

Vitamin B₁₂ deficiency from the perspective of a practicing hematologist

Ralph Green

Department of Pathology and Laboratory Medicine, University of California, Davis, Sacramento, CA

B₁₂ deficiency is the leading cause of megaloblastic anemia, and although more common in the elderly, can occur at any age. Clinical disease caused by B₁₂ deficiency usually connotes severe deficiency, resulting from a failure of the gastric or ileal phase of physiological B₁₂ absorption, best exemplified by the autoimmune disease pernicious anemia.

There are many other causes of B₁₂ deficiency, which range from severe to mild. Mild deficiency usually results from failure to render food B₁₂ bioavailable or from dietary inadequacy. Although rarely resulting in megaloblastic anemia, mild deficiency may be associated with neurocognitive and other consequences. B₁₂ deficiency is best diagnosed using a

combination of tests because none alone is completely reliable. The features of B₁₂ deficiency are variable and may be atypical. Timely diagnosis is important, and treatment is gratifying. Failure to diagnose B₁₂ deficiency can have dire consequences, usually neurological. This review is written from the perspective of a practicing hematologist. (*Blood*. 2017;129(19):2603-2611)

Introduction

Traditionally, vitamin B₁₂ deficiency has been considered to lie within the scope and expertise of hematologists. This assignment has deep historical roots, going back to the earliest recognition of the disease that acquired the eponymic title of Addisonian pernicious anemia following the somewhat vague description by the Guy's Hospital physician, Thomas Addison, of "a very remarkable form of general anemia occurring without any discoverable cause whatsoever." It was the astute clinical observations of Richard Cabot, William Osler, and others that brought the picture of the syndromic disease with its classical triad of associated jaundice, glossitis, and myeloneuropathy into sharper focus, as nicely recorded in William Castle's historical review of the disease.¹ Coller,² in his commentary to mark the 70th anniversary of *Blood*, wrote: "The most dramatic and far reaching event in hematology in the United States in the pre-*Blood* period was Minot and Murphy's 1926 report that feeding liver to patients with pernicious anemia could cure this otherwise fatal disorder. This dramatic breakthrough was an enormous stimulus to hematologic investigation." A quest for the active principle in liver that made it possible to "cure" pernicious anemia ushered in the era of Big Pharma in a race to identify and produce the compound that ultimately became known as vitamin B₁₂. Elucidation of the physiology of B₁₂ and its intricate mechanism of assimilation made it clear that there was a myriad of causes of B₁₂ deficiency.³

nonhematological complications, including increased risk of neural tube defect pregnancy, cognitive impairment, osteopenia, and vascular occlusive disease, perhaps attributable to the accumulation of homocysteine (Hcy) that occurs in B₁₂ deficiency.³ Even so, because the most conspicuous manifestations of established B₁₂ deficiency affect the blood and bone marrow and are a leading cause of macrocytic and megaloblastic anemia, it is ultimately the practicing hematologist who remains front and center of the clinical diagnosis and management of patients with suspected or confirmed B₁₂ deficiency.

This review is written from the perspective of a practicing hematologist who might suspect B₁₂ deficiency during a routine patient encounter or who might see a patient in consultation for anemia as part of a complex medical problem. An understanding of the normal physiology and its perturbations in disease is a key factor to the understanding of the causes and manifestations of B₁₂ deficiency. The clinical features in a given case of B₁₂ deficiency may range from the typical "textbook" picture through any 1 of a kaleidoscopic variety of atypical presentations that can befuddle the unwary.

Vitamin B₁₂ deficiency: the hematological perspective, past and present

Because of the often conspicuous hematological manifestations of B₁₂ deficiency, it remained largely within the domain of hematology. However, as ever more sensitive methods were developed to assess subtle degrees of deficiency of the vitamin,^{4,5} it became clear that the effects of B₁₂ deficiency were not restricted to the hematopoietic system but were often overshadowed by neurological complications and were sometimes entirely absent.⁶ Just as folate deficiency is associated with effects beyond anemia,⁷ B₁₂ deficiency also can be associated with

Pathobiology of B₁₂ deficiency

In an adult, the total body B₁₂ store is 3 to 5 mg, and the recommended daily intake (RDI) is 2.4 μg.⁸ Natural food sources of B₁₂ are restricted to food of animal origin. As a consequence, it is a micronutrient that is often in critically short supply, particularly among vegetarian or vegan populations who, through culture, poverty, or conviction, subsist on diets that lack or are poor in B₁₂. Were it not for efficient conservation of biliary B₁₂ through enterohepatic reabsorption,⁹⁻¹¹ symptomatic B₁₂ deficiency would occur more frequently among vegans.

Complex mechanisms are in place to render B₁₂ bioavailable, protect it during intestinal transit, and then absorb and retain the precious vitamin for cellular uptake^{3,12} (Figure 1). It is remarkable that B₁₂ is the required cofactor for only 2 biochemical reactions in

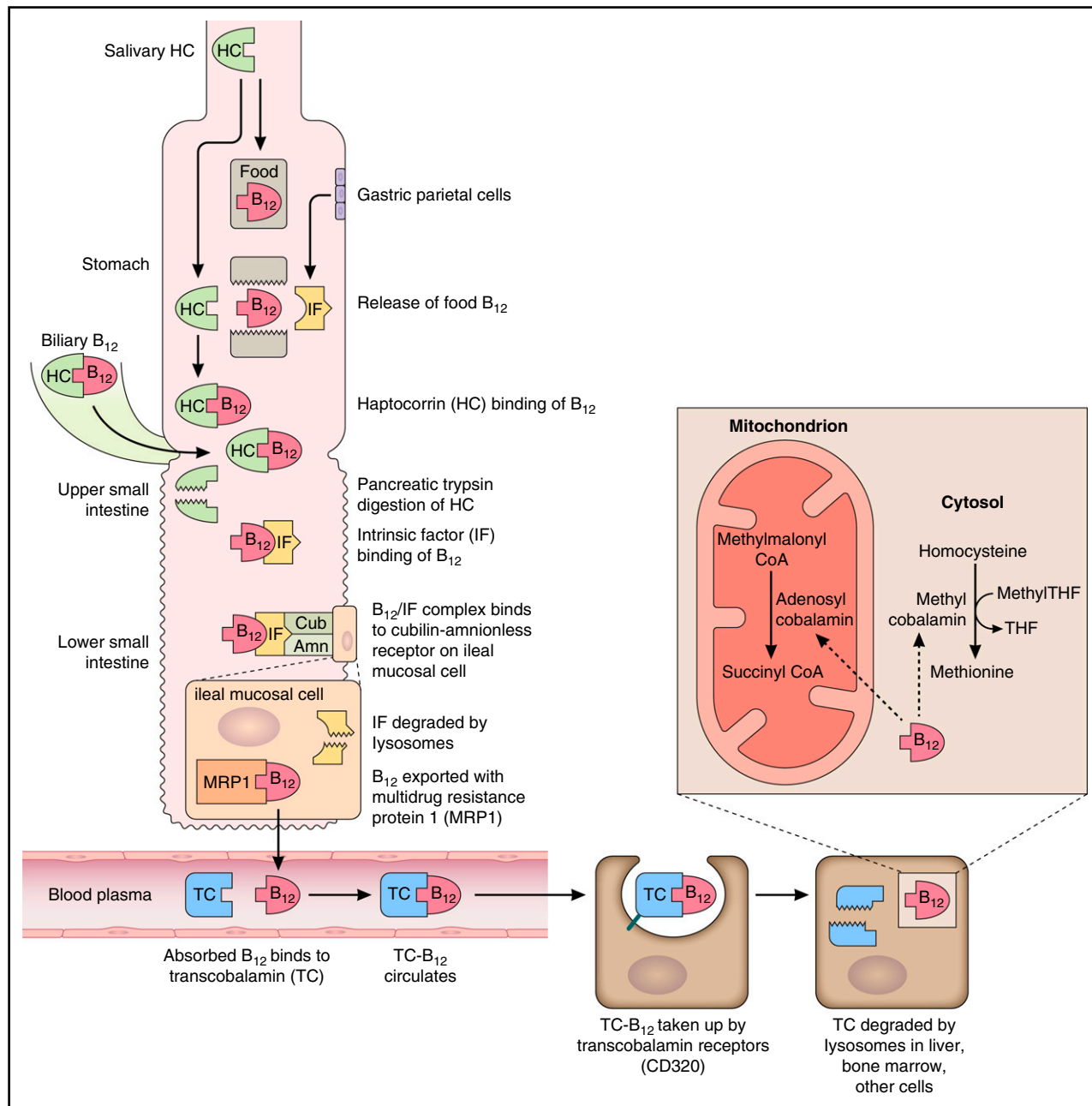


Figure 1. Normal pathway of B₁₂ absorption and cellular uptake. Food B₁₂ is released in the stomach and binds to salivary HC. In the small intestine, food B₁₂ and biliary B₁₂ are released from HC by pancreatic proteases and bind to intrinsic factor (IF). The IF-B₁₂ complex then binds to the cubilin (Cub)-amionless (Amn) receptor in the terminal ileum for internalization and release to plasma where it is bound by TC. TC delivers B₁₂ to the TC receptor (CD320) on cells, and following release in the cell, B₁₂ is reduced and converted to adenosylcobalamin in the mitochondria and methylcobalamin in the cytosol, where they serve as cofactors for the 2 B₁₂-dependent reactions. CoA, coenzyme A; THF, tetrahydrofolate. Professional illustration by Patrick Lane, ScEYence Studios.

humans,³ yet the effects of B₁₂ deficiency are not only profound but protean. The several possible reasons for the broad spectrum of manifestations fall into the broad categories of genetic variation and acquired comorbidities.

Depletion of body B₁₂ stores resulting from insufficient capture of the vitamin from dietary sources because of either inadequate intake or malabsorption eventually leads to a state of deficiency. When a certain threshold of deficiency is reached, the supply of B₁₂ becomes inadequate to support biochemical pathways requiring the vitamin, leading to disruption of the functional and ultimately the structural integrity of cells. Absent of any underlying perturbation of B₁₂-dependent

pathways that occur in individuals who harbor inborn errors involving intracellular B₁₂ assimilation and processing,^{13,14} the major determinant of the severity of B₁₂ deficiency and whether it leads to either megaloblastic anemia, demyelinating neurological disease, or both appears to be whether there is abrogation of the normal physiological axis of B₁₂ absorption. Normal B₁₂ absorption requires intact gastric production of intrinsic factor as well as a functioning cubam receptor for the B₁₂-intrinsic factor complex in the terminal ileum.^{3,12,15}

B₁₂ and folate are intimately connected through their cooperative roles in one-carbon metabolism, and the hematological complications seen in deficiency of either vitamin are indistinguishable. Both are

caused by impaired DNA synthesis that results in a prolongation of the S phase of the cell cycle¹⁶ and causes maturation arrest.¹⁷ Prolongation of the cell cycle is associated with delayed migration of the DNA replication fork and the annealing of DNA fragments synthesized from the lagging strand.¹⁸ The retardation of DNA replication in megaloblasts arises from failure of the folate-dependent conversion of deoxyuridine to deoxythymidine. The deoxyuridine triphosphate that accumulates is incorporated into DNA in place of thymidine 5'-triphosphate by the somewhat promiscuous DNA polymerase enzyme.¹⁹ The normal process of excision-repair of U-A mismatches in DNA fail for persistent lack of thymidine 5'-triphosphate. Repetitive iterations of defective DNA repair ultimately lead to DNA strand breaks, fragmentation, and apoptotic cell death.^{20,21}

The morphological appearances of these biochemical lesions are seen as megaloblastic changes in the marrow, which consist of red cell precursors that are larger than normal with a lack of synchronous maturation of the nucleus and cytoplasm (Figure 2). There is a preponderance of basophilic erythroblasts over more mature hemoglobinized forms, creating the appearance of a maturation arrest. The myeloid-to-erythroid ratio falls and may even show reversal (<1:1), due to varying degrees of both apparent erythroid hyperplasia caused by maturation arrest and granulocytic hypoplasia. Megaloblastic features in the granulocyte precursors consist of giant metamyelocyte and band forms containing large horseshoe-shaped nuclei (Figure 2). Megaloblastic megakaryocytes may be seen with abnormally large and polylobated nuclei, sometimes with detached lobes and absent cytoplasmic granulation.

All megaloblastic anemias display similar clinical features. Absent of any sudden acceleration in the rate of B₁₂ depletion, such as occurs following exposure to nitrous oxide,^{22,23} anemia develops slowly, and symptoms including weakness, palpitations, fatigue, light-headedness, and shortness of breath may not occur until anemia is fairly profound, because compensatory cardiopulmonary changes mitigate tissue hypoxia. The melding of severe pallor with jaundice caused by hemolysis produces a peculiar lemon-yellow skin color.

All formed blood elements are affected by the ineffective megaloblastic hematopoiesis, but erythrocytes show the most marked changes, both in size and in shape, with large oval macrocytes and prominent anisopoikilocytosis. In general, the degree of anemia corresponds with the severity of the red cell morphologic changes. When the hematocrit falls <20%, megaloblasts may appear in the blood. The morphologic features of megaloblastic anemia may be grossly exaggerated in splenectomized patients or in whom there is functional asplenia as occurs in celiac disease or sickle cell anemia when circulating megaloblasts and bizarre red cell morphology may be present.²⁴

The anemia is macrocytic (mean corpuscular volume 100-150 fl or more); mild macrocytosis may be the earliest evidence of a megaloblastic process, but because of longevity of red cells, there is a gradual shift in mean corpuscular volume as comingling occurs with older normocytic red cells. Anisocytosis becomes more marked, and the earliest measurable change in red cell indices is an increase in the red cell distribution width.

Neutrophils typically show hypersegmentation of their nuclei, beyond the usual 3 to 5 lobes, and may contain 6 or more lobes.²⁵ Hypersegmented neutrophils are an early sign of megaloblastosis and may persist for many days after treatment.²⁵ However, neutrophil hypersegmentation does not appear to be a sensitive indicator of mild B₁₂ deficiency.²⁶ Leukopenia and thrombocytopenia may be present but only rarely cause clinical problems. Thrombocytopenia may be severe, when it may be confused with thrombotic thrombocytopenic purpura.^{27,28} Platelet production is reduced to 10% of what

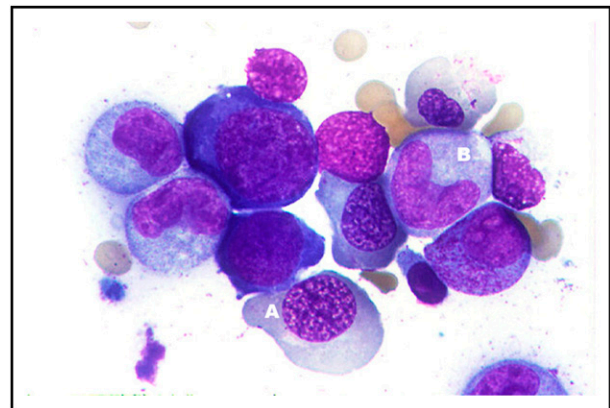


Figure 2. Photomicrograph of bone marrow in a patient with pernicious anemia. (A) Megaloblastic change in the nucleus of an erythroid precursors consisting of variegated finely granular chromatin ("salt-and-pepper" appearance) in contrast to the ground-glass texture of normal proerythroblasts. With progressive maturation, chromatin condensation occurs at a slower pace than normal, giving rise to darker aggregates that fuse nonhomogeneously and impart to the nucleus a characteristic lattice-like appearance. Undisturbed maturation of the cytoplasm as hemoglobin forms in a cell with an immature-appearing nucleus results in cells that are conspicuous for their lack of synchrony between nuclear and cytoplasmic development. (B) A megaloblastic ("giant") granulocyte precursor. Original magnification $\times 100$; Wright-Giemsa stain.

would be expected from the megakaryocyte mass,²⁹ reflecting ineffective thrombopoiesis, and platelets may be functionally abnormal.³⁰

Cytogenetic changes, when they occur, are nonspecific and show elongated and broken chromosomes, changes that are usually corrected within 2 days of treatment, although some abnormalities may remain for months.^{21,31}

Variations on the theme and the B₁₂-folate nexus

What determines the particular manifestations of B₁₂ deficiency in a given individual depends on several factors, some of which are understood, others not. Two clear examples of what influences the clinical presentation in a given patient are the rate of development and the degree of severity of deficiency. The extent to which absorption of B₁₂ is compromised, whether partial or complete and whether absorption is totally abrogated or whether it relates only to poor bioavailability of food B₁₂ is also important. Polymorphic differences in genes involved in the complex repertoire that comprises the pathways of B₁₂ absorption, assimilation, cellular metabolism, and plasma transport of the vitamin (Figure 1) are known to affect the susceptibility to develop B₁₂ deficiency.^{32,33} Whether these genetic factors can also influence the disease phenotype in B₁₂ deficiency is not well understood at this time. Another factor that may play a role in the susceptibility of an individual to B₁₂ deficiency is the composition of their gastrointestinal microbiome. Some microbiota are capable of degrading B₁₂, which may affect bioavailability of the vitamin and also lead to the generation of B₁₂ analogs.³⁴ B₁₂ analogs have been identified in plasma and tissues³⁵ and have been invoked as a possible cause of some of the manifestations of B₁₂ deficiency.³⁶ Host-microbial interactions have also been implicated as a possible initiating factor in autoimmune gastritis leading to pernicious anemia. In this proposed mechanism, chronic *Helicobacter pylori* infection may, through molecular mimicry of H⁺K⁺ ATPase, evoke a host immune response

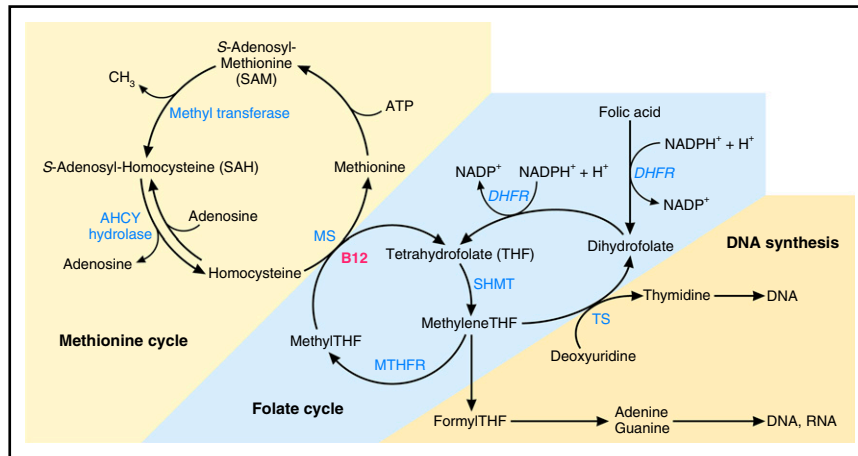


Figure 3. Intersections of B₁₂ and folate metabolism, the methionine cycle, folate cycle, and DNA synthesis showing the methyl folate “trap.” The key intersection of B₁₂ and folate occurs at the methionine synthase (MS) reaction in which the one-carbon methyl group of methyltetrahydrofolate (MethylTHF) is transferred to Hcy to form methionine. The cofactor for this reaction is B₁₂ in the form of methylcobalamin. The folate product tetrahydrofolate regains a one-carbon methylene group through the serine hydroxymethyltransferase (SHMT) reaction, and the resulting methylenetetrahydrofolate is essential for conversion of deoxyuridine to thymidine in the thymidylate synthase (TS) reaction. This reaction is rate limiting for DNA synthesis. In B₁₂ deficiency, folate becomes trapped as methylTHF. Administration of folic acid can temporarily overcome this block through dihydrofolate reductase (DHFR) reduction to tetrahydrofolate. The other product of the MS reaction, the essential amino acid methionine, after adenylation to S-adenosyl-methionine (SAM), serves as a universal methyl donor in numerous methyltransferase reactions. The product, S-adenosyl-homocysteine (SAH), undergoes reversible hydrolysis by the enzyme adenosyl-homocysteine hydrolase (AHCY hydrolase), yielding Hcy and thus completing the methionine or remethylation cycle. Not shown in this figure is the alternative transsulfuration pathway for Hcy disposal, which requires vitamin B₆.⁸ ATP, adenosine triphosphate; DHFR, dihydrofolate reductase; H⁺, proton; MTHFR, methylene tetrahydrofolate reductase; NADP⁺, NAD phosphate; NADPH⁺, reduced NAD phosphate. Professional illustration by Patrick Lane, ScEYence Studios.

that involves CD4⁺ T cells through a Fas-dependent mechanism³⁷ and leads to destruction of the gastric mucosa.^{38,39}

Nutrient-nutrient interactions are known to play a role in the manifestations of B₁₂ deficiency. The best known of these is concomitant iron deficiency, which can mask the macrocytosis typically seen in B₁₂ deficiency. The same is true for other microcytic disorders like α - or β -thalassemia trait.^{40,41}

An important B₁₂ nutrient interaction is with folate. In B₁₂ deficiency, there is disruption of normal folate cycling for regeneration of methylene-tetrahydrofolate, the form required to sustain synthesis of thymidine for DNA replication. Folate becomes effectively “trapped” as methylfolate,⁴² because B₁₂ is required for its conversion to tetrahydrofolate in the methionine synthase reaction (Figure 3). Trapping of methylfolate creates a state of functional folate deficiency. Supply of folic acid to a B₁₂-deficient patient can intermittently bypass this block through reduction of folic acid to dihydrofolate and then tetrahydrofolate, thereby partially or temporarily alleviating the anemia. Alleviation of the anemia masks the underlying B₁₂ deficiency and allows the neurological damage from B₁₂ deprivation to continue unabated. There is well-described evidence in the early literature that amounts of folic acid exceeding 1 mg daily given to patients with pernicious anemia were fraught with deleterious outcome.^{3,8} Although at first ameliorating hematological features of the disease, even at times with temporary improvement in neurological symptoms, continued administration of folic acid would precipitate or aggravate neurological complications, usually with subsequent recurrence of the anemia.^{8,43} Linked to these observations are reports of dissociation between neurological and hematological manifestations in B₁₂-deficient patients⁶ as well as an inverse correlation between the degree of anemia and the severity of neurological involvement.^{44,45} There is some evidence that this relationship might be related to higher serum folate concentrations in patients with exclusively or predominantly neurological manifestations.⁴⁴ More recently, and occurring in the wake of national folic acid fortification programs designed to reduce neural tube defect pregnancies, there have been several reports from longitudinal population studies that individuals with low serum B₁₂ levels, who had associated

high serum folate levels, had significantly higher levels of methylmalonic acid (MMA) and Hcy and were more likely to show cognitive decline and have lower hemoglobin concentrations than those with low B₁₂ but without high serum folate.⁴⁶⁻⁴⁸ Moreover, individuals with low serum B₁₂ and high serum folate had depressed levels of holotranscobalamin (holoTC), indicating an apparent depletion of circulating active B₁₂ when serum folate was high.⁴⁸ A report that the prevalence of anemia in patients with low B₁₂ levels before and after the introduction of folic acid fortification was unchanged argues against the proposition that food fortification may have caused an increase in masking the hematological complications of B₁₂ deficiency.⁴⁹ However, it is possible that if there is any deleterious effect of folate in B₁₂-deficient persons, this occurs only in individuals consuming amounts of folate well in excess of the recommended safe upper limit.⁸

Causes of B₁₂ deficiency

There are several causes and varying degrees of severity of B₁₂ depletion leading to deficiency (Table 1). From the hematological standpoint, it is convenient to divide the causes of B₁₂ deficiency into those that frequently lead to megaloblastic anemia or overt neurological complications and those that usually do not.^{3,50} The features that distinguish the severe from the mild category of B₁₂ deficiency are summarized in Table 2. The separation is based on pathophysiologic considerations and the degree of severity of the deficiency that occurs. The causes that are listed as severe usually involve disease processes that disrupt some aspect of the physiological pathway for B₁₂ absorption comprising intrinsic factor and the cubam receptor in the terminal ileum. Undiagnosed or untreated, these conditions ultimately advance to a level of depletion of B₁₂ that manifests the clinical features of B₁₂ deficiency, either hematological or neurological or both. The exemplar of this category of B₁₂ deficiency is pernicious anemia. The slow evolution of this disease reflects the rate at which the autoimmune process disables the manufacture of intrinsic factor in gastric

Table 1. Causes of vitamin B₁₂ deficiency

A. Severe deficiency	
1. Severe malabsorption (affecting the physiological intrinsic factor cubam receptor axis)	
a. Pernicious anemia (autoimmune gastritis)	
b. Total or partial gastrectomy	
c. Gastric bypass or other bariatric surgery	
d. Ileal resection or organ reconstructive surgery (ileal conduit diversion & ileocystoplasty)	
e. Inherited disorders affecting B ₁₂ absorption (affecting either intrinsic factor or the cubam receptor)	
2. Abuse of nitrous oxide	
3. Inherited metabolic	
a. Impaired ability to transport B ₁₂ (TC deficiency)	
b. Impaired ability to process B ₁₂ (8 distinct inborn errors of cobalamin metabolism resulting in homocystinuria and/or methylmalonic acidemia) with varying clinical spectra involving the nervous system and blood	
B. Mild to moderate deficiency	
1. Mild to moderate malabsorption (impaired ability to render food B ₁₂ bioavailable)	
a. Protein-bound vitamin B ₁₂ malabsorption	
b. Mild, nonimmune, chronic atrophic gastritis	
c. Use of metformin	
d. Use of drugs that block stomach acid	
e. Chronic pancreatic disease	
2. Dietary deficiency	
a. Adults: vegans/vegetarian diet, or diet low in meat and dairy products	
b. Infants: breastfeeding in infants with vitamin B ₁₂ -deficient mothers	

parietal cells leading to the inexorable depletion of the body B₁₂ store. Gastrectomy emulates abrogation of intrinsic factor production but with surgical suddenness. Similar temporal considerations apply in the case of ileal disease vs surgical resection. In the case of chemical inactivation of B₁₂ by nitrous oxide, depending on the frequency and duration of its use and the state of B₁₂ reserves, deficiency can develop either suddenly or insidiously.^{22,23}

The causes of mild B₁₂ deficiency, on the other hand, involve either a disruption of the ability to render natural dietary B₁₂ bioavailable or a primary dietary lack of B₁₂ that is obtained among unsupplemented vegans or, to a lesser extent, among vegetarians.⁵¹ There are several conditions that disrupt the normal processes, as discussed in the review by Nielsen et al¹² and depicted in Figure 1, whereby food B₁₂ is rendered bioavailable for absorption through the physiological intrinsic factor-cubam receptor pathway (Table 1). Disruption of the mechanisms to render dietary B₁₂ bioavailable all involve a failure of adequate gastric acid production, disrupting the proteolytic activity of peptic digestion.⁵² A similar failure of the digestive process applies in the case of chronic pancreatic disease,⁵³ in which the release of B₁₂ from salivary haptocorrin (HC) in the upper small intestine is disrupted through lack of bicarbonate and trypsin production.⁵⁴ There are some less common causes of B₁₂ deficiency that do not fit nicely into either

category, such as infestation with the fish tapeworm, *Diphyllobothrium latum*, in which the degree of deficiency and hence its clinical severity can vary considerably.⁵⁵

Diagnosis of B₁₂ deficiency

Two pathophysiologic processes contribute to the anemia resulting from B₁₂ deficiency. In addition to the ineffective erythropoiesis caused by intramedullary apoptosis of megaloblastic erythroid precursors,²⁰ the erythrocytes that are produced have increased rigidity associated with abnormal red cell membrane proteins leading to shortened red cell survival.^{56,57} The resulting hemolysis is associated with a 30% to 50% reduction in red cell lifespan. Plasma bilirubin is increased,⁵⁸ as is serum lactic dehydrogenase (LDH),⁵⁹ with LDH-1 predominating over LDH-2.⁶⁰ Serum AST levels are, however, often normal.⁶¹ There is an increase in serum erythropoietin levels, but the increase is relatively modest, compared with other anemias of similar severity.⁶² Another feature arising from the ineffective erythropoiesis is a block in iron utilization, resulting in increased serum iron and ferritin levels,⁶³ but with increased levels of soluble serum transferrin receptor, presumably related to hemolysis.⁶⁴ Corresponding to the increase in LDH, there may be an increase in serum muramidase caused by increased granulocyte turnover.⁶⁵

Serum B₁₂ levels

Although often used as the first-line screening test for B₁₂ deficiency, serum B₁₂ measurement used in isolation has a generally poor sensitivity and specificity for reliable detection of B₁₂ deficiency.^{4,5} A low serum B₁₂ level does not always indicate B₁₂ deficiency, and a serum B₁₂ within the reference range does not always connote normalcy. There are several reasons serum B₁₂ is not low in all patients with B₁₂ deficiency. In part, this relates to the distribution of B₁₂ on the 2 major plasma B₁₂ binding proteins. Normally, the major fraction (70% to 90%) of circulating B₁₂ is bound to HC, which is unavailable for immediate delivery to cells. The other 10% to 30% is bound to transcobalamin (TC), the functional B₁₂ transport protein. Consequently, if levels of the HC-bound fraction are conserved, the total serum B₁₂ level may lie within the normal reference range, despite lowered levels of the important TC-bound fraction. An extreme example of this is seen in a B₁₂-deficient patient with normal serum B₁₂ levels who has an underlying myeloproliferative disease with high HC levels.⁶⁶ In almost 50% of patients with low vitamin B₁₂ levels, levels of the biochemical markers, MMA and Hcy, were found to be normal, and these patients had no hematologic or neurologic response to B₁₂ replacement therapy, suggesting that the

Table 2. Severe and mild categories of B₁₂ deficiency

	Severe	Mild
Mechanism	Disruption of intrinsic factor/cubam absorption	Failure of gastric digestion and release of food B ₁₂
Enterohepatic reabsorption of biliary B ₁₂	Interdicted	Intact
Manifestations	Megaloblastic anemia and/or neurological complications	Megaloblastic anemia and serious neurological deficits rare; associated with more rapid cognitive decline
Rate of depletion	Rapid, and may be extreme	Slow, usually mild and usually limited
Treatment	Require lifelong regular B ₁₂ replacement, either monthly injection or daily high-dose oral B ₁₂	Responds to daily physiological dose supplements of oral B ₁₂

low B₁₂ values were false positive results.⁶⁷ Serum B₁₂ levels are usually normal in functional B₁₂ deficiency, resulting from exposure to nitrous oxide, which chemically inactivates the methylcobalamin at the active site of the methionine synthase during its catalytic cycle.⁶⁸ Serum B₁₂ levels are also usually normal in TC deficiency, and inborn errors of cobalamin metabolism.⁶⁹ Conversely, serum B₁₂ levels may be low in the presence of normal tissue B₁₂ in vegetarians,⁷⁰ in subjects taking megadoses of ascorbic acid,⁷¹ in inherited “benign” HC deficiency,^{72,73} and in a substantial proportion of patients with megaloblastic anemia resulting from folate deficiency (30%).⁴

Serum holoTC

The B₁₂ bound to HC comprises 70% to 90% of the total plasma B₁₂, yet is unavailable for cellular delivery. That TC is the critical plasma B₁₂ binding protein is underscored by the fact that inherited TC deficiency is associated with serious hematological and neurological sequelae and, if untreated, fatal outcome,⁷⁴ whereas HC deficiency has no morbid consequence.⁷³ Theoretically, measurement of the TC-bound fraction of the plasma B₁₂ (holoTC), also termed “active B₁₂,”⁷⁵ should be more relevant for assessing functional B₁₂ status, even though it constitutes only 10% to 30% of the total plasma B₁₂. Increasingly, holoTC measurement is being used for clinical assessment of B₁₂ status, either singly^{3,76,77} or in combination with the total serum B₁₂ with or without measurement of the metabolites MMA and Hcy.^{78–80} In addition, holoTC levels also reflect B₁₂ absorptive capacity.^{81–83}

Serum or plasma MMA and Hcy

Because they are the substrates of the 2 B₁₂-dependent reactions, elevated levels of MMA and Hcy are sensitive indicators of tissue B₁₂ deficiency. Their levels are high in >90% of B₁₂-deficient patients and increase before serum B₁₂ falls to subnormal levels.^{4,5} Even when there is no manifest evidence of clinical B₁₂ deficiency, and serum B₁₂ levels are not low, elevated levels of MMA and Hcy can be considered as sensitive biomarkers of a subclinical underlying state of B₁₂ deficiency, which may potentially progress to a state of manifest B₁₂ deficiency with its attendant clinical complications that may remain subtle, often being only neurological⁸⁴ or may become more exuberant.^{3,85,86} MMA measurements can be carried out on either plasma or serum, whereas Hcy is best measured in plasma, because cellular release of Hcy in a clotted blood sample can alter Hcy levels.^{87,88} Elevated plasma MMA and/or elevated Hcy are both indicators of B₁₂ deficiency in patients without impaired renal function or an inherited defect in cobalamin processing enzymes.^{4,13,14,89} Of the 2, MMA measurement is both more sensitive and more specific, and elevated MMA will persist for several days, even after B₁₂ treatment. MMA elevation is seen only in B₁₂ deficiency, unlike Hcy levels that also increase in folate and pyridoxine deficiencies, as well as in hypothyroidism.⁴ However, certain intestinal microbes synthesize propionate, a precursor of MMA, and when there is bacterial overgrowth in the small intestine, as occurs in blind loops following gastrointestinal surgery, microbial-derived MMA may contribute to elevations in plasma MMA.^{5,90} Although measurement of these metabolites may be used in population screening for B₁₂ deficiency, the finding of an isolated elevation of plasma MMA

should not be taken as proof of clinically attributable B₁₂ deficiency, absent of any ancillary measurements to support that diagnosis or any demonstration of a therapeutic response to the administration of B₁₂.^{90,91}

Assays of B₁₂ absorption and intrinsic factor antibodies

There is currently no approved test in routine clinical use to measure B₁₂ absorption since the Schilling test became obsolete. Lack of a validated B₁₂ absorption test hampers accurate diagnosis of pernicious anemia as the cause of B₁₂ deficiency and clinical investigations related to all causes of B₁₂ malabsorption.⁹² One possible test that shows promise, the Cobasorb test, is based on the measurement of the change in holoTC following oral administration of nonradiolabeled cobalamin.^{82,93,94} An alternative approach has been described using accelerator mass spectrometry to quantify ¹⁴C in the blood following an orally administered dose of [¹⁴C]-cyanocobalamin.⁹⁵

In absence of any test for B₁₂ absorption, definitive diagnosis of pernicious anemia is problematic and depends on the demonstration of circulating antibodies to intrinsic factor and gastric parietal cells. Antibodies to intrinsic factor can be of 2 types, varying according to the epitope on the intrinsic factor molecule to which they are directed. For diagnostic purposes, the so-called “blocking” type, directed against the B₁₂ binding site, is measured, as this type not only is highly specific for pernicious anemia but also is the species present in 70% of patients.⁹⁶ Antibodies to parietal cells, although present in 90% of patients with pernicious anemia, are less specific, as they can occur in simple atrophic gastritis and in autoimmune thyroid disease.⁹⁷

Prevention and treatment of B₁₂ deficiency

Regarding prevention of B₁₂ deficiency, the Institute of Medicine Food and Nutrition Board has defined the RDI for adults at 2.4 μg daily but with the caveat that individuals 51 years and older obtain most of this amount through consuming foods fortified with B₁₂ or in a B₁₂-containing supplement.⁸ This rider is added in consideration of the high prevalence of food B₁₂ malabsorption caused by gastric dysfunction among the elderly. Assuming that the lowest possible MMA level is consistent with optimal well-being, a large segment of the population may exist in a state of precarious B₁₂ balance, as evidenced by the fact that concentrations of serum MMA leveled off to a nadir in healthy individuals consuming 4 to 7 μg B₁₂ daily.⁹⁸ One of the possible implications of this finding is that individuals consuming less B₁₂ may have a narrow margin of safety in the event that they were to develop any condition that further compromised their state of B₁₂ repletion. Provided the physiologic intrinsic factor–cubam pathway for physiologic B₁₂ absorption is intact, a daily supplement of B₁₂ of 10 μg or more would suffice to prevent B₁₂ deficiency or to maintain adequate B₁₂ status in individuals with food B₁₂ malabsorption caused by gastric dysfunction, including atrophic gastritis or the chronic use of drugs that impair acid production, such as proton pump inhibitors.^{12,50} The defined RDI notwithstanding, it is important to recognize that individuals with pernicious anemia or any other condition that interdicts the physiological intrinsic factor cubam absorption pathway would not benefit from the additional Institute of Medicine recommendation.

It is worth noting that prospective interventional trials using Hcy-lowering vitamin supplements containing B₁₂ in subjects at high

risk through suboptimal baseline B vitamin status show a slowing of cognitive decline and of cerebral atrophy.⁹⁹ Considering that vitamin B₁₂ deficiency is the dominant modifiable cause of hyperhomocysteinemia in the post-folic acid fortification era,¹⁰⁰ it is reasonable to conclude that B₁₂ adequacy is important to maintain, and this becomes progressively more relevant with advancing age.

Concerning treatment of confirmed B₁₂ deficiency, well-defined guidelines have been enunciated,^{50,101} the details of which still apply. Some important principles need emphasizing. Where the cause of the deficiency is not known or irreversible, treatment must be lifelong. In general, the form and dosage of treatment depend first on whether the intrinsic factor-dependent pathway is intact or not. If not intact, then the choices lie between intramuscular injection of 1000 µg B₁₂ (cyanocobalamin in the United States; hydroxocobalamin in Europe) given every other day for 1 to 2 weeks followed by weekly injections for a month and then tapered to once a month indefinitely. Only ~10% of each B₁₂ dose is retained. The alternative to injected B₁₂ is high-dose oral B₁₂. Between 1% and 4% of an oral dose of B₁₂ is absorbed passively, even when the intrinsic factor-dependent pathway is abrogated.¹⁰² Consequently, oral replacement therapy with B₁₂, which was used successfully in the past,¹⁰³ has again come into vogue,¹⁰⁴ because of convenience and cost. In most instances, however, it would be prudent to “top up” a B₁₂-deficient patient through parenteral injection before switching to the oral route for maintenance, with due vigilance concerning compliance, particularly in the elderly. Because the passive route of absorption of B₁₂ applies to all mucosal surfaces, approved sublingual and intranasal formulations of B₁₂ are also available. It should be noted that patients with pernicious anemia at times report that the recommended treatment schedule is not adequate to relieve all their neurological symptoms and therefore often request, or may even treat themselves with, B₁₂ injections more frequently than the guidelines suggest. No biological basis for this apparent increased requirement for B₁₂ replacement is known, but because there are no

reports of adverse effects associated with excess B₁₂ intake, there is no reason to advise against this practice.⁸

Conclusion

Although considered an “old” disease, new information is constantly accruing about B₁₂ deficiency, the broad array of its effects, and methods for its diagnosis. B₁₂ deficiency primarily affects the hematopoietic system, but its effects extend to other tissues and organs, most notably the nervous system. The spectrum of clinical presentations is broad so that diagnosis depends first on a high index of suspicion and then on the judicious application of appropriate testing. Because B₁₂ deficiency is amenable to simple replacement therapy, diagnosis is critical. Several questions still remain unanswered concerning B₁₂ deficiency, including the possible harmful effects of high folate levels in subjects with low B₁₂ status, particularly with respect to neurological damage. Other newer areas of investigation that may provide better insights into the variability of expression of B₁₂ deficiency include genetic analysis and the effects of the microbiome.

Authorship

Contribution: R.G. wrote the manuscript.

Conflict-of-interest disclosure: The author declares no competing financial interests.

ORCID profiles: R.G., 0000-0002-9935-230X.

Correspondence: Ralph Green, Department of Pathology and Laboratory Medicine, University of California, Davis, 4400 V St, PATH Building, Sacramento, CA 95817; e-mail: rgreen@ucdavis.edu.

References

1. Wintrobe MM. Blood, pure and eloquent: a story of discovery, of people, and of ideas. New York: McGraw-Hill; 1980.
2. Collier BS. Blood at 70: its roots in the history of hematology and its birth. *Blood*. 2015;126(24):2548-2560.
3. Green R, Miller JW. Vitamin B12. In: Zemleni J, Suttie JW, Gregory JF, Stover PJ, eds. Handbook of Vitamins. 5th ed. Boca Raton, FL: Taylor & Francis; 2014:447-490.
4. Green R. Metabolite assays in cobalamin and folate deficiency. *Baillieres Clin Haematol*. 1995;8(3):533-566.
5. Lindenbaum J, Savage DG, Stabler SP, Allen RH. Diagnosis of cobalamin deficiency: II. Relative sensitivities of serum cobalamin, methylmalonic acid, and total homocysteine concentrations. *Am J Hematol*. 1990;34(2):99-107.
6. Lindenbaum J, Heaton EB, Savage DG, et al. Neuropsychiatric disorders caused by cobalamin deficiency in the absence of anemia or macrocytosis. *N Engl J Med*. 1988;318(26):1720-1728.
7. Green R, Miller JW. Folate deficiency beyond megaloblastic anemia: hyperhomocysteinemia and other manifestations of dysfunctional folate status. *Semin Hematol*. 1999;36(1):47-64.
8. Institute of Medicine. Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline. 446. Washington, DC: The National Academies Press; 2000.
9. Green R, Jacobsen DW, van Tonder SV, Kew MC, Metz J. Enterohepatic circulation of cobalamin in the nonhuman primate. *Gastroenterology*. 1981;81(4):773-776.
10. Green R, Jacobsen DW, Van Tonder SV, Kew MC, Metz J. Absorption of biliary cobalamin in baboons following total gastrectomy. *J Lab Clin Med*. 1982;100(5):771-777.
11. el Kholty S, Gueant JL, Bressler L, et al. Portal and biliary phases of enterohepatic circulation of corrinoids in humans. *Gastroenterology*. 1991;101(5):1399-1408.
12. Nielsen MJ, Rasmussen MR, Andersen CB, Nexø E, Moestrup SK. Vitamin B12 transport from food to the body's cells—a sophisticated, multistep pathway. *Nat Rev Gastroenterol Hepatol*. 2012;9(6):345-354.
13. Watkins D, Rosenblatt DS. Inborn errors of cobalamin absorption and metabolism. *Am J Med Genet C Semin Med Genet*. 2011;157C(1):33-44.
14. Froese DS, Gravel RA. Genetic disorders of vitamin B₁₂ metabolism: eight complementation groups—eight genes. *Expert Rev Mol Med*. 2010;12:e37.
15. Fyfe JC, Madsen M, Højrup P, et al. The functional cobalamin (vitamin B12)-intrinsic factor receptor is a novel complex of cubilin and amnionless. *Blood*. 2004;103(5):1573-1579.
16. Steinberg SE, Fonda S, Campbell CL, Hillman RS. Cellular abnormalities of folate deficiency. *Br J Haematol*. 1983;54(4):605-612.
17. Rondanelli EG, Gorini P, Magliulo E, Fiori GP. Differences in proliferative activity between normoblasts and pernicious anemia megaloblasts. *Blood*. 1964;24:542-552.
18. Wickremasinghe RG, Hoffbrand AV. Reduced rate of DNA replication fork movement in megaloblastic anemia. *J Clin Invest*. 1980;65(1):26-36.
19. Duthie SJ, McMillan P. Uracil misincorporation in human DNA detected using single cell gel electrophoresis. *Carcinogenesis*. 1997;18(9):1709-1714.
20. Koury MJ, Horne DW, Brown ZA, et al. Apoptosis of late-stage erythroblasts in megaloblastic anemia: association with DNA damage and macrocyte production. *Blood*. 1997;89(12):4617-4623.
21. Das KC, Mohanty D, Garewal G. Cytogenetics in nutritional megaloblastic anaemia: prolonged persistence of chromosomal abnormalities in lymphocytes after remission. *Acta Haematol*. 1986;76(2-3):146-154.
22. Amess JA, Burman JF, Rees GM, Nancekivill DG, Mollin DL. Megaloblastic haemopoiesis in patients receiving nitrous oxide. *Lancet*. 1978;2(8085):339-342.
23. O'Leary PW, Combs MJ, Schilling RF. Synergistic deleterious effects of nitrous oxide exposure and vitamin B12 deficiency. *J Lab Clin Med*. 1985;105(4):428-431.
24. Marsh GW, Stewart JS. Splenic function in adult coeliac disease. *Br J Haematol*. 1970;19(4):445-457.

25. Lindenbaum J, Nath BJ. Megaloblastic anaemia and neutrophil hypersegmentation. *Br J Haematol.* 1980;44(3):511-513.
26. Carmel R, Green R, Jacobsen DW, Qian GD. Neutrophil nuclear segmentation in mild cobalamin deficiency: relation to metabolic tests of cobalamin status and observations on ethnic differences in neutrophil segmentation. *Am J Clin Pathol.* 1996;106(1):57-63.
27. Green R. Anemias beyond B12 and iron deficiency: the buzz about other B's, elementary, and nonelementary problems. *Hematology Am Soc Hematol Educ Program.* 2012;2012:492-498.
28. Dalsania CJ, Khemka V, Shum M, Devereux L, Lachant NA. A sheep in wolf's clothing. *Am J Med.* 2008;121(2):107-109.
29. Harker LA, Finch CA. Thrombokinetics in man. *J Clin Invest.* 1969;48(6):963-974.
30. Obeid R, Geisel J, Schorr H, Hübner U, Herrmann W. The impact of vegetarianism on some haematological parameters. *Eur J Haematol.* 2002;69(5-6):275-279.
31. Lawler SD, Roberts PD, Hoffbrand AV. Chromosome studies in megaloblastic anaemia before and after treatment. *Scand J Haematol.* 1971;8(4):309-320.
32. Tanaka T, Scheet P, Giusti B, et al. Genome-wide association study of vitamin B6, vitamin B12, folate, and homocysteine blood concentrations. *Am J Hum Genet.* 2009;84(4):477-482.
33. Miller JW, Ramos MI, Garrod MG, Flynn MA, Green R. Transcobalamin II 775G>C polymorphism and indices of vitamin B12 status in healthy older adults. *Blood.* 2002;100(2):718-720.
34. Allen RH, Stabler SP. Identification and quantitation of cobalamin and cobalamin analogues in human feces. *Am J Clin Nutr.* 2008;87(5):1324-1335.
35. Kolhouse JF, Kondo H, Allen NC, Podell E, Allen RH. Cobalamin analogues are present in human plasma and can mask cobalamin deficiency because current radioisotope dilution assays are not specific for true cobalamin. *N Engl J Med.* 1978;299(15):785-792.
36. Kanazawa S, Herbert V. Noncobalamin vitamin B12 analogues in human red cells, liver, and brain. *Am J Clin Nutr.* 1983;37(5):774-777.
37. Marshall AC, Alderuccio F, Toh BH. Fas/CD95 is required for gastric mucosal damage in autoimmune gastritis. *Gastroenterology.* 2002;123(3):780-789.
38. Stopeck A. Links between *Helicobacter pylori* infection, cobalamin deficiency, and pernicious anemia. *Arch Intern Med.* 2000;160(9):1229-1230.
39. Hershko C, Ronson A, Souroujon M, Maschler I, Heyd J, Patz J. Variable hematologic presentation of autoimmune gastritis: age-related progression from iron deficiency to cobalamin depletion. *Blood.* 2006;107(4):1673-1679.
40. Green R, Kuhl W, Jacobson R, Johnson C, Carmel R, Beutler E. Masking of macrocytosis by alpha-thalassemia in blacks with pernicious anemia. *N Engl J Med.* 1982;307(21):1322-1325.
41. Spivak JL. Masked megaloblastic anemia. *Arch Intern Med.* 1982;142(12):2111-2114.
42. Herbert V, Zalusky R. Interrelations of vitamin B12 and folic acid metabolism: folic acid clearance studies. *J Clin Invest.* 1962;41:1263-1276.
43. Reynolds EH. What is the safe upper intake level of folic acid for the nervous system? Implications for folic acid fortification policies. *Eur J Clin Nutr.* 2016;70(5):537-540.
44. Heaton EB, Savage DG, Brust JC, Garrett TJ, Lindenbaum J. Neurologic aspects of cobalamin deficiency. *Medicine (Baltimore).* 1991;70(4):229-245.
45. Waters AH, Mollin DL. Observations on the metabolism of folic acid in pernicious anaemia. *Br J Haematol.* 1963;9:319-327.
46. Morris MS, Jacques PF, Rosenberg IH, Selhub J. Folate and vitamin B-12 status in relation to anemia, macrocytosis, and cognitive impairment in older Americans in the age of folic acid fortification. *Am J Clin Nutr.* 2007;85(1):193-200.
47. Selhub J, Morris MS, Jacques PF. In vitamin B12 deficiency, higher serum folate is associated with increased total homocysteine and methylmalonic acid concentrations. *Proc Natl Acad Sci USA.* 2007;104(50):19995-20000.
48. Miller JW, Garrod MG, Allen LH, Haan MN, Green R. Metabolic evidence of vitamin B-12 deficiency, including high homocysteine and methylmalonic acid and low holotranscobalamin, is more pronounced in older adults with elevated plasma folate. *Am J Clin Nutr.* 2009;90(6):1586-1592.
49. Mills JL, Von Kohorn I, Conley MR, et al. Low vitamin B-12 concentrations in patients without anemia: the effect of folic acid fortification of grain. *Am J Clin Nutr.* 2003;77(6):1474-1477.
50. Stabler SP. Clinical practice. Vitamin B12 deficiency. *N Engl J Med.* 2013;368(2):149-160.
51. Allen LH. Causes of vitamin B12 and folate deficiency. *Food Nutr Bull.* 2008;29(suppl 2):S20-34; discussion S35-27.
52. Carmel R, Aurangzeb I, Qian D. Associations of food-cobalamin malabsorption with ethnic origin, age, *Helicobacter pylori* infection, and serum markers of gastritis. *Am J Gastroenterol.* 2001;96(1):63-70.
53. Toskes PP, Deren JJ, Conrad ME. Trypsin-like nature of the pancreatic factor that corrects vitamin B12 malabsorption associated with pancreatic dysfunction. *J Clin Invest.* 1973;52(7):1660-1664.
54. Allen RH, Seetharam B, Podell E, Alpers DH. Effect of proteolytic enzymes on the binding of cobalamin to R protein and intrinsic factor. In vitro evidence that a failure to partially degrade R protein is responsible for cobalamin malabsorption in pancreatic insufficiency. *J Clin Invest.* 1978;61(1):47-54.
55. Nyberg W. The influence of diphyllobothrium latum on the vitamin B12-intrinsic factor complex. I. In vivo studies with Schilling test technique. *Acta Med Scand.* 1960;167:185-187.
56. Ballas SK. Abnormal erythrocyte membrane protein pattern in severe megaloblastic anemia. *J Clin Invest.* 1978;61(4):1097-1101.
57. Ballas SK, Pielichowski HJ, Stoll DB. Further characterization of the erythrocyte membrane protein abnormality in megaloblastic anemia. *J Med.* 1982;13(1-2):15-34.
58. Schilling RF, Harris JW. Liver function in untreated Addisonian pernicious anemia. *J Lab Clin Med.* 1952;40(5):718-725.
59. Emerson PM, Wilkinson JH. Lactate dehydrogenase in the diagnosis and assessment of response to treatment of megaloblastic anaemia. *Br J Haematol.* 1966;12(6):678-688.
60. Winston RM, Warburton FG, Stott A. Enzymatic diagnosis of megaloblastic anaemia. *Br J Haematol.* 1970;19(5):587-592.
61. Heller P, Weinstein HG, West M, Zimmerman HJ. Enzymes in anemia: a study of abnormalities of several enzymes of carbohydrate metabolism in the plasma and erythrocytes in patients with anemia, with preliminary observations of boen marrow enzymes. *Ann Intern Med.* 1960;53:898-913.
62. de Klerk G, Rosengarten PC, Vet RJ, Goudsmit R. Serum erythropoietin (EST) titers in anemia. *Blood.* 1981;58(6):1164-1170.
63. Hussein S, Laulicht M, Hoffbrand AV. Serum ferritin in megaloblastic anaemia. *Scand J Haematol.* 1978;20(3):241-245.
64. Carmel R, Skikne BS. Serum transferrin receptor in the megaloblastic anemia of cobalamin deficiency. *Eur J Haematol.* 1992;49(5):246-250.
65. Hansen NE, Karle H. Blood and bone-marrow lysozyme in neutropenia: an attempt towards pathogenetic classification. *Br J Haematol.* 1971;21(3):261-270.
66. Corcino JJ, Zalusky R, Greenberg M, Herbert V. Coexistence of pernicious anaemia and chronic myeloid leukaemia: an experiment of nature involving vitamin B12 metabolism. *Br J Haematol.* 1971;20(5):511-520.
67. Stabler SP, Allen RH, Savage DG, Lindenbaum J. Clinical spectrum and diagnosis of cobalamin deficiency. *Blood.* 1990;76(5):871-881.
68. Skacel PO, Hewlett AM, Lewis JD, Lumb M, Nunn JF, Chanarin I. Studies on the haemopoietic toxicity of nitrous oxide in man. *Br J Haematol.* 1983;53(2):189-200.
69. Hannibal L, Lysne V, Bjørke-Monsen AL, et al. Biomarkers and algorithms for the diagnosis of vitamin B12 deficiency. *Front Mol Biosci.* 2016;3:27.
70. Pawlak R, Lester SE, Babatunde T. The prevalence of cobalamin deficiency among vegetarians assessed by serum vitamin B12: a review of literature. *Eur J Clin Nutr.* 2014;68(5):541-548.
71. Herbert V, Jacob E, Wong KT, Scott J, Pfeffer RD. Low serum vitamin B12 levels in patients receiving ascorbic acid in megadoses: studies concerning the effect of ascorbate on radioisotope vitamin B12 assay. *Am J Clin Nutr.* 1978;31(2):253-258.
72. Carmel R. R-binder deficiency. A clinically benign cause of cobalamin pseudodeficiency. *JAMA.* 1983;250(14):1886-1890.
73. Carmel R. Mild transcobalamin I (haptocorrin) deficiency and low serum cobalamin concentrations. *Clin Chem.* 2003;49(8):1367-1374.
74. Cooper BA, Rosenblatt DS. Inherited defects of vitamin B12 metabolism. *Annu Rev Nutr.* 1987;7:291-320.
75. Clarke R, Sherlikar P, Hin H, et al. Detection of vitamin B12 deficiency in older people by measuring vitamin B12 or the active fraction of vitamin B12, holotranscobalamin. *Clin Chem.* 2007;53(5):963-970.
76. Nexø E, Hvas AM, Bleie Ø, et al. Holotranscobalamin is an early marker of changes in cobalamin homeostasis. A randomized placebo-controlled study. *Clin Chem.* 2002;48(10):1768-1771.
77. Obeid R, Herrmann W. Holotranscobalamin in laboratory diagnosis of cobalamin deficiency compared to total cobalamin and methylmalonic acid. *Clin Chem Lab Med.* 2007;45(12):1746-1750.
78. Miller JW, Garrod MG, Rockwood AL, et al. Measurement of total vitamin B12 and holotranscobalamin, singly and in combination, in screening for metabolic vitamin B12 deficiency. *Clin Chem.* 2006;52(2):278-285.
79. Brito AVR, Hertrampf E, Miller JW, et al. Vitamin B-12 treatment of asymptomatic deficient elderly Chileans improves conductivity in myelinated peripheral nerves, but high serum folate impairs B-12 status response assessed by the combined indicator of vitamin B-12 status. *Am J Clin Nutr.* 2016;103(1):250-257.

80. Fedosov SN, Brito A, Miller JW, Green R, Allen LH. Combined indicator of vitamin B12 status: modification for missing biomarkers and folate status and recommendations for revised cut-points. *Clin Chem Lab Med*. 2015;53(8):1215-1225.
81. Lindgren A, Kilander A, Bagge E, Nexø E. Holotranscobalamin—a sensitive marker of cobalamin malabsorption. *Eur J Clin Invest*. 1999;29(4):321-329.
82. von Castel-Roberts KM, Morkbak AL, Nexø E, et al. Holo-transcobalamin is an indicator of vitamin B-12 absorption in healthy adults with adequate vitamin B-12 status. *Am J Clin Nutr*. 2007;85(4):1057-1061.
83. Bor MV, Nexø E, Hvas AM. Holo-transcobalamin concentration and transcobalamin saturation reflect recent vitamin B12 absorption better than does serum vitamin B12. *Clin Chem*. 2004;50(6):1043-1049.
84. Green R. Typical and atypical manifestations of pernicious anemia. In: Besser M, Bhatt R, James VHT, Keen H, eds. *Thomas Addison and His Diseases: 200 Years On*. Vol 1. London: Bristol: Journal of Endocrinology; 1994:377-390.
85. Carmel R. Biomarkers of cobalamin (vitamin B-12) status in the epidemiologic setting: a critical overview of context, applications, and performance characteristics of cobalamin, methylmalonic acid, and holotranscobalamin II. *Am J Clin Nutr*. 2011;94(1):348S-358S.
86. Smith AD, Refsum H. Do we need to reconsider the desirable blood level of vitamin B12? *J Intern Med*. 2012;271(2):179-182.
87. Jacobsen DW, Gatautis VJ, Green R, et al. Rapid HPLC determination of total homocysteine and other thiols in serum and plasma: sex differences and correlation with cobalamin and folate concentrations in healthy subjects. *Clin Chem*. 1994;40(6):873-881.
88. Refsum H, Smith AD, Ueland PM, et al. Facts and recommendations about total homocysteine determinations: an expert opinion. *Clin Chem*. 2004;50(1):3-32.
89. Carmel R, Green R, Rosenblatt D, Watkins D. Update on cobalamin, folate, and homocysteine. *Hematology Am Soc Hematol Educ Program*; 2003:62-81.
90. Green R. Screening for vitamin B12 deficiency: caveat emptor. *Ann Intern Med*. 1996;124(5):509-511.
91. Solomon LR. Cobalamin-responsive disorders in the ambulatory care setting: unreliability of cobalamin, methylmalonic acid, and homocysteine testing. *Blood*. 2005;105(3):978-985, author reply 1137.
92. Carmel R. The disappearance of cobalamin absorption testing: a critical diagnostic loss. *J Nutr*. 2007;137(11):2481-2484.
93. Morkbak AL, Heimdal RM, Emmens K, et al. Evaluation of the technical performance of novel holotranscobalamin (holoTC) assays in a multicenter European demonstration project. *Clin Chem Lab Med*. 2005;43(10):1058-1064.
94. Greibe E, Nexø E. Vitamin B12 absorption judged by measurement of holotranscobalamin, active vitamin B12: evaluation of a commercially available EIA kit. *Clin Chem Lab Med*. 2011;49(11):1883-1885.
95. Carkeet C, Dueker SR, Lango J, et al. Human vitamin B12 absorption measurement by accelerator mass spectrometry using specifically labeled (14)C-cobalamin [published correction appears in *Proc Natl Acad Sci U S A*. 2006;103(27):10526]. *Proc Natl Acad Sci USA*. 2006;103(15):5694-5699.
96. Kapadia CR, Donaldson RM Jr. Disorders of cobalamin (vitamin B12) absorption and transport. *Annu Rev Med*. 1985;36:93-110.
97. Irvine WJ, Davies SH, Teitelbaum S, Delamore IW, Williams AW. The clinical and pathological significance of gastric parietal cell antibody. *Ann N Y Acad Sci*. 1965;124(2):657-691.
98. Bor MV, von Castel-Roberts KM, Kauwell GP, et al. Daily intake of 4 to 7 microg dietary vitamin B-12 is associated with steady concentrations of vitamin B-12-related biomarkers in a healthy young population. *Am J Clin Nutr*. 2010;91(3):571-577.
99. Smith AD, Refsum H. Homocysteine, B vitamins, and cognitive impairment. *Annu Rev Nutr*. 2016;36:211-239.
100. Green R, Miller JW. Vitamin B12 deficiency is the dominant nutritional cause of hyperhomocysteinemia in a folic acid-fortified population. *Clin Chem Lab Med*. 2005;43(10):1048-1051.
101. Carmel R. How I treat cobalamin (vitamin B12) deficiency. *Blood*. 2008;112(6):2214-2221.
102. Berlin H, Berlin R, Brante G. Oral treatment of pernicious anemia with high doses of vitamin B12 without intrinsic factor. *Acta Med Scand*. 1968;184(4):247-258.
103. Ungley CC. Absorption of vitamin B12 in pernicious anaemia. I. Oral administration without a source of intrinsic factor. *BMJ*. 1950;2(4685):905-908.
104. Kuzminski AM, Del Giacco EJ, Allen RH, Stabler SP, Lindenbaum J. Effective treatment of cobalamin deficiency with oral cobalamin. *Blood*. 1998;92(4):1191-1198.