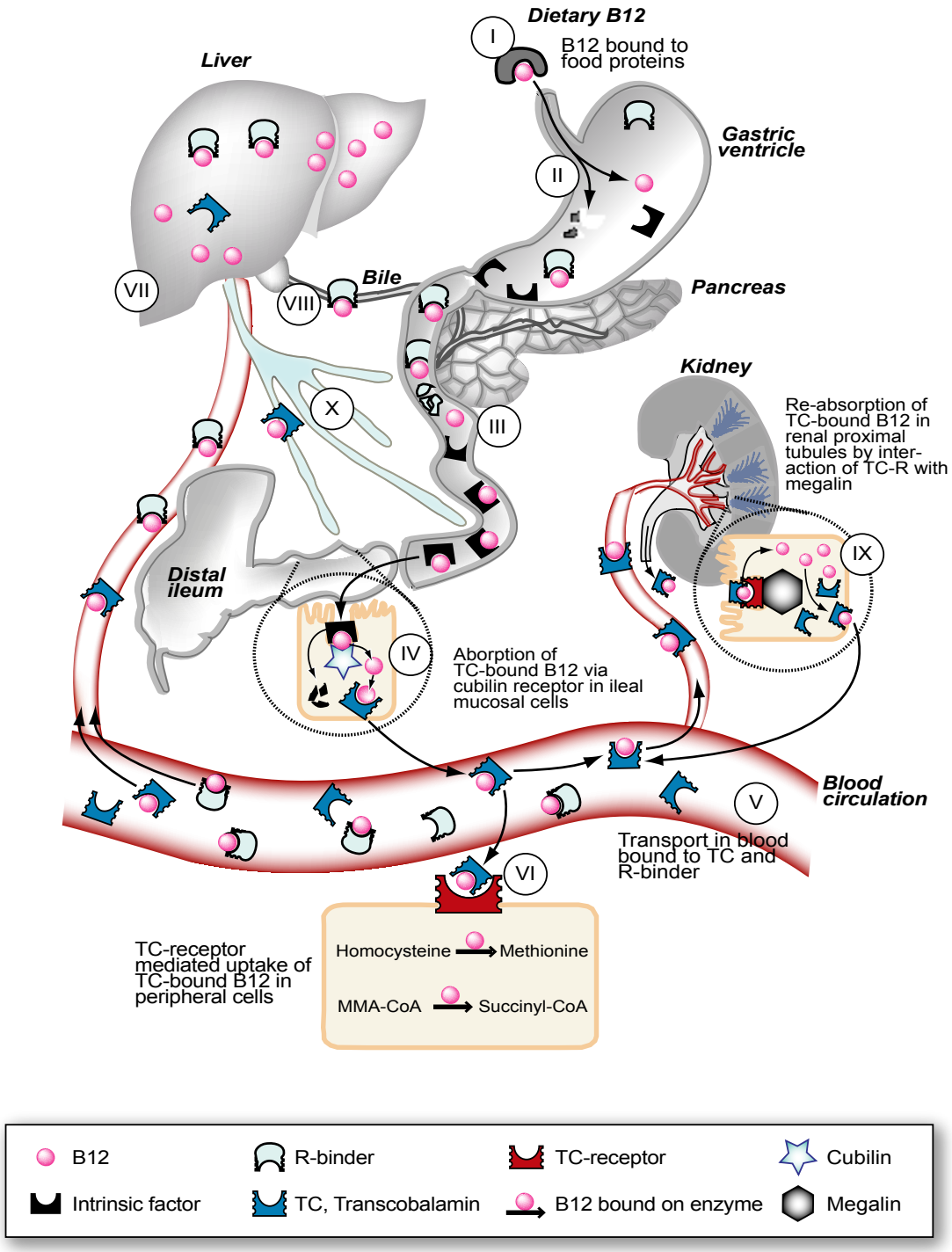

Figure 1. Cobalamin absorption and metabolism.Dietary cobalamin (vitamin B12) is normally protein-bound (**I**) and provided by food products of animal origin. Pepsin and low pH in the gastric ventricle degrade food proteins, resulting in release of cobalamin (**II**). Free cobalamin is then bound to R-binder, which is produced by the salivary glands and parietal cells. R-binders are degraded by pancreas proteases, and cobalamin (both newly ingested and cobalamin bound to R-binder in the bile) is released again and binds with high affinity to intrinsic factor produced in the stomach (**III**). Intrinsic factor has high affinity to cobalamin at neutral or alkaline pH of the pancreatic juice. In the mucosal cells of the distal 80 cm of the ileum, the cobalamin-intrinsic factor complex is recognized by cubilin receptors (in a functional complex with the amnionless molecule) (**IV**). Cobalamin enters the blood circulation bound to TCII. There, the majority of cobalamin (70-80%) is bound to R-binder and only a minor portion (20-30%) is bound to transcobalamin II (TC-II) (**V**). TC-II-bound cobalamin (holoTC) is the biologically active fraction of total cobalamin in serum, as only this fraction is taken up by the majority of cells in the body. Cellular up-take of holoTC is mediated by transcobalamin (TC)-receptors (**VI**). Cobalamin absorbed in the intestine subsequently enters the liver (**VII**) via the portal system (X). Within the cells, holoTC-molecules are degraded and cobalamin enzymatically converted into its two coenzyme forms, methylcobalamin (co-factor for the methionine-synthase enzyme) and adenosylcobalamin (co-factor for the methylmalonyl-CoA mutase in mitochondria) (**VI**). There is extensive enterohepatic circulation transporting 3-5 times more cobalamin than is newly absorbed from food. Cobalamin and cobalamin analogues are bound to R-binders in the bile (**VIII**). The kidneys seem to have a more important role for cobalamin homeostasis than earlier recognized. HoloTC is filtered in the glomeruli and quantitatively reabsorbed in the proximal tubuli in a process involving the TC-receptor and megalin (IX). Cobalamin leaves tubulus cells at the basal membrane bound to TC-II.

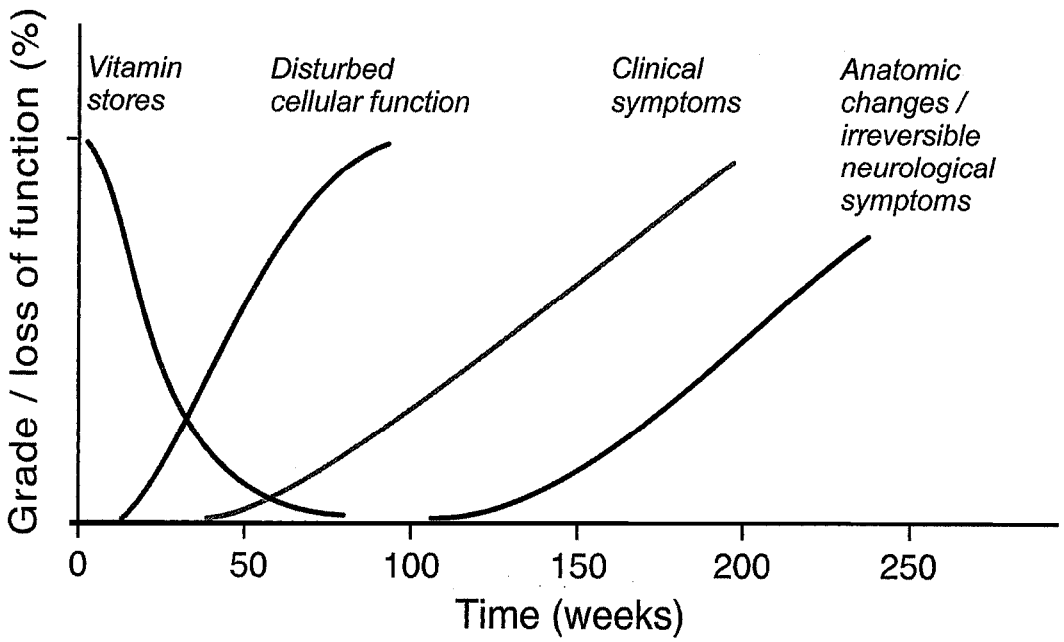

Figure 2 Development of cobalamin deficiency. Development of cobalamin deficiency is a slowly progressing continuum, which may take several years. At an early stage, cobalamin stores are reduced. The first signs of cobalamin deficiency are impaired cellular metabolism resulting in increased blood concentrations of the metabolites, homocysteine, and methylmalonic acid. Later, clinical symptoms may become overt. Some neurological symptoms already may become irreversible 1 year after the first clinical signs.

Table 1 Clinical Manifestations of Cobalamin Deficiency

Symptom Categories	Manifestations
Nervous system symptoms	Peripheral neuropathy
	Demyelinating disease of the spinal cord
Psychiatric disorders	Dementia
	Cognitive impairment/psychosis/confusion
	Depression
Hematological disorder	Macrocytic anemia
	Megaloblastic change in bone marrow
	Neutrophil hypersegmentation
Hearing impairment	Tinnitus
	Hearing loss
Eye symptoms	Amblyopia
	Reduced vision
Dermatological symptoms	Vitiligo
	Hyperpigmentation
Urogenital tract disorders	Infertility
	Cervical dysplasia
	Incontinence
Gastrointestinal disease	Hunter glossitis/abnormalities of taste
	Unexplained diarrhea
	Irritable bowel syndrome
	Inflammatory bowel syndrome
	Vomiting (infants)

pathology—before accepting the diagnosis of cobalamin deficiency.[17] Likewise, hematologists may seek the presence of typical hematological signs, including anemia.[19] Often, diagnostic strategies are closely related to the criteria used for definition of cobalamin deficiency. However, the common practice is unfortunate in that the same parameter that serves as a "gold standard" for definition of the disease is also applied as a diagnostic test in clinical routine. In these cases, circular reasoning may occur (Fig. 3).[10] Variable diagnostic criteria may also hamper objective intercomparison of different diagnostic strategies.[20]

Diagnosis of cobalamin deficiency is based on four lines of evidence; that is, measurement of vitamin concentrations in blood or other matrices, identifying the cause of vitamin B_{12} deficiency (in Western countries it is mainly intestinal malabsorption), application of functional tests to evaluate metabolic consequences, and finally, monitoring response to treatment. Therapeutic trials of vitamin B12 supplementation may also serve as a confirmatory test of cobalamin deficiency.

With the advent of functional tests for clinical routine testing in the early 1990s, a paradigm shift in the understanding of cobalamin deficiency occurred. It is now possible to diagnose early stages of deficiency even before the development of clinical symptoms. The definition or diagnosis of a disease purely based on abnormal biochemical parameters is, however, problematic[3] and invokes the danger of circular reasoning, especially if the diagnostic approach no longer includes clinical criteria (Fig. 3). Another challenge is how

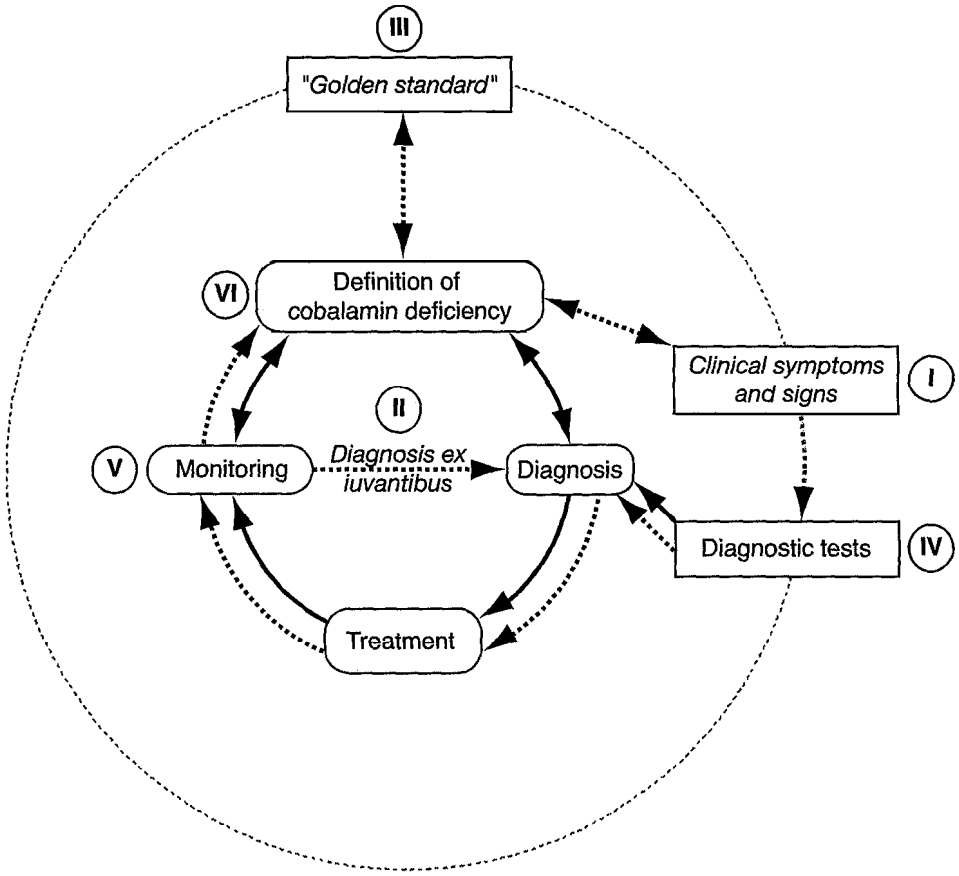

Figure 3 Circular reasoning. The ideal ~~diagnostic approach to a~~ clinical disorder, like cobalamin deficiency, is indicated by dashed lines: Observation of clinical signs and symptoms **(I)** is usually the primary indication of diagnostic testing. If tests are positive, a (tentative) diagnosis is made. Patients are treated for the disorder, and treatment effects are monitored. A marked response to treatment may confirm the tentative diagnosis (*diagnosis ex iuvantibus*) **(II)**. The observation of treatment effects is also important because it confirms that the expected clinical findings are in accordance with the definition of the disease. Sometimes, it may be necessary to check whether the clinical definition and diagnostic approach still are appropriate by application of an external, independent gold standard method, if such exists **(III) (outer circle)**. The circular reasoning **(solid lines)** occurs when diagnosis is made solely on the basis of pathological diagnostic tests **(IV)** in patients without clinical symptoms and signs. Furthermore, if the same tests are used for evaluation of treatment effects **(V)**, the diagnostic circle **(inner circle)**, including definition **(VI)** of the disorder, may become completely detached from the clinical picture **(outer circle)**. This situation can occur when new, highly sensitive markers are used and an independent external gold standard for validation of the diagnostic procedures is lacking, as in cobalamin deficiency.

to respond to and follow up after abnormal test results of new, sensitive markers of cobalamin deficiency in asymptomatic patients.[10] Moreover, in those cases with no clinical symptoms or signs before the start of supplementation, an evaluation of treatment effects by other than surrogate markers is not possible.[10]

WHO SHOULD BE TESTED?

The decision to test and start treatment will depend on the likelihood of the disease; the accuracy, costs, and risks of the test; the costs and adverse effects of treatment; and the potential negative consequences of withholding treatment in an unrecognized, diseased patient (Fig. 4).[21]

For cobalamin deficiency, it is difficult to increase the probability of disease by selecting individuals on the basis of clinical signs or symptoms alone. In a Swedish study that evaluated an extensive list of symptoms and signs of cobalamin deficiency, only changes in the tongue mucosa and angular stomatitis could predict abnormal total homocysteine (tHcy) and serum folate levels.[9] All other symptoms and clinical findings traditionally linked to cobalamin or folate deficiency were unable to predict pathological test outcomes. A recent study confirms the relatively high predictive value of sore mouth for pernicious anemia.[22] However, the threshold to test is normally lowered when investigating elderly patients[23] and patients belonging to risk groups of cobalamin deficiency, including those with certain gastrointestinal diseases, dietary habits, lifestyles, and the use of some drugs (Table 2). In addition, the costs and risks of B_{12} supplementation are rather low, and the price paid for diagnostic procedures may easily exceed the expenses of a longer treatment period (Fig. 4, panel D).[16] Opportunistic screening of asymptomatic,

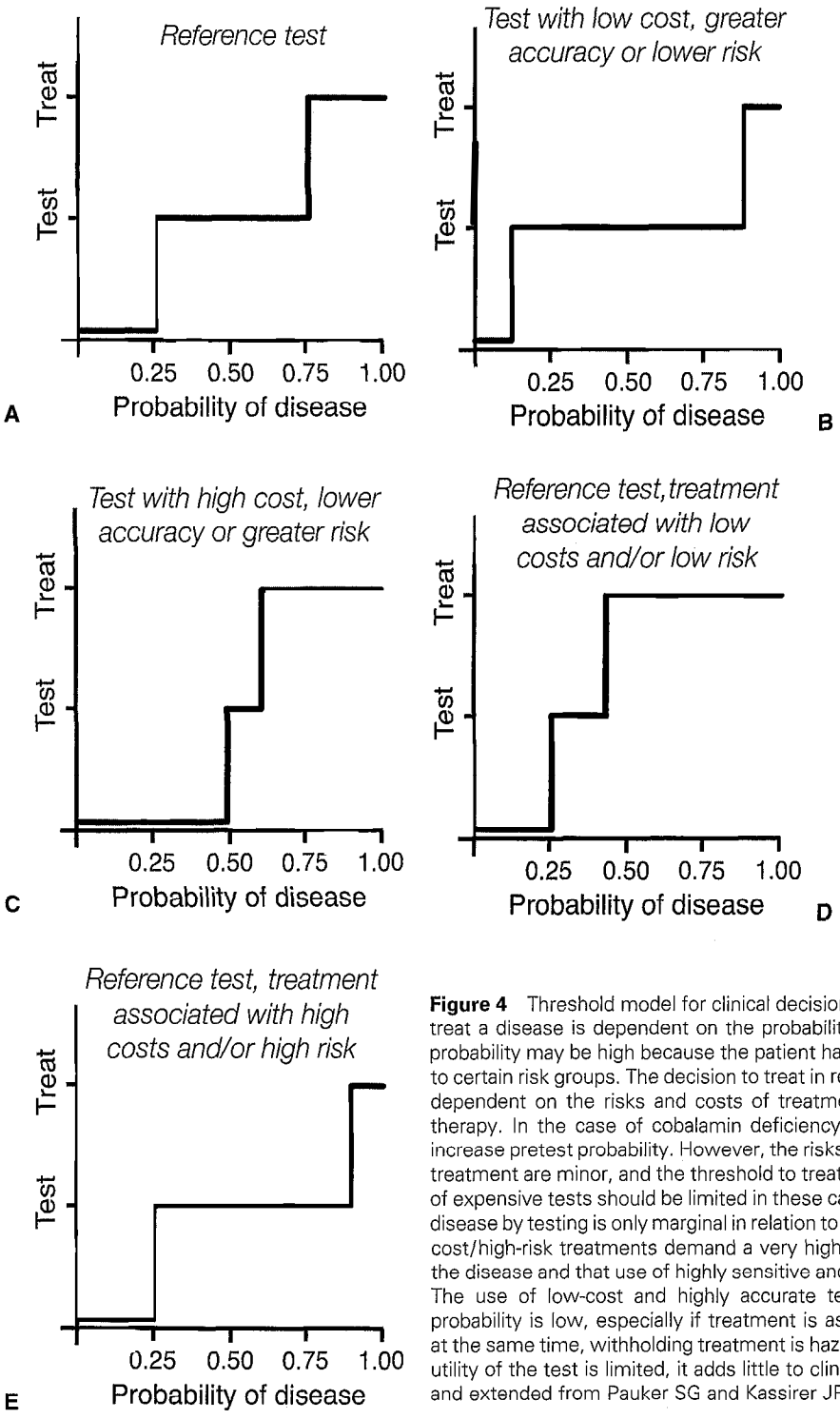

Figure 4 Threshold model for clinical decision-making. The clinical decision to test or treat a disease is dependent on the probability of the disease (panel A). The pretest probability may be high because the patient has pathognomonic symptoms or belongs to certain risk groups. The decision to treat in relation to the probability of the disease is dependent on the risks and costs of treatment and on the dangers of withholding therapy. In the case of cobalamin deficiency, there are few clinical signs that may increase pretest probability. However, the risks and costs in association with cobalamin treatment are minor, and the threshold to treat should be rather low (panel D). The use of expensive tests should be limited in these cases, as the increase in probability of the disease by testing is only marginal in relation to the treatment threshold. However, high-cost/high-risk treatments demand a very high probability that the patient actually has the disease and that use of highly sensitive and specific tests can be justified (panel E). The use of low-cost and highly accurate tests may be indicated even if pretest probability is low, especially if treatment is associated with high risks and costs but, at the same time, withholding treatment is hazardous (panel B). If, however, the clinical utility of the test is limited, it adds little to clinical decision-making (panel C). (Adapted and extended from Pauker SG and Kassirer JP[21])

nonrisk patients with new, sensitive markers should be avoided, and all diagnostic procedures should be applied with a clear objective.[24] However, irrespective of diagnostic procedure, the threshold for treatment of suspected cobalamin deficiency, especially among the elderly, should be rather low[25] (Fig. 4, panel D).

DIAGNOSTIC PROCEDURES

Medical History

Medical history may identify patients belonging to certain risk group that have a higher a priori probability of cobalamin deficiency (Fig. 4, Table 2).

Table 2 Risk Factors for Development of Cobalamin Deficiency

Categories	Subgroups/Comments
Age	Prevalence of cobalamin deficiency increases with age
Positive family history	Juvenile pernicious anemia
	Polymorphisms in cobalamin metabolism
	Imerslund Gräsbeck
Diet	Strict vegetarians
	Microwave heating of food
	Malnutrition
Lifestyle	Heavy smoking
	Chronic alcoholism
Increased demands	Pregnancy
	Hemolytic anemia
	Hemodialysis
	Chronic inflammatory diseases
Autoimmune diseases	Sjögren's syndrome
	Polyglandular autoimmune syndrome
Gastrointestinal disease	Atrophic gastritis/food cobalamin malabsorption
	Gastric surgery
	Small intestinal bacterial overgrowth
	Pelvic irradiation
	Inflammatory bowel syndrome
Drugs	Nitrous oxide
	Antacids/proton-pump inhibitors
	Metformin
	Cholestyramine

A positive family history of pernicious anemia could indicate congenital forms of the disease. Different types have been described ranging from juvenile pernicious anemia[26] to congenital intrinsic factor deficiency.[27] Furthermore, gastrointestinal surgery,[28] chronic inflammatory bowel disease,[29] small bowel bacterial overgrowth,[30] pelvic irradiation,[31] or polyglandular autoimmune syndromes[32] increase the probability of cobalamin deficiency.

Blood Tests

HEMATOLOGICAL TESTS

Hematological signs, especially macrocytic anemia, have traditionally been the cornerstone of cobalamin diagnostics,[3] but this idea has been reevaluated during the last few years.[33] The concept that patients suffering from cobalamin deficiency may have neuropsychiatric symptoms without macrocytosis or anemia was not widely accepted until the publication of the article by Lindenbaum et al. in 1988.[8] Even though macrocytosis is considered a diagnostic, increased mean corpuscular volume (MCV) values are in most cases related to other conditions like drugs and alcohol abuse. In a clinical study of 300 consecutive patients with elevated MCV values, cobalamin and folate deficiencies were responsible for macrocytosis in only about 6% of cases.[34] Thus, the positive predictive value of elevated MCV is low, whereas the negative predictive value is considered rather high.[35] However, concomitant evaluation of red cell distribution width could improve the diagnostic specificity.[36] Absence of macrocytosis in B_{12} deficiency may be encountered in patients with concurrent iron deficiency,[33] but it is unlikely that macrocytosis can by masked by high serum folate concentrations, which is important in connection with folic acid food fortification.[37]

Evaluation of peripheral blood smear may serve as an additional test. Detection of neutrophil hypersegmentation and macroovalocytosis was the only ancillary test in addition to metabolite elevations that could predict response to cobalamin treatment.[18] Still, neutrophil hypersegmentation is not a specific finding and may also be encountered in iron-deficiency anemia.[38] It is regarded as being not sufficiently sensitive, compared with functional markers, for the diagnosis of early stages of cobalamin deficiency.[39]

Sometimes biochemical signs of hemolysis such as elevated lactic dehydrogenase[40] and lowered haptoglobin[41] are observed in deficient patients. The diagnostic sensitivity and specificity of these tests are, however, limited.[18]

SERUM COBALAMIN

Novel commercial assays for determination of serum cobalamin are regularly developed for new instrumental platforms. Paradoxically, increased use of patent-protected modern, integrated workstations; proprietary reagents; and restricted economic resources in clinical chemistry have hampered an intra- and interlaboratory assessment of new generations of cobalamin assays. Published data are sparse on validation of the newer chemiluminescence assays, which have outperformed the former methods.[10] In addition, the diagnostic utility of serum total cobalamin has been questioned because total cobalamin in blood may not accurately reflect intracellular B_{12} status in the different tissues. Because of the lack of specificity, the lower cutoff limits for serum cobalamin have been reduced at the expense of sensitivity.[10] As a result, total cobalamin in serum is now considered an unreliable indicator of functional cobalamin status.[42,43]

Furthermore, total cobalamin may be strongly influenced by folate deficiency,[44] sex hormones,[45–49] hematological diseases,[50] and genetic polymorphisms.[51,52] There are several conditions under which artificially high or low total vitamin B_{12} concentrations can be measured, which strongly affects the specificity of the test in these patients (Table 3).

Table 3 Causes of False Positive or Negative Results of Tests of Cobalamin Deficiency

Test	Elevated Values of the Diagnostic Marker Caused by other Conditions than B_{12} Status	Low Values of the Diagnostic Marker Caused by other Conditions than B_{12} Status	Physiological Changes
Serum cobalamin	Liver disease (including alcoholic liver cirrhosis) Myeloproliferative disease Transcobalamin II deficiency Small intestinal bacterial overgrowth Hemolysis	Grave/severe folate deficiency Grave/severe iron deficiency Oral contraceptives Mild/severe haptocorrin deficiency Myeloma	Physiological reduction during pregnancy? Physiological reduction with increasing age? Physiologically lower levels during pregnancy (?)
Holo-transcobalamin	Impaired renal function Liver disease (?)	TC-II polymorphism? Oral contraceptives (?) High folate status (?)	Sex-differences? Age-related changes in TC-II receptor (?)
Serum folate	B_{12} deficiency (early stage) Hemolysis	Acute alcohol intoxication MTHFR-polymorphism (?)	MTHFR-polymorphism results in intracellular changes of folate distribution Long-term fasting results in higher values
Red blood cell-folate	Alcoholism	Alcoholism	Higher/lower values are encountered during pregnancy
Mean corpuscular volume	Alcoholism Miscellaneous drugs Hemolysis/posthemorrhage Hypothyroidism Chronic obstructive lung disease Cold agglutinins Severe hypoglycemia	Concomitant iron deficiency Hemoglobinopathies	
Methylmalonic acid (MMA)	Renal insufficiency Volume contraction Small intestinal bacterial overgrowth Inherited metabolic defects	Antibiotic treatment related changes in bowel bacterial flora with reduced propionic acid production	Physiological elevation during pregnancy?
Total homocysteine (tHcy)	Incorrect blood sampling/processing Age, sex, lifestyle factors Inborn errors of homocysteine metabolism MTHFR-polymorphism Renal insufficiency Hypothyroidism Vitamin B_9, B_6, B_2 deficiency Miscellaneous drugs Alcohol abuse	High folate-intake in vegetarians Various drugs like • Nitrous oxide • Penicillamine • N-acetylcysteine • Estrogens • Antiestrogens Early diabetes with hyperfiltration and without renal impairment	Lower values during pregnancy, in early childhood before puberty

HOLOTC

Most cobalamin in serum is bound to haptocorrin, or R-binder. Only a minor portion, 20–30%, is bound to transcobalamin (TC), and this fraction is essential for the transport of cobalamin from the ileum to the majority of tissues and for cellular uptake through a receptor-mediated process involving a specific receptor, TC-R[53] (Fig. 1). The TC-R also seems to be important for reabsorption of holoTC filtrated through the glomerulus[54] (Fig. 1).

HoloTC may be a better marker of recently absorbed cobalamin[55] and has been considered a more sensitive marker of cobalamin status compared with total cobalamin.[56]

HoloTC is strongly correlated with total cobalamin[57] and was found not to give additional information on B_{12} status compared with total cobalamin in a group of psychogeriatric patients.[58] The dose–response curves of the relationship between holoTC and the metabolic markers homocysteine and methylmalonic acid indicate

a stronger association and more distinct cutoff values for holoTC compared with total cobalamin.[59] In addition, in some clinical studies, holoTC does seem to be better correlated with clinical endpoints than total cobalamin.[31,55]

However, other factors than cobalamin status may affect the holoTC levels, and possibly its relation to the metabolic makers. These include a common genetic polymorphism 776 (776C → G) of the transcobalamin (TC) gene and impaired renal function and liver disease,[60–62] as well as female sex hormones.[59] The influence on holoTC from factors that are unrelated to cobalamin status may weaken the utility of holoTC as a diagnostic marker. Moreover, the fact that holoTC (like serum cobalamin) cannot be used to monitor response to cobalamin supplementation is another limitation of this marker.

Recently, a commercial test for analysis of holoTC based on solid phase absorption of holoTC to magnetic beads and RIA determination of TC-II-bound cobalamin has become available,[63] but this assay is time consuming and cumbersome, requires a large sample volume, and involves many manual steps. The analytical precision in the lower, clinically important concentration range was poor but has been improved recently by modifications of the assay.[64] Moreover, a new automated version of the holoTC test that requires considerably less volume is currently under development.

Further research is necessary to evaluate the usefulness and limitations of holoTC in clinical practice.[56]

Tests of Gastrointestinal Function

GASTRIN

Food cobalamin malabsorption results from impaired release of the vitamin from food proteins. Normally, this process requires sufficient amounts of gastric acid and pepsin.[65] Chronic atrophic gastritis, especially type A (autoimmune) gastritis, in which mainly the fundus and corpus of the gastric ventricle are involved, results in hypochlorhydria, which may cause hyperplasia of antral G-cells.[66] As a consequence, gastrin production is increased. Hypergastrinemia in pernicious anemia patients, which can be quite substantial (four to eight times above normal limits),[67] is, however, only rarely used as diagnostic tool in cobalamin deficiency and may be considered an ancillary test that is not specific for pernicious anemia.[68]

Pepsinogen A and C

Together with gastrin, serum pepsinogen A (PG I) and pepsinogen C (PG II) are surrogate markers of gastritis.[69] PG I is secreted in the gastric corpus and fundus, whereas PG II is mainly produced by both corpus and antrum cells.[70] Atrophic gastritis involving corpus/fundus, which can lead to food cobalamin malabsorption, normally reduces PG I more than PG II, which results in a lowering of the PG I:PG II ratio. PG I combined with gastrin, or the PG I:PG II ratio, has been proposed as a first-line test of suspected cobalamin deficiency.[17] However, there are causes and mechanisms of food-cobalamin malabsorption other than atrophic gastritis and achlorhydria. Thus, the idea that absence of laboratory signs of atrophic gastritis excludes cobalamin deficiency is erroneous.[15] Moreover, PG II and, to a lesser extent, PG I concentrations may also rise in connection with superficial gastritis, as observed in *Helicobacter pylori* infections,[71] which further limits the diagnostic accuracy of these markers.[69]

SCHILLING TEST

The Schilling test was developed in the early 1950s when labeled cobalamin became available.[72] It is a test of intestinal absorption of cobalamin and is based on determination of the fraction of orally ingested labeled cobalamin that is excreted/recovered in the 24-hour urine sample. For many years, the Schilling test was considered the gold standard for the diagnosis of cobalamin deficiency.[24] However, the test is cumbersome and involves intake of radioactive material and a 24-hour sampling of urine, which makes it less suitable for screening in clinical routine. Moreover, the supply of radioactive-labeled cobalamin has become problematic, as many manufacturers have ceased production. Furthermore, in patients with gastric or serum antibodies against intrinsic factors, misleading results may occur.[73,74] For these reasons, the test has been largely abandoned from clinical practice during the recent years.

FECAL ELASTASE

Fecal elastase is a relatively new marker of chronic pancreatitis.[75] Chronic pancreatitis may be caused by vitamin B_{12} malabsorption.[76] The clinical usefulness of fecal elastase for the diagnosis of cobalamin malabsorption resulting from pancreatic insufficiency remains to be determined.

LACTULOSE BREATH TESTING

The lactulose breath test is used for diagnosis of small intestinal bacterial overgrowth (SIBO).[77] Especially in the elderly, chronic atrophic gastritis may facilitate SIBO and thereby result in intestinal B_{12} malabsorption caused by competition with bacteria.[78] The breath test has, however, only in a few cases been used in the diagnosis of B_{12} deficiency.[79,80]

Antibodies

Antibodies against parietal cells, including gastric H^+, K^+-ATPase, pepsinogen, and intrinsic factor, may be

used as confirmatory or exclusion tests.[81] In general, parietal cell antibodies are characterized by high sensitivity and low specificity, whereas antibodies against intrinsic factor have low sensitivity but high specificity for pernicious anemia.[24] In patients negative for both parietal and intrinsic factor antibodies, pernicious anemia is highly unlikely.[74]

Assessment of Cobalamin-Dependent Metabolism

Methylmalonic acid (MMA) and total homocysteine (tHcy) serve as metabolic markers of cobalamin status, which is explained by the fact that cobalamin is a cofactor of methionine synthase and methylmalonyl-CoA mutase[5] (Fig. 1). Very recently, a third marker, methylcitric acid, showed a negative correlation with cognitive function[14] and was superior to tHcy and MMA in this respect. All these metabolic markers have in common the tendency to increase in patients with impaired renal function[82] (Table 3).

In general, when evaluating the diagnostic utility of these tests, it is important to distinguish between a pure marker of cobalamin status and a marker that is also involved in the pathogenesis of the disease or symptoms. Ellinson et al. investigated the relationship between different serum markers of cobalamin/folate deficiency and signs of cognitive impairment.[83] Figure 5 shows a summary of the relationship between metabolic markers, serum vitamin B_{12} and serum folate, and cognitive function based on the recent results of Ellinson et al.[83] and Garcia et al.[14] This figure illustrates the interrelations of MMA, Hcy, methylcitric acid, cobalamin, and cognitive function. The observations indicate that both homocysteine and methylcitric acid, but not MMA, may be involved in the pathogenesis of cognitive impairment. This notion is corroborated by the in vitro findings of Kölker et al. in MMA-aciduria.[84] They found that not MMA but, rather, methylcitric acid, malonic acid, and propionyl CoA exert inhibitory effects on the mitochondrial respiratory chain. Thus, extremely high MMA concentrations found in methylmalonic acidurias do not seem to exhibit direct toxic effects. Correspondingly, the moderate elevations of MMA concentrations encountered in cobalamin deficiency are unlikely to be directly involved in the pathogenesis of neurological symptoms. This may explain why MMA is not related to cognitive function in some clinical studies.[14] However, lack of direct association of elevated MMA with some clinical endpoints of cobalamin deficiency must not be interpreted as lack of diagnostic sensitivity and specificity of the test.[85]

Elevated tHcy is a very sensitive indicator of clinical cobalamin deficiency[42]; however, it has poor specificity.[10] tHcy levels are dependent on at least four B-vitamins—vitamin cobalamin, folate, vitamin B_6, and vitamin B_2—and is also affected by other factors like renal function and lifestyle[86] (Table 3). However, in countries with mandatory food folic acid fortification, cobalamin deficiency has become the most important

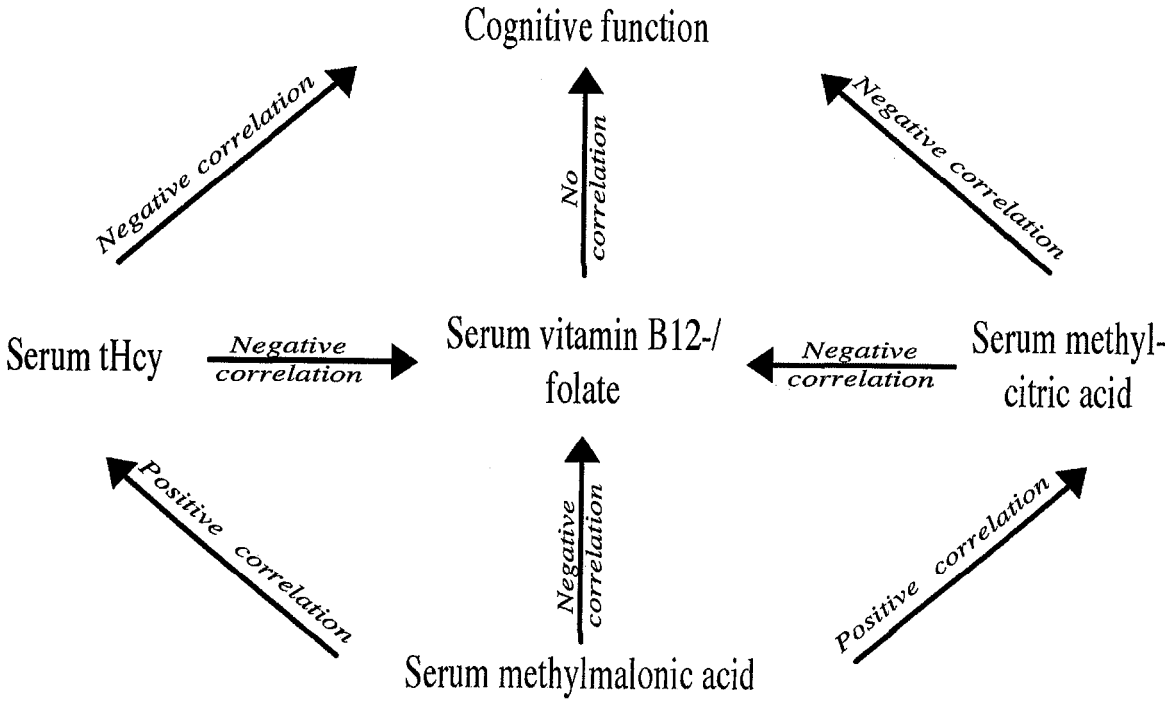

Figure 5 Summary of the relationship between serum markers of cobalamin deficiency and cognitive function. (Adapted and extended from Ellinson et al.[83] and Garcia et al.[14])

cause of hyperhomocysteinemia, if impaired renal function can be excluded.[87] Thus, the predictive value of elevated tHcy has increased in these countries.

MMA is a marker as least as sensitive as tHcy for clinical cobalamin deficiency but is more specific.[10,42] In addition, MMA is subject to confounding such as impaired renal function, but to a lesser degree than tHcy (Table 3).

The deoxyuridine suppression test (dUST) is based on the ability of deoxyuridine to suppress the incorporation of radioactive-labeled thymidine into DNA.[88] Deoxyuridine may function as a metabolic precursor for the synthesis of thymidine, a process that is dependent on the availability of folate and cobalamin. The test is normally performed using incubated bone marrow cells, but PHA-stimulated peripheral blood lymphocytes have also been used, even though the diagnostic utility of dUST in lymphocytes has been questioned.[89] By sequentially adding either folate or cobalamin in vitro, it is possible to correct an abnormal test result and distinguish between cobalamin and folate deficiency.[90] The test is very sensitive but is rather expensive and invasive because of the need for bone marrow samples. Furthermore, the test requires a high grade of laboratory expertise, making it less suitable for clinical routine use.

Among the functional tests, dUST appears to be the most sensitive compared with both tHcy and MMA.[91] All functional tests may become abnormal in early stages of cobalamin deficiency before macrocytic anemia develops.[15] However, the diagnostic specificity of these tests has been questioned, especially as dUST is technically demanding, which may cause false-positive results.[3]

An overview of the relative specificities of cobalamin (-fraction) measurement, tests of cobalamin malabsorption, and functional tests is provided in Table 4.

GENETIC POLYMORPHISMS

Inborn errors with severe defects of cobalamin absorption, such as the Imerslund-Gräsbeck syndrome or congenital intrinsic factor deficiency,[27] and metabolism, such as severe deficiencies of methionine synthase (cblG), methionine synthase reductase (cblE), and the methylenetetrahydrofolate reductase, are rare and usually present during infancy or early childhood; they are not discussed here.[10] In contrast, recent progress in genetics has resulted in the discovery of far more common polymorphisms like the TC-II 776C → T, MTHFR 677C → T, and 1298A → C, MTR 2756 A → G, MTRR 66A → G. Although the clinical significance of these polymorphisms is still debated, they certainly can interfere with traditional tests of cobalamin status. Recent publications indicate that heterozygous transcobalamin I (haptocorrin) deficiency may result in falsely low cobalamin values in a substantial number of cases in subjects selected by low cobalamin values.[52]

The clinical usefulness of testing for the increasing number of polymorphisms involved in cobalamin metabolism is uncertain. However, data on these polymorphisms may help to understand abnormal or paradoxical test results of traditional cobalamin markers and may aid in assessing the risk of cobalamin dysfunction and requirements in certain individuals.[10]

COMPARISON OF NEW DIAGNOSTIC MARKERS OF COBALAMIN DEFICIENCY

In the absence of a clear definition and an independent reference standard for diagnosis of cobalamin deficiency, it is difficult to compare the diagnostic utility of laboratory tests objectively.[16]

However, in prospective studies, the metabolites tHcy and MMA have been demonstrated to be sensitive markers that are able to predict clinical response to cobalamin therapy.[18] Furthermore, these metabolites were the earliest parameters to rise when cobalamin treatment was withheld,[42] and MMA was the best predictor/discriminator of dietary group in a study of macrobiotic children and matched controls.[92]

In addition, functional tests, especially tHcy and MMA, allow an early (after 1–2 weeks) evaluation of treatment response.[93] This is in contrast to total cobalamin[10] and, most likely, holoTC,[55] which rise instantly after supplementation, regardless of restoration of cellular metabolism.

Table 4 Relative Diagnostic Specificities of Different Markers of Cobalamin Deficiency

Specificity	Methods for Evaluation of Intake and Blood Concentrations of Cobalamin	Tests of Cobalamin Malabsorption	Tests of Cobalamin Function
Low	Dietary B_{12}-intake Serum total cobalamin	Breath tests for detection of SIBO*/*Helicobacter pylori* infections	BME,** total blood count
Intermediate	Holo-transcobalamin (holoTC)	Gastrin, pepsinogen A, pepsinogen A/C-ratio	tHcy
High	HoloTC/total TC ratio, holo-haptocorrin/total haptocorrin ratio	Gastroscopy Schilling test	MMA/uMMA dUST

*SIBO: Small intestinal bacterial overgrowth; **BME: Bone marrow examination

Table 5 Performance and Characteristics of Different Markers of Cobalamin Deficiency

Test	Sensitivity	Specificity	Interference Robustness*	Monitoring Treatment Response	Clinical Utility**	Costs	Availability	Comments	Total Score
Total cobalamin	2	2	1	0	3	3	3	Established test	14
Holo-TC	3	2	2	0	3	2†	1†	Automated, low volume test under way	13
DUST	3	3	3	3	1	1	1	Invasive, technically demanding procedures	15
THcy	3	2	1	3	2	2	3	Dependent on several B-vitamins and life style factors	16
MMA	3	3	2	3	3	1	2	High assay complexity	17
Pepsinogen I/II	2	1	2	0	3	2	1	Influenced by superficial gastritis and *H. pylori* infection	11
Gastrin	2	1	2	0	2	2	2	Food intake and stress increase gastrin production	11

Score is given on a scale from 0 (worst) to 3 (best). For sensitivity, specificity, measuring of treatment response, utility and availability, 0 = no, 1 = low, 2 = intermediate, and 3 = high. For interference robustness and costs, 1 = high degree of confounding or high cost, whereas 3 = essentially no interference or very low cost. *Interference robustness includes the risk of confounding or interference of assay results by other factors than cobalamin status. **Clinical utility includes easiness of sampling, preanalytical variability, invasiveness of procedure, and inconvenience for patient †Costs and availability will most likely be improved with the advent of new automated, low-volume test.

A comparison of the diagnostic utilities of new diagnostic markers is provided in Table 5.

DIAGNOSTIC STRATEGIES

The diagnostic approach must be individualized and optimized according to the characteristics of the study population and according to risk groups and the clinical symptoms of the patients.[16]

The costs and availability of diagnostic markers and the experience of the investigator will also influence the choice of first-line diagnostic tests.[10] Although testing for cobalamin deficiency in certain risk- and age-groups may be indicated even in asymptomatic patients,[94] unmotivated, opportunistic screening should be avoided.

The pretest probability of cobalamin deficiency is crucial when determining the thresholds for testing and for treating patients.[21] If tests of high diagnostic accuracy and low costs are available, the threshold to test should be lowered (Fig. 4, panel A). As cobalamin supplementation is cheap and virtually without risk of serious adverse effects, the threshold to treat patients also should be rather low (Fig. 4, panel D).

If the patient presents with typical clinical signs or symptoms of cobalamin deficiency, the pretest probability is already high (Fig. 4, panel C). In this case, extensive and expensive testing does not seem to be justified because one may decide to start treatment of the patient solely on the basis of clinical findings without testing. A single test, either total cobalamin or holoTC, normally will suffice for treatment decision.[10,16]

In patients with clinical findings that are not typical for cobalamin deficiency, but in whom cobalamin deficiency should be ruled out as one of many differential diagnoses, pretest probability is low (Fig. 4, panel B). In this case, not confirmation but, rather, exclusion of cobalamin deficiency is the primary goal. Therefore, the use of more sensitive and specific tests is indicated, and functional tests, primarily MMA and tHcy, should be applied.[10]

Asymptomatic subjects belonging to a risk group of cobalamin deficiency (Table 2) have an intermediate pretest probability (Fig. 4, panel D). In this case, one should try to limit the expenses of diagnostic procedures, because the costs and adverse effects of prophylactic cobalamin supplementation are minor. Use of serum cobalamin and serum folate as primary tests may meet the diagnostic requirements. If low values are found the patient should be treated, and in the case of borderline values, additional testing of metabolites or holoTC may be indicated.[16]

In asymptomatic, nonrisk subjects in whom low cobalamin is discovered by chance in the course of opportunistic screening, MMA testing should be the first choice because of the high sensitivity and specificity of the test. If MMA is elevated, additional testing will be

necessary to find the cause of impaired cobalamin status.[10] If normal MMA values are found, no further testing is necessary, as cobalamin deficiency now can be ruled out with a high degree of certainty.

In case algorithms are used to guide the diagnostic strategies,[16,17,33,95–98] it is important to bear in mind that there is an error propagation for each new test included in the decision tree, and that the squares of the CVs of the different tests included in the algorithm are additive. Multiple tests could therefore introduce new layers of uncertainty rather than clarification of clinical situation.[99]

CONCLUSIONS

Several novel diagnostic markers of cobalamin deficiency have become available during the last couple of years, and the role of the established tests has been reevaluated.

The novel diagnostic strategies include assays for determination of cobalamin fractions in blood (in particular holoTC), tests of gastrointestinal dysfunction (including antibodies), tests of metabolic function, and different genetic polymorphisms. However, only a few of these tests, such as tHcy, methylmalonic acid, holoTC, and possibly methylcitric acid, have practicalities that make them attractive as first-line tests in routine clinical practice. Other tests, like hematological status, gastrointestinal function tests—including the Schilling test, and genetic polymorphisms may be used as ancillary markers, for "reflex testing," or as tools for finding the cause of cobalamin deficiency.

Tests for elucidation of the cause of cobalamin deficiency are important but may only have a limited influence on initial treatment decisions and are not useful as primary screening tests. However, most causes of cobalamin deficiency are chronic in nature, cannot be cured, and require lifelong supplementation.[100] A confirmation of the diagnosis and clarification of the cause of the disease is thus desirable because the accumulated costs of lifetime cobalamin treatment may be considerable.

Functional tests, such as tHcy and MMA, have been available for several decades and are the best option to assess cobalamin deficiency and to monitor the effects of treatment. Although, nowadays, tHcy analysis is offered by the majority of laboratories, the more specific MMA test is not, and this, in addition to higher costs, has prohibited general use of MMA as a first-line test for cobalamin deficiency.

The holoTC-test has not yet been sufficiently evaluated in clinical practice, but preliminary results are promising. HoloTC is most likely superior to conventional assays of total cobalamin. Once low-volume, automated holoTC assays are generally available at a reasonable price, holoTC has the potential to replace total cobalamin assays.

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