Title

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Journal

Journal of Nutrition, 148(suppl_4)

ISSN

0022-3166

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Publication Date

2018-12-01

DOI

10.1093/jn/nxy201

Peer reviewed

The Journal of Nutrition
Supplement: Biomarkers of Nutrition for Development (BOND) Expert Panel Reviews, Part 6

Biomarkers of Nutrition for Development (BOND): Vitamin B-12 Review

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Abstract

This report on vitamin B-12 (B12) is part of the Biomarkers of Nutrition for Development (BOND) Project, which provides state-of-the art information and advice on the selection, use, and interpretation of biomarkers of nutrient exposure, status, and function. As with the other 5 reports in this series, which focused on iodine, folate, zinc, iron, and vitamin A, this B12 report was developed with the assistance of an expert panel (BOND B12 EP) and other experts who provided information during a consultation. The experts reviewed the existing literature in depth in order to consolidate existing relevant information on the biology of B12, including known and possible effects of insufficiency, and available and potential biomarkers of status. Unlike the situation for the other 5 nutrients reviewed during the BOND project, there has been relatively little previous attention paid to B12 status and its biomarkers, so this report is a landmark in terms of the consolidation and interpretation of the available information on B12 nutrition. Historically, most focus has been on diagnosis and treatment of clinical symptoms of B12 deficiency, which result primarily from pernicious anemia or strict vegetarianism. More recently, we have become aware of the high prevalence of B12 insufficiency in populations consuming low amounts of animal-source foods, which can be detected with ≥1 serum biomarker but presents the new challenge of identifying functional consequences that may require public health interventions. *J Nutr* 2018;148:1995S–2027S.

Keywords: BOND, vitamin B-12, B-12 biomarkers, serum B-12, cobalamin, transcobalamin, homocysteine

Background

Vitamin B-12 (B12, cobalamin) is a dietary essential nutrient for humans. It is the generic term for all corrinoids (i.e., compounds containing the corrin nucleus) exhibiting the qualitative biological activity of cyanocobalamin. Cyanocobalamin is the common name of the B12-active corrinoid (also called cobalamin) with a cyanide ion (CN–) at the β-position of the cobalt atom.

Historically, cobalamin deficiency was recognized and studied in the clinical setting, when patients presented with symptoms caused by pernicious anemia [an autoimmune condition in which the parietal cells in the stomach are attacked, resulting in lack of intrinsic factor (IF) necessary for effective B12 absorption], malabsorption, or strict vegetarianism. However, it

The Biomarkers of Nutrition for Development (BOND) project was supported in part by the Bill and Melinda Gates Foundation, PepsiCo, the Office of Dietary Supplements (ODS), NIH, the former Division of Nutrition Research Coordination (DNRC), NIH, and the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD), NIH.
The vitamin B-12 review was written in response to an invitation from NICHD, NIH within the US Department of Health and Human Services (DHHS). The content represents the views of the Vitamin B-12 Expert Panel (EP) and other invited contributors and does not necessarily reflect the opinions of the NICHD, the NIH, or the DHHS. In addition, individual members of the EP may not endorse all statements in this report. The original EP consisted of JWM, LdG, IHR, ADS, HR, and DJR, led by LHA as chair. The BOND project thanks the European Recommendations Aligned (EURRECA) program, the Micronutrient Genomics Project (MGP), the WHO, and the CDC for their partnership.

Author disclosures: LHA, JWM, LdG, IHR, and DJR, no conflicts of interest. ADS consults for E. Merck and Nestlé Health Science; HR consults for E. Merck. HR and ADS are named as inventors on patent US9364497B2 and on a patent application pending PCT/GB2015/050786. ADS is named as inventor on patents US6008221 and US6127370.
Address correspondence to DJR (e-mail: raitend@mail.nih.gov).
Abbreviations used: AI, Adequate Intake; BOND, Biomarkers of Nutrition for Development; B12, vitamin B-12; CBS, cystathionine-synthase; cB12, combined marker of vitamin B-12 status; CVD, cardiovascular disease; EAR, Estimated Average Requirement; EURRECA, EURopean micronutrient RECommendations Aligned; holoTC, holotranscobalamin; IF, intrinsic factor; IOM, Institute of Medicine; LMIC, low- and middle-income country; LNS, lipid-based nutrient supplement; MCV, mean corpuscular volume; MMA, methylmalonic acid; NCD, noncommunicable disease; NTD, neural tube defect; PPI, proton pump inhibitor; SAM, *S*-adenosylmethionine; SCCD, subclinical cobalamin deficiency; SRM, standard reference material; tHcy, total homocysteine; UL, Tolerable Upper Intake Level.

Manuscript received May 15, 2017. Initial review completed July 6, 2017. Revision accepted August 2, 2018.
First published online 0, 2018; doi: https://doi.org/10.1093/jn/nxy201.

is now recognized that there is a high prevalence of deficiency in many population groups, especially those with lower incomes. Worldwide, the current challenges are to improve the definition of deficiency and to identify the functional consequences of subclinical cobalamin deficiency (SCCD), which is far more prevalent than clinically evident severe deficiency.

To address these challenges, dependable biomarkers of status and tools for measuring exposure, function, and the effects of interventions are needed. In addition, valid approaches to interpret and deploy these measurements are needed that can be utilized in various settings. This review covers the relevant aspects of cobalamin biology, exposure, health impact, and assessment, including the value and technical aspects of current and new approaches to assessment.

Historical Overview

The discovery of B12 required skills from multiple disciplines, including clinical medicine, chemistry, nutrition, crystallography, microbiology, and pharmacy, and resulted in several Nobel Prize awards. This interesting story has been described by Chanarin (1) among others. **Text Box 1** outlines a brief history of B12.

Text Box 1 A brief history of B12

- The initial discovery resulted from the need to find a cause and treatment of pernicious anemia, first described by Thomas Addison in 1849 (2).
- A cure was discovered in 1926 when consumption of lightly cooked liver resulted in correction of anemia and prevention of death, although at the time it was believed that proteins and iron in the liver were the curative factors (3).
- In the late 1940s, 2 groups announced the discovery of a new vitamin, purified and crystallized from liver, that induced and maintained remission of pernicious anemia (4).
- The structure of the vitamin was solved in 1956 by Dorothy Hodgkin, an X-ray crystallographer (5).
- B12 was synthesized in 1973 by Robert Woodward (6).
- William Castle summarized his research in 1929 showing that the underlying cause of pernicious anemia involved lack of a gastric IF; gastric juice from healthy individuals substantially reduced the amount of liver needed to treat pernicious anemia (7).
- SCCD characterized by B12 depletion emerged as a significant public health problem as evidenced by biochemical indications of deficiency rather than anemia or clinical symptoms such as neuropathy (8).
- The prevalence of SCCD has generated interest in the development, application, and testing of biomarkers of B12 status during the past 20–30 y.
- Although serum cobalamin concentration was and is still used as the main status indicator, it is becoming more common to utilize 2–4 biomarkers simultaneously, or 2–4 biomarkers combined, especially in population status surveys and research (9).
- The highest prevalence of abnormal B12 status biomarkers is found in:
  - individuals and populations that have a low consumption of animal source foods
  - the elderly
- The current challenge is to interpret the biomarker values, apply valid cutoffs, and use the information correctly as a guide to appropriate interventions.

Chemistry and Biology of B12

B12 and B12 analogs

Cobalamins are members of a family of cobalt-containing compounds found in nature and called corrinoids. In addition to cobalamins, the corrinoid family includes cobamides and cobinamides, which are chemically modified analogs of the cobalamins. The general cobalamin molecule is a complex, organometallic compound that consists of *1*) a planar corrin ring with a cobalt atom coordinated in the center, and *2*) a ribose-3-phosphate-dimethylbenzimidazole structure that extends below the plane of the corrin ring. Covalently bound to the cobalt atom and extending above the corrin ring is a variable ligand group that distinguishes the various supplemental and active forms of the vitamin. Possible ligands include cyano and hydroxyl, which constitute forms of B12 commonly used in dietary supplements (cyanocobalamin and hydroxocobalamin), and methyl and 5′-deoxyadenosyl, which constitute active cofactor forms of the B12 in cells (methylcobalamin and 5′-deoxyadenosylcobalamin). B12 analogs are found in circulation (10) and in feces (11).

The sources of B12 analogs may be diet or gut bacteria. However, it is unclear how B12 analogs are absorbed at the intestinal level, or whether they have biological activity or inhibit B12-dependent reactions.

B12 absorption and intestinal transport

Cobalamins are absorbed by an active physiologic process and by passive diffusion. In contrast to the physiologic absorptive process, B12 absorption by passive diffusion occurs throughout the absorptive surface of the gastrointestinal tract. Only ~1–2% of an oral dose is absorbed passively, and thus this represents only a small fraction of overall B12 absorption, except when B12 is consumed in supraphysiologic supplemental doses (>50 μg) (1). Passive absorption of B12 can also occur in mucous membranes, including those in the mouth and the nose (12).

In the receptor-mediated active process (**Figure 1**), B12 absorption from first ingestion by mouth to appearance in the circulation takes 3–4 h to complete. The essentials of B12 absorption are outlined in **Text Box 2**.

Text Box 2 Essentials of B12 absorption

B12 in the stomach

- Dietary B12 is released from protein in the stomach by the action of gastric enzymes facilitated by the low pH of the stomach.
  - Low gastric pH is maintained by the production of hydrochloric acid from the gastric parietal cells.
  - The parietal cells also produce IF.
- After release from food, B12 is bound to salivary R-binder (haptocorrin).
- The R-B12 complex and IF then travel to the upper small intestine, where the higher pH causes release of B12 from haptocorrin, and haptocorrin is then digested by pancreatic trypsin.

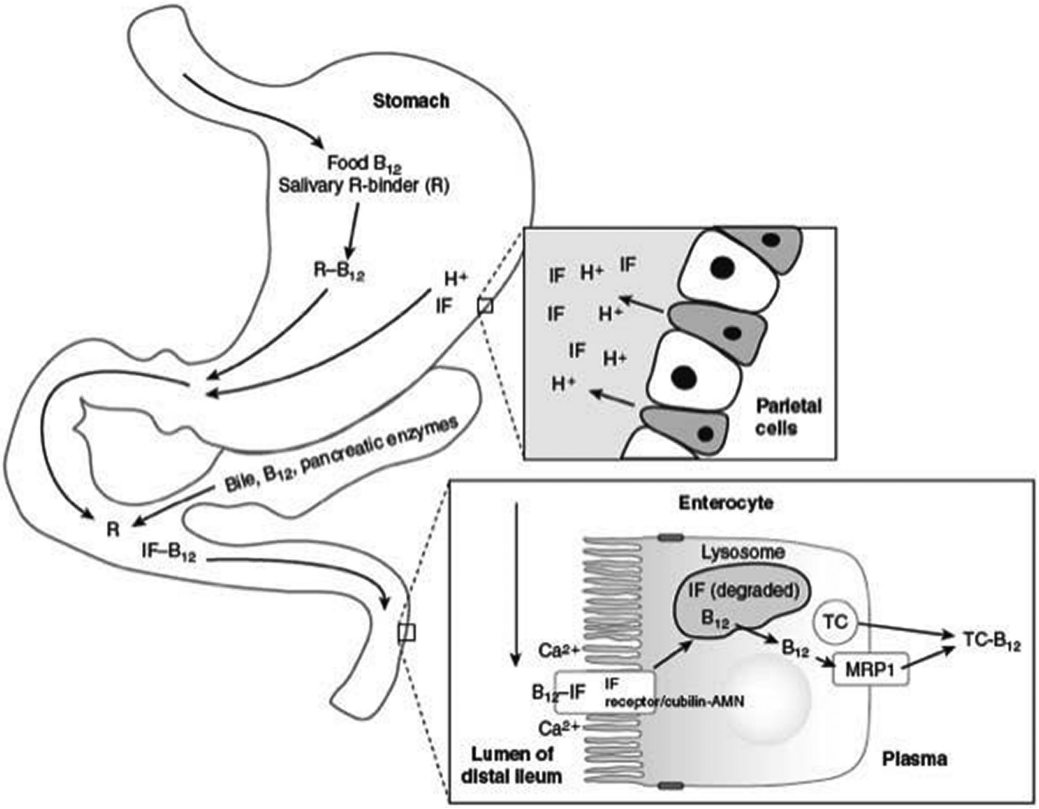

FIGURE 1 Digestion and absorption of dietary cobalamins. In the receptor-mediated active process for B12 absorption, dietary B12 is released in the stomach and bound to salivary R-binder. The R-B12 complex and IF are transported to the ileum where B12 is released from the R-binder and binds to IF. The IF-B12 complex enters the ileal enterocyte, IF is degraded, and the B12 is released through MRP1 to plasma, where it binds with and is transported on TC. More details are provided in Text Box 2. Reproduced with permission from reference 14. AMN, amnionless; B12, vitamin B-12; IF, intrinsic factor; MRP1, multidrug resistance protein 1; TC, transcobalamin.

- The binding of B12 to IF occurs in the more favorable, less acidic conditions in the small intestine.

B12 absorption

- The primary site of intestinal absorption of B12 is the terminal ileum.
- Absorption occurs via receptor-mediated calcium-dependent endocytosis of the IF-B12 complex.
- The IF-B12 receptor consists of 2 components:
  - cubilin
  - receptor-associated protein
- Genetic defects in cubilin/receptor-associated protein–mediated B12 absorption underlie autosomal recessive megaloblastic anemia, also known as Imerslund-Grasbeck disease.

B12 in the enterocyte

- Upon internalization within the ileal enterocyte, the IF-B12 complex enters a lysosome where IF is degraded and the B12 is released.
- The B12 is either metabolized to its active cofactor forms for use within the enterocyte or is processed for release into the portal circulation.
- B12 exits unbound through the ABC drug transport protein, ABCC1 (also known as multidrug resistance protein 1), and then binds with unsaturated transcobalamin in the circulation (13).

An important aspect of ileal B12 absorption is that the number of IF-B12 receptors is limited on the apical membrane of the enterocyte. This restricts the capacity for physiologic absorption of B12 and explains why although 50% of a 1-μg oral dose of B12 is absorbed by the physiologic absorptive process, the percentage of the dose absorbed decreases with increasing amount of B12 consumed (1, 14). In addition, after receptor-mediated absorption of B12, there is a refractory period of ~6 h during which further uptake of IF-B12 is restricted while apical surface receptor density is regenerated (1).

Enterohepatic circulation

Another important component of B12 absorption is enterohepatic circulation. It is estimated that between 0.5 and 5.0 μg of B12 is excreted in bile per day. This biliary B12 is readily reabsorbed across the ileal enterocyte and, thus, enterohepatic circulation represents a mechanism by which B12 is conserved by the body. The efficiency of biliary B12 absorption is such that B12 depletion and deficiency may take years after the onset of dietary B12 deficit. However, in conditions that cause B12 malabsorption (such as pernicious anemia), B12 depletion and deficiency can be very rapid (<1 y) (15–17). Further details are provided in the section on bioavailability.

Plasma transport

B12 transport in the plasma is accomplished by 2 proteins, haptocorrin and transcobalamin.

Haptocorrin. The characteristics of haptocorrin are listed in **Text Box 3**. Plasma haptocorrin is typically ~80–90% saturated with B12 and carries between 70% and 80% of total circulating B12. However, haptocorrin is not primarily responsible for delivering B12 to extrahepatic tissues because there are no haptocorrin receptors located on most cells. Instead, haptocorrin-B12 is taken up by asialoglycoprotein receptors in the liver.

Text Box 3 Characteristics of haptocorrin

- Haptocorrin forms are found in various bodily fluids, including:
  - plasma
  - breast milk
  - gastric juice
  - bile
  - saliva (also known as R-binder)
- The haptocorrins are glycoproteins that differ in carbohydrate modifications to their structures.
- Historically, it was believed that there were 2 primary plasma haptocorrin proteins, known as transcobalamin I and transcobalamin III. Today, transcobalamins I and III are referred to collectively as haptocorrin.
- Haptocorrins bind both B12 and B12 analogs.
- Haptocorrin may also sequester B12 from bacteria and thus may have an antimicrobial function.

Because haptocorrin binds both B12 and B12 analogs, asialoglycoprotein receptor–mediated uptake of haptocorrin into liver may be a mechanism by which B12 analogs are removed from the circulation and excreted in the bile. IF is highly specific for B12 and does not bind B12 analogs. Thus, B12 analogs are not reabsorbed by the IF-receptor–mediated process and are excreted in the stool. However, the original source of B12 analogs in the circulation is likely diet or gut microbiota, which suggests they are absorbed by a currently unknown absorptive process (11).

Transcobalamin. The transport protein responsible for delivery of B12 to all tissues is transcobalamin, previously known as transcobalamin II. Transcobalamin is typically ~10–20% saturated and carries only 20–30% of circulating B12. B12 absorbed in the ileal enterocyte by the IF-dependent mechanism appears in the portal venous blood bound to transcobalamin to form holotranscobalamin (holoTC). The amount of increase in serum holoTC concentrations after oral ingestion of B12 may be indicative of the absorptive capacity for the vitamin. Genetic deficiency of transcobalamin is associated with severe B12 deficiency (18). In contrast, genetic deficiency of haptocorrin appears to have little or no effect on functional B12 status (19, 20).

Uptake of the transcobalamin-B12 complex into tissues occurs by receptor-mediated endocytosis via the transcobalamin receptor (TcblR/CD320) (21). HoloTC then enters acidic lysosomes in which the transcobalamin protein is degraded and the B12 is released (**Figure 2**). The B12 is then converted to its cofactor forms.

Metabolic functions of B12 cofactors

Adenosyl-B12 serves as a cofactor for mitochondrial methylmalonyl-CoA mutase, and methyl-B12 serves as a cofactor for cytosolic methionine synthase (see Figure 2). The mitochondrial enzyme is important for the metabolism of odd-chain fatty acids and ketogenic amino acids, whereas the cytosolic enzyme is important for the remethylation of homocysteine (a putative vascular and neurotoxin), synthesis of methionine for protein synthesis and *S*-adenosylmethionine (SAM) for methylation capacity, and maintaining DNA synthesis (20). **Text Box 4** highlights the respective roles of these 2 cofactors.

Text Box 4 Metabolic roles of B12 cofactors (11)

5′-Deoxyadenosylcobalamin

- Serves as a cofactor for the mitochondrial enzyme, methylmalonyl CoA mutase.
- Methylmalonyl CoA mutase catalyzes the conversion of methylmalonyl CoA to succinyl CoA, an intermediate step in the conversion of propionate to succinate.
- This conversion is an important step in the oxidation of odd-chain fatty acids and in the catabolism of ketogenic amino acids.

5-Methylcobalamin

- Serves as a cofactor in folate-dependent conversion of homocysteine to methionine, catalyzed by the enzyme methionine synthase.
- Methionine is required for incorporation into proteins and for the synthesis of the universal methyl donor, SAM.
- The methionine synthase reaction also converts methyltetrahydrofolate to tetrahydrofolate:
  - Tetrahydrofolate is subsequently converted to methylenetetrahydrofolate after condensation with formate or by a one-carbon transfer during conversion of serine to glycine.
  - Methylenetetrahydrofolate can be reduced again to form methyltetrahydrofolate, or can serve as the one-carbon source for the de novo synthesis of thymidylate from deoxyuridylate, required for DNA replication.

Homeostatic controls and nutrient-nutrient interactions

By virtue of its fundamental cofactor role in the conversion of homocysteine to methionine and the de novo synthesis of thymidylate, B12 works in concert with several other nutrients, most prominently folate, but also vitamin B-6 (as pyridoxal-5′-phosphate), riboflavin (as FAD), and choline. These nutrient interrelations and homeostatic controls of one-carbon metabolism are described extensively in the Biomarkers of Nutrition for Development (BOND) review of folate (22). Additional levels of homeostatic control of homocysteine and one-carbon metabolism not discussed extensively in the folate review include allosteric regulation by SAM, a critical component of amino acid metabolism whose functions are highlighted in **Text Box 5**.

Text Box 5 Characteristics of SAM

- SAM is an allosteric inhibitor of methylenetetrahyrofolate reductase (MTHFR), the enzyme that catalyzes

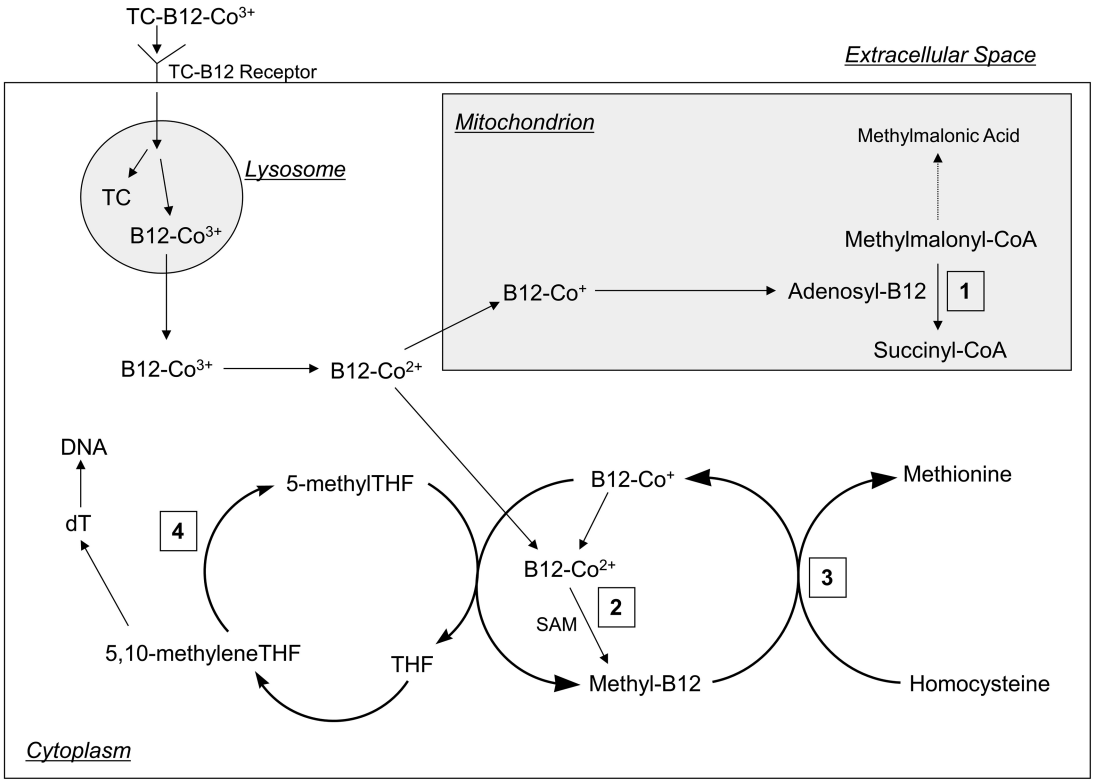

FIGURE 2 B12 cellular uptake and metabolism. In mitochondria, B12 as 5-deoxyadenosylcobalamin is a cofactor for methylmalonyl-CoA mutase, which catalyzes conversion of methylmalonyl CoA to succinyl CoA. In cytoplasm as 5-methylcobalamin, it is a cofactor for methionine synthase, which catalyzes conversion of homocysteine to methionine. Enzymes: 1, methylmalonyl-CoA mutase; 2, methionine synthase reductase; 3, methionine synthase; 4, methylenetetrahydrofolate reductase. More details are provided in Text Box 4. Reproduced with permission from reference 20. B12, vitamin B-12; SAM, *S*-adenosylmethionine; TC, transcobalamin; THF, tetrahydrofolate.

the FAD-dependent reduction of methylenetetrahydrofolate to methyltetrahydrofolate (23).

- It serves as an allosteric activator of cystathionine β-synthase (CBS), the enzyme that initiates pyridoxal-5′-phosphate–dependent homocysteine catabolism through cystathionine synthesis and subsequent formation of cysteine and glutathione.
- A putative function of this allosteric control by SAM is as a sensor of dietary methionine supply (24) as evidenced by an increase in cellular SAM concentrations after ingestion of methionine.
  - The rise in SAM promotes homocysteine catabolism and decreases recycling of homocysteine to form methionine.
  - After a period of fasting or low protein (i.e., methionine) intake, cellular SAM concentrations decrease and recycling of homocysteine to methionine is promoted whereas homocysteine catabolism is diminished.
  - Importantly, disruption of this homeostatic mechanism, owing to B12, folate, or vitamin B-6 deficiencies, or genetic defects in key enzymes, results in the accumulation of the homocysteine precursor *S*-adenosylhomocysteine within cells, and increased homocysteine in the blood.

Other homeostatic mechanisms affecting homocysteine and one-carbon metabolism involve oxidative stress and hormonal regulation by insulin, estrogen, and thyroid hormone. Aside from potential health implications, these relations have possible value for the assessment of the 3 central nutrients in these pathways. *S*-adenosylhomocysteine and homocysteine can serve as sentinel markers or bioindicators of the functional impact of deficiencies of vitamins including folate, vitamin B-6, and B12. The following is a brief coverage of each of these aspects of B12 and metabolic homeostasis.

Oxidative stress. Oxidative stress influences homocysteine metabolism by promoting catabolism of the amino acid. The activities of the enzymes that convert homocysteine to methionine (methionine synthase and betaine-homocysteine methyltransferase) are decreased, whereas the activity of cystathionine β-synthase is increased, under cellular oxidative stress (25). The putative purpose of this regulation is to increase the synthesis of the antioxidant glutathione.

Endocrine interactions. Insulin influences homocysteine metabolism by affecting the expression of key enzymes. Most prominently, CBS is inhibited by insulin, which causes reduced overall catabolism of homocysteine. In models of type 1 diabetes, when insulin production is impaired, homocysteine concentrations in the blood actually decrease owing to increased CBS expression and increased homocysteine catabolism (26).

Only when diabetes progresses to affect kidney function does the blood concentration of homocysteine rise above normal.

Lack of thyroid hormone (hypothyroidism) (27) and lack of estrogen (menopause) cause blood homocysteine to increase (28). The mechanisms by which these latter hormones affect homocysteine metabolism are less clear than for insulin, but taken together, these observations indicate that hormonal control is important for homeostatic control of these pathways. Importantly, hormonal status may affect the requirements for the B vitamins, including B12, for maintaining low blood homocysteine concentrations.

Implications of the interactions of folate and B12

Whether excess folic acid intake and consequent high folate status exacerbate biochemical and clinical manifestations of B12 deficiency is controversial. With the advent in the mid- to late-1990s of government-mandated folic acid fortification programs to prevent neural tube defects (NTDs), first in the United States and Canada and now globally in >70 countries, an increase in the prevalence of individuals exceeding the Tolerable Upper Intake Level (UL) for folic acid intake (1000 μg/d) (15) has been reported (29). The prevalence exceeds 10% in the subset of the US population that takes supplements containing folic acid (30). The accumulating evidence indicating the potential public health implications of this interaction is presented in **Text Box 6.** The available evidence has raised some concerns, particularly in populations with a high prevalence of low B12 status in which folic acid exposure is high owing to fortification policies and supplement use.

Text Box 6 Possible interactions between high folate status and B12 deficiency

- Epidemiologic studies indicate that although the risk of anemia and cognitive impairment is increased in B12-deficient individuals, the risk of these conditions is accentuated if folate status (based on plasma folate concentrations) is high (31).
- Biochemical indicators of B12 deficiency, including elevated blood concentrations of homocysteine and methylmalonic acid (MMA) and low blood concentrations of holoTC, are also accentuated by high folate status (32–34).
- The Pune Maternal Nutrition Study, in which offspring exposed in utero to low B12 and high folate had increased adiposity and insulin resistance at age 6 y, suggested an influence of B12-folate imbalance on epigenetic programming (35). However, folic acid supplementation during their mother's pregnancy did not further increase the risk of insulin resistance in 6- to 8-y-old Nepalese children beyond that of maternal B12 deficiency alone (36).
- Recent evidence suggests that improvement in B12 status in deficient individuals after intramuscular B12 supplementation is attenuated by high folate status (37).
- New data from the Human Nutrition Research Center on Aging in Boston show that elderly persons with a genetic variant in the transcobalamin gene *TCN2* (transcobalamin 776C→G) who consume high amounts of folate (twice the RDA of 800 μg Dietary Folate Equivalents), mostly due to supplements containing folic acid, are 7 times more likely to have neuropathy (38).
- Additional investigations are needed to clarify whether there are any negative effects of high folate status on B12 metabolism, and specifically in B12-deficient individuals and population groups.

Functions affected by B12

B12 cofactors are essential to the normal metabolism and function of a number of organ systems. The most well-established functional roles are summarized in **Text Box 7**. The following is a brief description of these and some other potential roles of B12 in human biology. Aside from highlighting the functional role of B12 in these systems, this overview will also point out the strengths and weaknesses of available tools for evaluating these relations.

Text Box 7 Functions of B12 cofactors in organ systems

- RBC synthesis and prevention of megaloblastic anemia.
- Neurologic function including prevention of neuropathy and demyelination.
- Cognitive function and prevention of dementia.
- Prevention of hyperhomocysteinemia.

Hematology

The classical clinical manifestation of B12 deficiency is macrocytic or megaloblastic anemia, which is characterized by enlarged RBCs and hypersegmented neutrophils. The megaloblastic anemia of B12 deficiency is essentially identical to that caused by folate deficiency. **Text Box 8** includes some of the salient points regarding the relations among B12, folate, and megaloblastic anemia (39).

Text Box 8 B12, folate, megaloblastic anemia, and the "folate trap"

- Folate, in the form of methylenetetrahydrofolate, is a required substrate for the conversion of uridylate to thymidylate and the subsequent incorporation of thymidine into DNA.
- When folate is deficient, DNA synthesis in the blood cell precursors of the bone marrow is inhibited, which prevents mitosis while allowing for cytoplasmic maturation.
- This results in enlarged, but reduced numbers of, circulating RBCs (i.e., megaloblastic anemia).
- B12 and the "folate trap:"
  - When B12 is deficient, the conversion of homocysteine and methyltetrahydrofolate to methionine and tetrahydrofolate is inhibited. Folate is trapped as methyltetrahydrofolate and therefore cannot serve as a substrate for thymidine synthesis.
  - Thus, a functional folate deficiency is produced and megaloblastic anemia ensues.
- As both RBC and white blood cell precursors are dependent on folate and B12, pancytopenia and disturbances in both cellular and humoral immunity may occur.

Aside from the characterization of these key biological relations, the fact that both folate and B12 result in a similar clinical outcome points to the limitation in reliance on a single bioindicator (40), such as RBC morphology, in attempting to make a differential diagnosis, and the need to complement the clinical outcome/bioindicator with sensitive and specific biomarkers of the nutrients in question, i.e., folate and B12.

Neurologic function

In addition to megaloblastic anemia, the other classical pathophysiologic manifestation of B12 deficiency is neuronal demyelination affecting both the peripheral and central nervous systems (41). There are several theories of the cause of demyelinating syndrome, including a deficiency of SAM and consequent inhibition of methylation reactions, which are required for membrane phospholipid metabolism and metabolism of neurotransmitters (42). Alternatively, it has been proposed that the myeloneuropathy of B12 deficiency may result from disrupted odd-chain fatty acid metabolism caused by inhibition of the conversion of methylmalonyl CoA to succinyl CoA (42). Neurologic symptoms of B12 deficiency in humans and rats are associated with alterations in cytokines and epidermal growth factor in cerebrospinal fluid and serum, which are corrected by B12 administration, and have been postulated to play a role in neuropathy (43).

The long tracts of the posterior and lateral columns of the spinal cord are particularly vulnerable to B12 deficiency, resulting in loss of vibration and position sense, as well as loss of motor function often manifested as gait ataxia. Notably, the neurologic manifestations of B12 deficiency can precede or occur in absence of the hematologic consequences. Historically, B12 deficiency was typically suspected when megaloblastic anemia was the presenting symptom; in the absence of the anemia, the neurologic pathophysiology of B12 deficiency often went undiagnosed until permanent neurologic damage had occurred.

There have been a few randomized controlled trials on the benefits of supplementation on neurologic function in people with marginal B12 status. A recent study on elderly subjects with marginal B12 status residing in the United Kingdom failed to see benefits of 12 mo of B12 supplements on a battery of electrophysiologic measures of peripheral and central nerve function (44), although this trial has been criticized for use of insensitive outcome measures (45). In contrast, B12 treatment of Chilean elderly subjects with serum B12 <120 pmol/L at screening improved conductivity in myelinated peripheral nerves (37).

This scenario again reinforces the need for complementarity in the assessment of suspected B12 insufficiency that includes both bioindicators of function, as well as sensitive and specific biomarkers of the vitamin. To prevent or reverse the neurologic damage of B12 deficiency, treatment with B12 supplements (i.e., intramuscular injections or high oral doses) must be initiated early after the onset of deficiency (within 6 mo to 1 y).

Cognition and dementia

The concept of a reversible dementia due to cobalamin deficiency is widely acknowledged. However, in contrast to the dramatic improvement in hematologic signs after treatment of patients with pernicious anemia, there is limited evidence that cobalamin treatment improves cognitive status in patients with dementia. Low B12 status is associated with an increased risk of pathologically confirmed Alzheimer disease (46, 47) but by the time the patient has moderate to severe dementia, the brain is probably too damaged to benefit from cobalamin treatment (48). Reversal of cognitive deficits has been observed in B12-deficient patients with cognitive impairment, but not dementia (49). Thus, all patients with cognitive impairment should have their cobalamin status investigated.

There are several possible reasons for apparently contradictory reports in the literature about whether low cobalamin status contributes to cognitive decline and dementia. **Text Box 9** summarizes the current state of the evidence with regard to both potential mechanisms and response to interventions.

Text Box 9 B12 and cognition: current understanding of mechanisms and clinical evidence

- Even serum B12 in the "normal" range (i.e., >150 pmol/L) may be associated with cognitive deficit or with the risk of future cognitive decline (50–54).
- It is now recognized that serum B12 is not as sensitive a marker of functional cobalamin status as are markers such as holoTC, MMA, and total homocysteine (tHcy).
- Possible interactions between B12 status and clinical or biological context have been recognized including: folate status; concomitant depression; ApoE genotype; choline status; and homocysteine status.
  - Clinical depression is common in the elderly. In 2 elderly populations, low cobalamin status was only associated with cognitive impairment in people who had depression (55, 56).
  - The importance of *APOE* genotype has been shown in 2 studies, 1 in Caucasians (55) and 1 in Chinese (57). In both cohorts, only those who carried the E4 allele of ApoE showed an association between low cobalamin status and impaired cognition.
  - In the same Caucasian cohort, low cobalamin status became a significant risk factor for cognitive impairment in those with low choline status (58).
  - The importance of plasma tHcy as a marker for a subgroup was shown in a randomized trial (VITACOG) on elderly people with mild cognitive impairment:
    - In VITACOG, a combination of B vitamins (800 μg folic acid, 500 μg B12, and 20 mg vitamin B-6/d) slowed brain atrophy and cognitive decline over a 2-y period (59, 60), but only those whose baseline plasma tHcy was above the median (~11 μmol/L) benefitted from the B vitamins.
    - A further analysis showed that the B-vitamin treatment markedly slowed (by 90%) the rate of atrophy of the regions of the brain that are affected by Alzheimer disease, but only in those subjects with elevated tHcy.
    - Further data analysis showed that the key vitamin in the mixture was B12 (61).
    - High tHcy was the strongest predictor of cognitive impairment in a population-based cross-sectional study of 839 elderly (62).

Analysis of evidence from clinical trials

- There have been few randomized trials in which B12 alone has been given to assess its effect on cognitive function (63).
- Many were underpowered, too short in duration, and often carried out on the wrong type of subjects such as

normal elderly who are not showing cognitive decline; thus, no conclusions can be drawn.
- The conclusion of Cochrane reviewers in 2003, that new trials are needed (64), is still valid.
- There have been similar limitations in trials of combinations of B vitamins (folic acid, B12, and B-6). Meta-analyses (65, 66) concluded that B-vitamin treatment does not improve cognitive function, but these studies included a number of trials where no effect would be anticipated (67).
- Another trial of B vitamins (400 μg folic acid/d, 100 μg B12/d) reported that cognitive decline was slowed in elderly subjects with depression, but the trial did not include a comparison group without depression (68).

This review of the relation between B12 insufficiency and cognitive impairment would be incomplete without reference to the growing body of literature that describes adverse effects of high folic acid intake on cognitive function in people with marginal or insufficient B12 status. The exacerbation of neurologic damage by high doses of folic acid was observed early in the history of this vitamin and that experience was briefly described above. In addition to some beneficial effects of good folate status on cognitive function referred to elsewhere, there have been several reports that high intake of folic acid or high blood concentrations of folate may be detrimental to some cognitive functions. The following is a brief summary of the extant evidence with regard to the folate-B12-cognition nexus:

- The Chicago Health and Aging Project reported that those in the higher quintiles of total folate intake, mostly as folic acid, had a faster rate of cognitive decline (69).
- Analysis of the NHANES cohort in the United States revealed that those with low B12 status and high folic acid status were most likely to demonstrate cognitive impairment (31).
- Two reports from Australia have shown that high folate status is related to poor cognition, especially when the population is divided into those with low, normal, or high serum B12 concentrations (70, 71).

These and other observations have been reviewed recently (72). Taken together, these reports demonstrate that the greatest risk of cognitive impairment in those with low or insufficient status of B12 may exist in those (mostly elderly) who are exposed to high intakes of folic acid, which is not the natural form but rather a synthetic form of that vitamin used in supplements or fortification.

In summary, the results of clinical trials and observational studies are consistent with the view that cobalamin plays an important role in cognition, but that its effects may be more noticeable in certain subgroups of the population.

Depression

The association of low B12 status or intake with depression is not as well-established as its relation to cognition. The evidence can be summarized as follows:

- Several, but not all, cross-sectional studies have reported an association (73–78).
- Two prospective studies found associations between low B12 status or intake and later onset of depression (79, 80).
- Trials of cobalamin supplementation in relation to depression are few and mostly negative; the only positive result was from a trial that included folic acid and vitamin B-6 as well as cobalamin (81).

Notably, an association between low folate status and depression has long been recognized (82). The associations between folate, B12, and depression may be related to SAM, which has antidepressant properties (83), and as highlighted above, both folate and B12 are required for the synthesis of SAM. Although there have been calls for the addition of cobalamin and folic acid to standard antidepressant treatments (84), a large community study showed no interaction (85). More research is clearly needed and it will be necessary to examine subgroups of the population, as in the studies on cognition.

NTDs and pregnancy outcome

Although folic acid fortification and periconceptional folic acid supplements are responsible for dramatic reductions in the incidence of NTDs, a residual rate of NTDs remains. Because of the shared roles of B12 and folate in one-carbon metabolism, it is reasonably predicted that low B12 status could be responsible for some of the residual incidence. Indeed, case-control studies confirm an association between low maternal B12 status and risk of NTDs in offspring (86–88). In Canada, where flour has been fortified with folic acid since 1997, a case-control study in midpregnancy revealed that women in the lowest quartile for holoTC (<55.3 pmol/L) had 3 times the risk of an NTD delivery compared with those in the highest quartile (89). A meta-analysis of published reports also found that low maternal serum B12 is a risk factor for NTDs (90). However, unlike the situation for folic acid, there have been no randomized controlled trials of B12 supplements to assess the influence on NTD risk.

Maternal B12 nutrition, lactation, and child development

The risks of B12 insufficiency are especially strong during the period before conception, throughout pregnancy and lactation, and for infants and young children:

- Low maternal serum B12 and elevated tHcy during pregnancy result in low B12 and high tHcy in cord blood (91).
- Elevated tHcy in pregnancy is associated with an increased risk of low birth weight that is not explained by folate status (92, 93).
- Infants born to B12-deficient vegan women are at high risk of being born with low stores of the vitamin, and of developing serious clinical signs of deficiency during the first year of life, including developmental delays, only about half of which are reversed by B12 supplementation (94).
- Human milk concentrations of B12 are strongly related to maternal status during pregnancy and postpartum, and can be extremely low even in nonvegetarian populations in developing countries who consume inadequate amounts of animal source foods (95–97). Clearly, poor maternal status affects fetal and infant status, the influence of which is apparent into at least early childhood. The Institute of Medicine (IOM) has concluded that infants of vegan mothers should be supplemented with the Adequate Intake (AI) of B12 starting at birth because their stores will be low, as will breast-milk concentrations of the vitamin (15).

Noncommunicable diseases

Noncommunicable diseases (NCDs) continue to rise and contribute to significant morbidity and mortality in the United States and globally. B12 status has been linked to several of the most prominent NCDs, as summarized briefly in **Text Box 10**.

Text Box 10 B12 and NCDs

Cardiovascular disease

- The specific role of B12 status in cardiovascular health is unclear.
- Hyperhomocysteinemia is an independent risk factor for cardiovascular disease (CVD).
- Because B12, as well as folate and vitamin B-6, is required for the metabolism of homocysteine, deficiencies of these vitamins cause hyperhomocysteinemia.
- This suggests that lowering blood homocysteine with B-vitamin supplements should reduce risk of CVD. B-vitamin supplements effectively reduce plasma tHcy, but such reductions have not typically translated into reduced CVD risk in randomized clinical trials (98, 99).
- The one possible exception is stroke, for which some benefit of B-vitamin supplements and homocysteine lowering has been observed (100).
- It remains unclear if B12 status itself (independent of folate and vitamin B-6) is an important determinant of CVD risk.

Cancer

- Unlike folate, the influence of B12 status on cancer risk has not been studied extensively.
- The best evidence for a role of B12 in cancer is in breast cancer (101).
- One prospective study found that low B12 status was associated with increased risk of breast cancer in postmenopausal women (102).
- A second prospective study found an association in premenopausal women, but not postmenopausal women (103).
- The putative mechanisms by which low B12 status might contribute to increased cancer risk are the same as for folate, i.e., impaired synthesis of thymidine required for DNA synthesis leading to DNA strand breaks due to uracil misincorporation, and disruption of DNA methylation leading to alterations in genome stability and altered patterns of gene expression.

Bone health

- Osteoporosis has long been established to be a consequence of untreated pernicious anemia, although an influence of reduced gastric acid secretion in this condition cannot be ruled out (104).
- In mice lacking gastric IF, B12 deficiency lowered serum growth hormone and diminished liver production of taurine and subsequently of IGF-I, which reduces osteoblastic proliferation and function (105).
- Several, but not all, observational studies in the elderly have shown relations of serum B12 concentration with various measures or markers of bone mineral density (106, 107).
- A 2-y, randomized, vitamin D-controlled intervention trial on Dutch elderly with elevated tHcy concentrations provided 500 μg B12 and 400 μg folic acid/d, but there was no overall effect of this treatment on bone (108).
- A meta-analysis of 4 prospective studies on 7475 people reported a 4% decrease in bone fractures per 50 μmol/L increase in serum B12, which reached borderline significance (109).
- However, although a meta-analysis of 8 studies on 11,511 people found an increased fracture risk of 4%/μmol tHcy, there were too few studies evaluating the risk specifically associated with serum B12 or folate (110).

Clinical considerations

B12 deficiency is caused either by malabsorption secondary to gastrointestinal disease, aging, and surgery, or, most commonly, by consuming low amounts of animal source foods. Clinical presentation of B12 deficiency is more likely to occur as a result of medical conditions (e.g., pernicious anemia, malabsorption) whereas inadequate intake results in abnormal biomarkers, associated SCCD, and more subtle functional effects.

Clinical stages of B12 insufficiency

The major clinical symptoms of B12 deficiency result from impairment of the functions for which the vitamin is required (**Text Box 11**). The organ systems primarily affected include the blood and nervous system.

Text Box 11 Stages of B12 insufficiency

- Events in the blood:
  - Increase in plasma MMA concentration and urinary MMA excretion
  - Decrease in serum holoTC and serum B12
  - Increase in plasma tHcy concentration
  - Reduction in RBC B12
  - Hypersegmentation of nuclei in neutrophils
  - Megaloblastic anemia—abnormally large RBCs with large nuclei; increased mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration
- Megaloblastic changes in bone marrow.
- Infertility and recurrent fetal loss.
- Weakness and fatigue.
- Demyelination of neurons.
- Reduced conductivity of peripheral and central neurons.
- Peripheral neuropathy, abnormal gait, and position sense.
- Subacute combined degeneration (myelopathy).
- Brain atrophy.
- Dementia, depression, memory loss, and psychosis.

As discussed above, it is not unusual for the neurologic complications to precede the appearance of hematologic changes and in fact, for reasons that are poorly understood, patients tend to present with either hematologic or neurologic symptoms in many cases (111). Thus, the presence of megaloblastic anemia alone is not a reliable way to diagnose B12 deficiency.

Importantly, B12 deficiency has to be relatively severe before anemia appears. **Text Box 12** is a summary of the current approaches and considerations in the clinical diagnosis of B12 deficiency.

Text Box 12 Clinical considerations in the clinical diagnosis of B12 deficiency

Assessment of megaloblastic anemia

- Is readily made with a Complete Blood Count, which includes:
  - MCV and mean corpuscular hemoglobin concentration, both of which will be elevated in this condition.
- Nutrient interactions to be considered:
  - Iron deficiency anemia (microcytosis, which can present in a substantial number of B12-deficient patients because both can be caused by low meat intake) can obscure the macrocytosis (112).
  - Folate status must be considered because:
    - Both folate and B12 deficiencies cause megaloblastic anemia, so it is important to distinguish the cause of the anemia before treatment.
    - Folic acid supplements may ameliorate the anemia of B12 deficiency by providing folate for thymidine and DNA synthesis.
- Alternatively, megaloblastic erythrocytes can be identified by microscopy.

Clinical attributes of neurologic symptoms of B12 deficiency

- Neurologic complications result from demyelination of peripheral and central nerves.
- Because some of the symptoms respond rapidly to intramuscular injection with therapeutic doses of B12 (112), other mechanisms are likely involved.
- There is evidence that the neurologic damage of B12 deficiency is exacerbated by folic acid supplements (31, 42, 70), although this remains controversial.
- Methods used to assess neurologic function in suspected B12 deficiency include:
  - vibratory sensation
  - nerve conduction velocity
  - visual and auditory evoked potentials
  - tests of abnormal gait
  - detection of peripheral neuropathy
  - MRI, which detects signal changes in the lateral and posterior columns of the spinal cord and in subcortical white matter, among other abnormalities

Dietary B12

Humans are unable to synthesize B12 and are therefore fully dependent on dietary intake, supplements, or fortified foods. The USDA National Nutrient Database for Standard Reference, release 25, is the most up-to-date and extensive source of data on the B12 content of foods. It is supported by the federal government and updated regularly with new analytic data on the composition of foods and supplements. **Table 1** and **Text Box 13** summarize current data regarding the B12 content of some foods.

TABLE 1 B12 content of selected foods[1]

Food	Serving	μg/serving
Clams, cooked	3 oz	84
Liver, beef, cooked	3 oz	71
Fish, trout	3 oz	3.5
Salmon, cooked	3 oz	5
Breakfast cereal fortified with 100% Daily Value	1 serving	6
Tuna in water	3 oz	2.5
Beef sirloin, broiled	3 oz	1.4
Milk, low-fat	1 cup	1.2
Yogurt, fruit, low-fat	8 oz	1.1
Cheese, Swiss	1 oz	0.9
Egg, cooked	1 large	0.6
Chicken breast, cooked	3 oz	0.3

[1]Values from reference 113. 3 oz = ~100 g, 8 oz = ~227 g, 1 cup = 237 mL.

Text Box 13 Food sources of B12

- The only commonly consumed natural food sources are animal source foods, including meat, fish, dairy, eggs, and liver.
- Some dried green (*Enteromorpha* spp.) and purple (*Porphyra* spp., nori) seaweeds may contain substantial amounts of B12, as a result of a symbiotic relation with bacteria.
- Spirulina does not; the corrinoid in blue-green algae is biologically inactive (114).
- Tempeh may contain some of the vitamin synthesized by bacteria and mold produced during fermentation, but fermented soybeans and fermented Korean vegetables do not.
- Per 100 g, a few foods stand out as being especially high in the vitamin, including shellfish and liver (Table 1).

The B12 content of foods is commonly measured by a microbiological assay, but this is relatively time-consuming and expensive. The USDA now uses a combination of capillary electrophoresis and inductively coupled plasma MS to measure B12 in foods and supplements. This method provides values for the individual cobalamins found in foods and for cyanocobalamin, the form usually used in supplements and for food fortification.

Bioavailability

To understand the true value of dietary sources and establish recommended intakes, it is critical to understand the bioavailability of B12. For setting B12 recommendations the IOM referred to older studies which measured the absorption of B12 with the use of isotopes (15, 18).

Collectively those studies showed that ~50% of a 1-μg oral dose was retained, 20% of a 5-μg dose, and ~5% of a 25-μg dose (16, 17) (**Figure 3**). After 4–6 h, there is no inhibitory effect of the first dose on subsequent doses (115). At higher dose levels (e.g., 500 μg), only 1% is absorbed (116). This occurs because the B12-IF complex is saturated at low doses but 1% can be passively absorbed, even in the absence of IF, and can therefore provide benefit to patients with pernicious anemia (117).

In a systematic review by the EURopean Micronutrient RECommendations Aligned (EURRECA) group on bioavailability of B12 from different food sources, 8 papers were

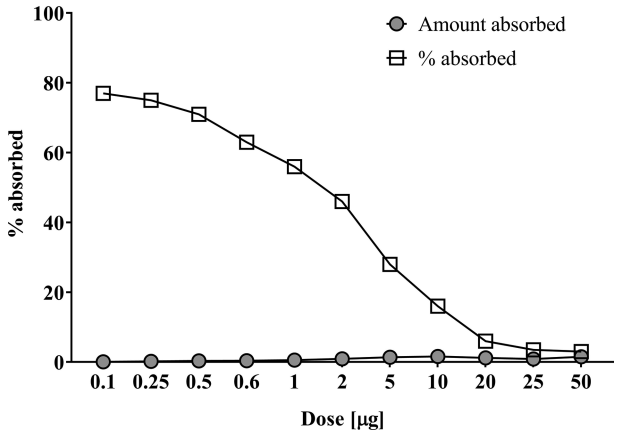

FIGURE 3 Relation between the intake and percentage absorbed from a dose of B12 (17) based on data from Chanarin (16). Reproduced with permission from reference 16. B12, vitamin B-12.

identified that addressed absorption of B12 from a specific food product alone or in combination with a meal free of B12 (118). Study populations included healthy subjects and subjects with a disease not affecting B12 absorption, and only individuals with average daily intakes ≤6 μg were included. Overall, absorption ranged from 4.5% (from liver providing 38 μg) to 83% (from mutton providing 3 μg); from 24% to 36% for egg products (dose 0.3–0.94 μg); from 52% to 83% for lean meat (dose 0.54–5.11 μg); from 30% to 42% for fish (dose 2.1–13.1 μg); and from 4.5% to 49% for liver products (dose 0.5–38 μg). Daily losses of the vitamin in these studies were measured by loss in body radioactivity after administration of a dose of labeled B12, or B12 excretion in bile corrected for estimated reabsorption (118). In general, absorption of 50% of the vitamin from the diet is assumed for estimating B12 requirements (16, 119). However, because there is a maximum amount of B12 that can be absorbed per meal, it is possible that adjustment for this reality may improve the strength of associations between intake and both status and functional outcomes. In the EURRECA analysis, the amount absorbed was estimated by the equation: log absorption = 0.7694 × log intake − 0.9614 (118). This equation does not account for the potentially more efficient absorption that could occur when intake is spread across a day, which is the usual situation, and a polynomial fit to the data is actually better and shows the reduced efficiency of absorption at higher intakes. A more simple approach may be to divide the amount of B12 in B12-rich foods by 5, given that only 11% is absorbed from liver, for example (95, 115). These methods for estimating absorption efficiency from intake need further testing.

Recommended intakes

The IOM has several values for recommended intakes in the United States and Canada, for most life stage and gender groups (15):

- Estimated Average Requirement (EAR): the median usual intake that meets the requirements of 50% of the population. An intake less than the EAR is considered to be inadequate.
- RDA: the EAR plus 2 SD. It represents the average daily dietary intake level sufficient to meet the nutrient requirements of 97.5% of the population.
- AI: the amount of a nutrient consumed by a group with no evidence of inadequacy.
- UL: the maximum daily intake level at which no risk of adverse effects is anticipated for almost all individuals in the general population, including sensitive individuals, when the nutrient is consumed over long periods of time.

Text Box 14 is a summary of key points in the IOM's recommendations on B12 intakes. Examples of recommended intakes of dietary B12 around the world are provided in Table 2.

Text Box 14 Key points with regard to IOM B12 recommendations

- For adults the recommendations are based on the daily amount needed to maintain normal hematologic values and serum B12 within the normal range, in patients with inability to absorb B12 due to pernicious anemia.
- The steps in this calculation were to estimate the average amount required from intramuscular injections, account for the lack of reabsorption of B12 from bile, and correct for an average bioavailability of 50% based on absorption of the radiolabeled vitamin from food.
- No UL was set because there have been no reported adverse effects of high intakes or large intramuscular injections of B12.
- Absorption of the crystalline B12 added to cereals and fortified foods, and used in supplements, is likely to be closer to 60%.
- Elderly should consume most of their B12 in the crystalline form, i.e., from supplements and/or fortified foods, because this form is likely to be better absorbed by those with food-cobalamin malabsorption.
- The EAR and RDA for pregnant women are increased by 0.2 μg/d to cover fetal deposition of B12.
- The AI for infants is based on an estimated 300 pmol/L in breast milk.
- Recommendations for children are extrapolations downwards from adults.
- Recommended intakes for lactating women are increased to cover the secretion of B12 in breast milk.

The IOM Review Committee estimated that food-cobalamin malabsorption may occur in 10–30% of the elderly, and that this group should therefore consume the majority of their B12 from fortified foods or supplements, i.e., in the crystalline form. There would be no point in increasing the recommended amount of B12 consumed from unfortified food because the assumption is that it would be poorly absorbed, and no countries in Table 2 advise a higher intake for the elderly. The FAO/WHO essentially adopted the IOM recommendations (120).

In Europe, B12 recommendations (RDAs) for adults vary from 1.5 to 4.0 μg/d (Table 2). This variability is due to the selection of different CVs in requirements (10–20%) and of different indicators of B12 status adequacy for maintaining health (e.g., maintenance of hematologic status or basal losses). These recommendations were collated within the EURRECA network of excellence (118, 126).

The European Food Safety Authority recently published its Scientific Opinion on Dietary Reference Values for cobalamin (123). These values, also shown in Table 2, are substantially higher than those from other countries based primarily on the observation that reported intakes of 4 μg/d are consistent with reference ranges for serum B12 and holoTC in healthy adults, and with tHcy and MMA concentrations below the cutoffs for inadequacy. All of the European Food Safety

TABLE 2 Selected examples of B12 intake recommendations worldwide (μg/d)[1]

Age/gender/life-stage	Dietary Reference Intakes, United States/Canada (15) RDA/AI	Recommended Nutrient Intakes, FAO/WHO (120) RNI	Nutrient Reference Values, Australia/New Zealand (121) RDI	Dietary Reference Values, United Kingdom (122) RNI	Recommended Dietary Allowances, European Community (123) AI	Nordic Nutrition Recommendations (124) RI	Indian Recommended Dietary Allowances (125)
Infants							
0–6 mo	0.4[2]	0.4[2]	0.4[2]	0.3	—	—	
7–12 mo	0.5[2]	0.5[2]	0.5[2]	0.4	1.5[2]	0.5	0.2
Children							
1–3 y	0.9	0.9	0.9	0.5	1.5[2]		0.2–1.0
4–8 y	1.2	1.2	1.2	0.8	1.5[2]	1.3	0.2–1.0
Males							
9–13 y	1.8	1.8	1.8	1.0	3.5[2]	2.0	0.2–1.0
≥14 y	2.4	2.4	2.4	1.5	4.0[2]	2.0	1.0
Females							
9–13 y	1.8	1.8	1.8	1.0	3.5[2]	2.0	0.2–1.0
≥14 y	2.4	2.4	2.4	1.5	4.0[2]	2.0	1.0
Pregnancy (all ages)	2.6	2.6	2.6	1.5	4.5[2]	2.0	1.2
Lactation (all ages)	2.8	2.8	2.8	2.0	5.0[2]	2.6	1.5

[1] AI, Adequate Intake; RDI, Recommended Dietary Intake; RI, Recommended Intake; RNI, Recommended Nutrient Intake for FAO/WHO and Reference Nutrient Intake for United Kingdom.
[2] Indicates an AI that is estimated to cover the needs of all in the group, but lack of data prevents being able to specify the percentage actually covered by this intake.

Authority values are AIs. The AIs for infants and children are extrapolated downwards from the values for adults and increased in pregnancy based on fetal accretion of B12, and in lactation for secretion of B12 into breast milk. The Nordic Nutrition Recommendations for B12 intake, last published in 2012, are unchanged from those set in 2004; the experts found no strong scientific data to suggest that changes were needed (124). The Recommended Dietary Allowances for Southeast Asia do not provide a value for B12 (127).

Dietary intake of B12

With the use of data from NHANES from 1999–2000, B12 intake was estimated from one 24-h recall interview of a total of 8604 participants (128). All ages were included and smaller population subgroups (adolescents, elderly, Mexican Americans, African Americans, low-income persons, and pregnant women) were oversampled to increase precision. For all ages combined, the mean intake of B12 was 4.6 μg/d (median 3.4 μg/d), ranging from 3.1 μg/d at <6 y old to 5.1 μg/d at 40–59 y old.

Data from 8860 adults in the NHANES 2003–2006 survey were analyzed to compare the prevalence of inadequate intakes in supplement users and nonusers (129). Men consumed a mean of 6.6 μg from food and women consumed 4.1 μg. Approximately 12% of women had inadequate intakes of B12 (<EAR) unless they consumed supplements, in which case the prevalence of inadequacy was <1%. Intake by users of supplements was 66 μg for men and 72 μg for women.

Results from European surveys are summarized in Table 3 and include the following:

- A meta-analysis based on 9 surveys including 28,015 adults and elderly people (130), revealing that mean B12 intakes ranged from 3.8 to 9.3 μg/d in men and from 3.5 to 8.8 μg/d in women.
- The prevalence of inadequate intake was <10% in studies from Denmark (with the exception of women), Germany, Portugal, Spain, Sweden (men), and the United Kingdom (women).
- Men in the nutrition surveys from Finland and women in Ireland had a prevalence of intake inadequacy between 21% and 30%, as did elderly women from the United Kingdom and Finland.

It is possible that differences in the methodology for measuring B12 intake can explain some of the differences among estimates.

B12 intakes are low in most low- and middle-income countries (LMICs) (131, 132), primarily due to limited availability and affordability of animal source foods, but in some cases low intake is due to the avoidance of these sources of B12 for religious or cultural reasons.

Prevalence and causes of inadequate B12 status

The ability to achieve an adequate status of any essential nutrient, as determined by biochemical assessment, is dependent on several processes, including ingestion, digestion, metabolism, and incorporation into dependent biological systems. These processes are affected by numerous factors that will be briefly reviewed in the following sections.

The major causes of poor B12 status are a low intake and malabsorption from food. On a global basis, deficiency due to low intake is much more common. Unless intake is very low, dietary deficiency is more likely to result in changes in biomarkers reflecting SCCD than in clinical symptoms of deficiency.

Prevalence of B12 deficiency

In the most recent NHANES to report B12 status data in the United States, the prevalence of deficiency was estimated based on several different cutoffs for both serum B12 and MMA (133). In the total sample, prevalence of low serum B12 was 2.8% based on a cutoff of <148 pmol/L, 10.5% based on <200 pmol/L, and 25.6% based on <258 pmol/L. Similarly, prevalence of deficiency was different based on cutoffs for MMA of >376 nmol/L (2.3%) or >271 nmol/L (5.8%). Prevalence of high MMA was substantially higher at age

TABLE 3 B12 (μg/d) intake and prevalence of inadequate intake (percentage of population below EAR) in Europe by gender and population group[1]

				Men (EAR = 1.4 μg/d)			Women (EAR = 1.4 μg/d)		
Country	Study	Study year	Food intake method	*n*	Mean ± SD	% < EAR	*n*	Mean ± SD	% < EAR
Adults (age 19–64 y)									
DE	German National Nutrition Survey II	2005–2007	DH	4912	6.6 ± 3.7	8.0	6016	4.4 ± 2.1	7.7
DK	Danish National Survey of Dietary Habits and PA	2000–2002	7 dDR	1283	5.8 ± 3.3	9.1	1486	4.3 ± 2.6	13.2
ES	ENCAT 2002–2003	2002–2003	adj 2 × 24 h	706	5.0 ± 1.0	0.0	875	4.0 ± 0.8	0.1
FI	National FINDIET 2007 Survey	2007	adj 48 h	730	6.6 ± 6.5	21.2	846	4.5 ± 3.4	18.1
GR	EPIC study	1994–1999	FFQ	500	5.3 ± 11.4	36.6	451	3.8 ± 9.7	40.2
IR	SLAN 2007	2007	FFQ	662	5.4 ± 3.7	14.0	717	4.1 ± 3.6	22.7
PT	EPIPORTO	1999–2003	FFQ	917	9.3 ± 4.1	2.7	1472	8.8 ± 4.0	3.2
SE	Riksmaten 1997–1998	1997–1998	7 dDR	517	6.8 ± 3.8	7.8	575	5.9 ± 5.4	20.2
UK	Health Survey for England	2000–2001	7 dDR	219	6.2 ± 4.3	13.2	259	6.1 ± 3.7	10.2
Elderly (age > 64 y)									
DE	German National Nutrition Survey II	2005–2007	DH	1469	5.9 ± 2.5	3.6	1562	4.3 ± 2.0	7.4
DK	Danish National Survey of Dietary Habits and PA	2000–2002	7 dDR	165	6.0 ± 3.3	8.2	164	4.8 ± 2.7	10.4
ES	ENCAT 2002–2003	2002–2003	adj 2 × 24 h	163	3.8 ± 0.6	0.0	179	3.5 ± 0.5	0.0
FI	National FINDIET 2007 Survey	2007	adj 48 h	229	6.5 ± 6.0	19.8	234	5.2 ± 4.8	21.4
PT	EPIPORTO	1999–2003	FFQ	246	8.2 ± 3.8	3.7	339	7.5 ± 4.1	6.8
SE	Riksmaten 1997–1998	1997–1998	7 dDR	64	8.0 ± 3.9	4.5	58	7.4 ± 4.1	7.2

[1] Reproduced with permission from reference 130. adj, adjusted for intraindividual variability; dDR, days dietary record; DE, Germany; DH, diet history; DK, Denmark; EAR, estimated average requirement; ENCAT, Evaluation of Nutritional Status in Catalonia; EPIC, European Prospective Investigation into Cancer and Nutrition; ES, Spain; FI, Finland; GR, Greece; IR, Ireland; PA, physical activity; PT, Portugal; SE, Sweden; SLAN, Survey on Lifestyle and Attitudes to Nutrition; UK, United Kingdom.

≥60 y, i.e., 7.7% with a cutoff of >376 nmol/L, and 15.9% with >271 nmol/L. Figure 4 shows the prevalences for each marker separately and combined across age groups.

No prevalence data on B12 deficiency were described in the EURRECA reports from Europe. In studies from individual countries across Europe, most data are focused on the elderly, and include:

- Netherlands: 24% of 105 healthy, free-living, older subjects aged 74–80 y had mild B12 deficiency, as defined by plasma cobalamin concentrations <260 pmol/L plus elevated plasma MMA (>0.32 μmol/L) (134).
- Norway: the Hordaland study evaluated B12 status in 1935 participants aged 71–74 y, and found that 5.9% had serum B12 <200 pmol/L (55).

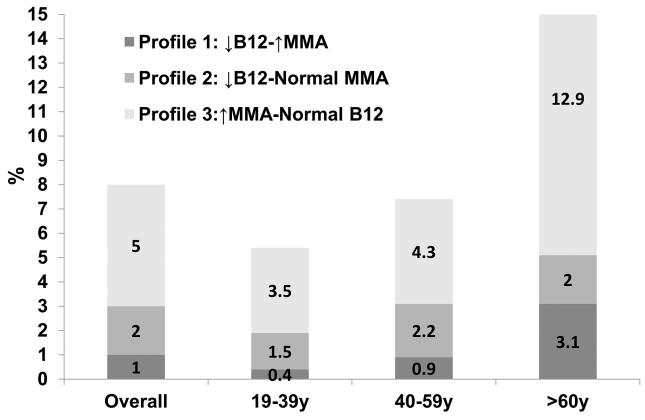

FIGURE 4 Prevalence of abnormal B12 status biomarkers in the US population. Data were generated from NHANES 1999–2004 for the overall adult population and by the age groups listed. The cutoff value between low and normal serum B12 is 148 pmol/L and between normal and elevated MMA is 271 nmol/L. The figure demonstrates that the prevalence of "B12 deficiency" depends on which biomarkers are used (alone or in combination) and how cutoff values are defined. Reproduced with permission from reference 133. B12, vitamin B-12; MMA, methylmalonic acid.

The current prevalence of worldwide B12 deficiency is uncertain. Figure 5 summarizes B12 data from selected national and larger nonrepresentative studies. Data were extracted predominantly from 3 previous reviews (135–137) plus additional published (97, 138–142) and unpublished studies. Notably, the prevalence of low B12 status (based on conventional adult cutoffs for deficiency and marginal status combined) varies widely, with some countries exceeding 40% in different subpopulations (children, young adults, women of childbearing age, pregnant women, and older adults). Validation of cutoffs is still needed to improve assessment of the global prevalence of B12 deficiency, especially in infants, young children, and pregnant women.

Factors associated with inadequate intake of B12

It is well known that strict vegetarianism (veganism) will lead to B12 deficiency unless supplements or fortified foods are consumed. For a healthy, well-nourished person who changes abruptly from an omnivorous diet to one containing no or very small amounts of animal source foods, biomarkers of status

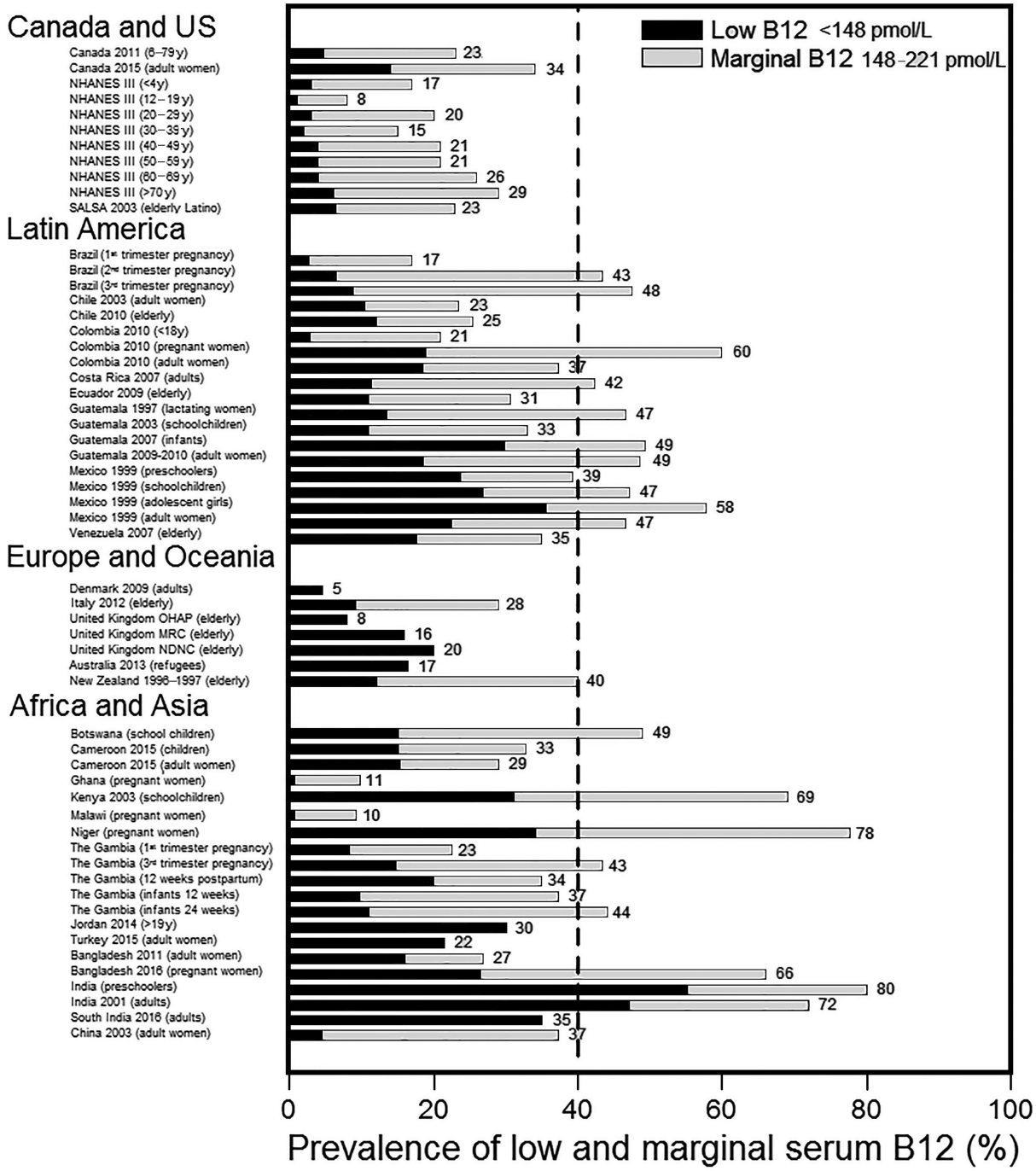

FIGURE 5 Prevalence of low (<148 pmol/L) and marginal (148–221 pmol/L) plasma or serum B12 concentrations in selected representative national surveys and large nonrepresentative studies. B12, vitamin B-12; MRC, Medical Research Council; NDNC, National Diet and Nutrition Survey; OHAP, Oxford Healthy Aging Project; SALSA, Sacramento Area Latino Study on Agin.

will not change for several years. This is because the body B12 store is ~2–3 mg, of which ~0.5–5 μg is secreted into bile daily (more when stores are higher), from which 50% of the vitamin is reabsorbed. However, in a person who initially has low stores, deficiency can appear more quickly. Deficiency will also appear most rapidly if the enterohepatic recirculation is impaired or prevented by intestinal disorders, such as ileal disease.

It is less commonly recognized that even moderate restriction of animal source foods will affect B12 status. For example, it has been reported that omnivores have better B12 status than those who avoid meat, poultry, and fish (lacto-ovo vegetarians), and they in turn have better status than lacto-vegetarians, with vegans having the poorest status (143). Indeed, a high proportion of adult lacto-ovo vegetarians in Germany had low serum B12 (26% <156 pmol/L); 77% had low holoTC and 68% had elevated MMA (143). In a cohort in Boston, serum B12 was correlated with total daily intake of the vitamin across the range of intake regardless of age, and intake of milk, supplements, and fortified cereal protected against lower serum B12 concentrations (144). Thus, in general, the prevalence of insufficiency is inversely related to the intake of animal source foods.

Worldwide, the percentage of energy contributed daily by animal source foods is often very low (145). It is likely that >25% of a population's energy has to be consumed as dairy

products, eggs, fish, meat, or poultry to support a low prevalence of B12 deficiency. Several studies conducted in LMICs have highlighted these important associations:

- In countries such as Kenya and India, where animal products constitute ~5–10% of energy, prevalences of B12 deficiency are very high:
  - >70% in Kenyan schoolchildren (146)
  - 27% (serum B12 <150 pmol/L) in New Delhi preschoolers (147)
  - 67% of 442 adult men in Pune, India, had serum B12 <150 pmol/L; in the urban middle class, 81% had serum B12 below this value (148). Vegetarians had a 4-times greater risk of low serum B12 than omnivores. Low B12 was related to tHcy and low hemoglobin but macrocytosis was rare
- In Nepal:
  - 41% deficiency (serum B12 <150 pmol/L) plus 16% depletion (150–200 pmol/L) was reported in children 6–35 mo old with acute diarrhea: serum MMA and tHcy started to rise when serum B12 fell below 150–200 pmol/L (149)

By contrast, in the United States animal source foods provide >40% of the dietary energy and low serum B12 is uncommon. In Kenyan children, the risk of low plasma B12 (<148 pmol/L) was >6 times higher for those in the lowest compared with the highest tertile, even though overall intake in the population was very low (150). Supplementation with animal source foods significantly reduced the prevalence of deficiency.

Developmental perspective on B12 intake and status

The period of pregnancy through early childhood has important implications for B12 exposure and status as evidenced by the following:

- B12 deficiency can start in early infancy as a result of low fetal stores at birth if the mother is deficient during pregnancy (94).
- Deficits will continue or be exacerbated by low maternal intake during lactation with consequent low amounts of the vitamin in the mother's milk (96, 97).
- During the period of complementary feeding and in childhood, low intakes of animal source foods—and especially of meat—are common in poor populations.
- Human milk is much lower in B12 than in formulas [~300 (range 150–700) pmol/L in milk from well-nourished women compared with 800–1200 pmol/L in formulas].
- In all studies, including higher-income countries and LMICs, where the status of breastfeeding relative to formula-feeding infants has been compared, B12 status is poorer in the breastfed infants based on differences in ≥1 B12 status biomarker (151).
- Consumption by partially breastfed Guatemalan infants of unfortified cow milk, which contains more B12 than breast milk, especially in Guatemala where breast-milk concentrations are very low, was a predictor of better infant B12 status (152).

Malabsorption

Less commonly, affecting 2–4% of adults >60 y of age (153), B12 deficiency is caused by pernicious anemia, an autoimmune disease with a genetic component that destroys the gastric mucosa, resulting in lack of IF and consequent lack of ileal B12 absorption. Prevention of deficiency under these circumstances requires lifelong oral or intramuscular high doses of B12. Pernicious anemia can also be caused by long-term gastric atrophy or atrophic gastritis and eventual destruction of the gastric parietal cells and loss of gastric acid. The latter impairs the release of B12 from food, and can also cause bacterial overgrowth with competition for the vitamin (154).

Atrophic gastritis and malabsorption of B12 from food can result from long-term chronic infection with *Helicobacter pylori* (155) and/or accompanying bacterial overgrowth in the small intestine. For example, serum B12 concentrations were substantially lower in Palestinian patients with gastric disease due to *H. pylori* infection compared with those with gastritis who were noninfected (156). Atrophic gastritis is commonly believed to be an important contributor to B12 deficiency in the elderly (156–158), possibly affecting 10–30% of the older population (15), although this is somewhat controversial (159).

Food-bound cobalamin malabsorption is relatively rare in younger healthy persons but increases with aging (160). Factors associated with food-cobalamin malabsorption were investigated in a prospective study of 202 subjects (43 volunteers and 159 patients) (160). Based on the egg yolk cobalamin absorption test, food-cobalamin malabsorption was significantly correlated with *H. pylori* infection (especially in severe malabsorption), age, markers of gastritis such as serum gastrin, and Latin-American and African-American compared with Asian-American ethnicity (an association independent of *H. pylori* infection). The investigators concluded that there are multiple causes of food-cobalamin malabsorption of which gastritis is one.

There are a number of approaches to test for B12 malabsorption, as listed in **Text Box 15**.

Text Box 15 Tests to assess B12 malabsorption

- Malabsorption from food can be detected by dosing with food containing the vitamin labeled with radioactive cobalt, and after excretion of the label in urine.
- In food-bound cobalamin malabsorption the vitamin can usually be absorbed normally in its crystalline form such as in supplements or fortified food.
- Schilling's test relies on measuring urinary excretion of an oral ^{57}Co-labeled test dose of B12 after intramuscular injection of nonlabeled B12 administered ~2 h postdose.
  - It was usually performed with the crystalline form which would not diagnose malabsorption of the vitamin from food.
  - It could, however, be modified to detect food-cobalamin malabsorption by mixing the labeled B12 with egg yolk (the egg yolk cobalamin absorption test) or chicken serum before administration, an approach that was used most often in clinical research.
  - Neither the Schilling test nor the egg yolk cobalamin absorption test modification are used as clinical diagnostic procedures today (161).
- Alternatives to Schilling's test:
  - Assessment of IF antibody can often diagnose pernicious anemia with >95% specificity, but with only 50–70% sensitivity.
  - Elevated serum gastrin or low pepsinogen can often diagnose gastric dysfunction.
  - The CobaSorb test (162, 163) is based on the fact that newly absorbed B12 circulates in the plasma

as holoTC. By giving three 9-μg doses of crystalline cyanocobalamin in water over the period of a day, and measuring the increase in plasma holoTC on the following day, malabsorbers can be detected.
    - The test has to be given before subjects are treated for B12 deficiency, otherwise the anticipated large increase in holoTC will not occur. The CobaSorb test measures relative absorption—the actual percentage absorbed cannot be quantified.
    - By administering a very low dose ^{14}C vitamin labeled by bacterial synthesis (164), excretion of the label in urine and feces can provide a quantitative estimate of the percentage absorbed, i.e., bioavailability. The labeled vitamin has been incorporated into chicken eggs in vivo as a test for food-bound absorption (LH Allen et al., USDA, ARS Western Human Nutrition Research Center, Davis, CA, 2018), or used in its crystalline form (164).

Gastrointestinal disease or resection

B12 deficiency can result from gastrointestinal malabsorption associated with conditions such as inflammatory bowel disease (Crohn disease and ulcerative colitis) and HIV infection (165). In an evaluation of >500 patients, the risk of B12 deficiency among those with Crohn disease was 33% compared with 16% in those with ulcerative colitis (166). Celiac disease, tropical sprue, bacterial overgrowth (154), bypass or extensive resection of the ileum (167), and total or partial gastrectomy can also cause deficiency. The odds of postoperative B12 deficiency after Roux-en-Y gastric bypass are >3 times higher compared with sleeve gastrectomy (168).

Medications

Clinically the potential for nutrient-drug interactions is an important consideration because it may pertain to specific conditions and across developmental stages. The following are some examples where B12 status may be compromised by the use of some therapeutic medications and drugs:

- Gastric acid suppression medications such as histamine 2 receptor antagonists and proton pump inhibitors (PPIs) can impair the release of B12 from food. In some studies only PPIs increased the risk of B12 deficiency in the elderly (169). In a large case-control study in older patients, B12 deficiency was more common with PPI or histamine 2 receptor antagonist use for >2 y, especially with higher dosages (>1.5 PPI pills/d) (170).
- Metformin treatment of diabetes is associated with significantly lower serum B12 concentrations. In the NHANES 1999–2006 surveys the geometric mean serum B12 concentrations were 318 and 387 pmol/L in metformin users and nonusers, respectively (171). However, tHcy was not increased by metformin. It now appears from animal studies that metformin causes B12 to accumulate in the liver, thus lowering serum concentrations and not causing true deficiency (172, 173).
- Chronic exposure to the anesthetic gas, nitrous oxide, can cause an acquired, functional B12 deficiency (174–176). Nitrous oxide causes the irreversible oxidation of methylcobalamin, the cofactor form of B12 required for the conversion of homocysteine to methionine by the enzyme methionine synthase. Initially, this causes blood homocysteine concentrations to rise, and if prolonged, can precipitate B12 deficiency–associated neuropathy, particularly in individuals who already have low B12 status.
- Highly active antiretroviral therapy substantially reduces the prevalence of low serum B12 in HIV-infected adults, likely by improving intestinal physiologic function, including B12 absorption (177).

Other medications and drugs that affect B12 status biomarkers are described in **Table 4**. Some of these affect each of the 4 biomarkers differently.

Inborn errors of intracellular B12 metabolism

Genetic anomalies have been identified that affect all aspects of B12 absorption, transport, and metabolism, and range from gene deletions and mutations that cause severe but rare B12 deficiency disorders, to single nucleotide polymorphisms that have mild and subtle effects. Several mutations affecting proteins involved in cellular B12 uptake, intracellular transport, and activation have been identified (**Figure 6**). The key characteristics of these mutations are highlighted in **Text Box 16**.

Text Box 16 Genetic abnormalities affecting B12 absorption and plasma transport

Congenital IF deficiency

Mutations in the IF gene (gene locus 11q13) can result in total absence of IF or an inactive or unstable IF protein:

- Causes B12 malabsorption
- Causes megaloblastic anemia in infants and young children

Imerslund-Grasbeck syndrome or autosomal recessive megaloblastic anemia

Mutations in the IF-receptor complex genes:

- Autosomal recessive disorder
- Causes of B12 malabsorption, and megaloblastic anemia at 1–5 y
- Various mutations have been identified in cubilin and an associated protein called amnionless (AMN)
- Because cubilin has other functions primarily in the kidney, 90% of patients with Imerslund-Grasbeck syndrome show nonspecific proteinuria, but otherwise normal renal function

Congenital transcobalamin deficiency

- Transcobalamin (gene locus 22q11.2-qter) deficiency in infants
- Usually manifests as severe megaloblastic anemia within a few weeks of birth
- Serum B12 usually normal, because most is bound to haptocorrin
- Rare but should be suspected in infants with unexplained megaloblastic anemia. Failure to institute adequate B12 therapy may lead to neurologic damage
- In some cases, the protein is present in normal amounts, but unable to bind B12 or the transcobalamin receptor
- Some patients have immune deficiency with reduced amounts of serum immunoglobulins

Congenital haptocorrin deficiency

- Patients with congenital haptocorrin (gene locus 11q11-q12) deficiency display no apparent overt adverse clinical effects of their deficiency
- B12 absorption normal, but total serum B12 concentrations below normal (185)

TABLE 4 Preanalytic factors influencing biomarkers of B12 status[1]

	Serum B12	Serum holoTC	MMA	tHcy
Participant				
Fasting (178)		Not required for individual or population	Not required after normal diet but several high doses in 24 h increase values	Increased by 21% if <3 compared with 8 h. Increased by 10–15% 6–8 h after large, protein-rich meal. Lowest in morning, highest in evening
Pregnancy (178)	Reduced by ~20%	Unchanged	Reduced by ~15%	Reduced by ~39%
Infancy	Values low at ~6 mo	Values low at ~6 mo	Concentrations increase at ~6 mo	
Aging	Not affected by age in healthy people	Not affected by age. Less affected by age than MMA	Substantial gradual increase after 50 y	Increases with age
Lifestyle factors				
Smoking	Lower concentrations in smokers	Not affected by smoking (179)		Increases with smoking
Alcohol use	5% decrease from 0 to 30 g alcohol/d (180)		No effect of moderate alcohol use	
Exercise				Increases with endurance training (181)
Biological variation, % (182)				
Within-person	14.6		18.7	12.2
Between-person	43.6		41.0	37.1
Sample collection				
Venous compared with capillary blood, anticoagulants	Lithium heparin can produce gelatinous serum and elevated B12 values	Serum recommended because EDTA plasma increases values in some studies		
Exposure to light	Protection from light usually recommended although evidence for effect is weak	Protection from light is recommended		
Sample processing				
Clotting time	In 4 participants, increased by 55 pmol/L from 0.5 to 24 h; increase significant by 6 h (183)			Plasma is recommended; serum concentrations will be increased ≤20% by clotting at room temperature for 30–60 min because tHcy is released from RBCs. Separate from RBCs within 1 h or keep whole blood in evacuated EDTA tubes on ice <8 h
Sample storage				
Stability, freeze-thaw stability	Stable under refrigeration and freezing	Stable under refrigeration and freezing		Stable for days at room temperature, weeks refrigerated, and years frozen. Excellent freeze-thaw stability
Inflammation (178)	No effect	No effect	No effect	No effect
Renal function (178)	Increase of 36 pmol/L between normal and stage 3–5 chronic kidney disease	Increased in chronic kidney disease but less so than serum B12, tHcy, or MMA	Increase of 95 nmol/L between normal and stage 3–5 chronic kidney disease	Increase of 3.9 μmol/L between normal and stage 3–5 chronic kidney disease
Disease		Elevated by conditions that have macrophage activation, liver disease, and autoantibodies against transcobalamin	Increased by bacterial overgrowth in small intestine owing to conversion from propionic acid from bacteria. Can occur if low gastric acid secretion	Elevated in renal disease, hypothyroidism, severe pre-eclampsia
Genetic polymorphisms	Affected by polymorphisms in *FUT2, CUBN, CD320, TCN1, TCN2, PON1, CBS,* and *DNMT2* (184)	Lower (by 20% for total TC) for *TCN2* 776C→G genotype. Not influenced much by other *TCN2* genotypes in most studies		Affected by polymorphisms in *CBS, MTRR, TCN2,* and *MTHFR*
Medications	Decreased by histamine 2 receptor antagonists, proton-pump inhibitors, metformin, and chronic exposure to nitric oxide			Increased by L-dopa treatment of Parkinson disease, folate antagonists such as methotrexate, metformin, H2 receptor antagonists, and omeprazole. Reduced by postmenopausal estrogens, tamoxifen

[1]B12, vitamin B-12; *CBS*, cystathionine β-synthase; *CD320*, transcobalamin receptor; *CUBN*, cubilin; *DNMT2*, DNA methyltransferase 2; *FUT2*, fucosyltransferase 2; holoTC, holotranscobalamin; MMA, methylmalonic acid; *MTHFR*, methylenetetrahydrofolate reductase; *MTRR*, methionine synthase reductase; *PON1*, paraoxonase 1; TC, transcobalamin; *TCN1*, haptocorrin; *TCN2*, transcobalamin; tHcy, total homocysteine.

Single nucleotide polymorphisms

A highly prevalent polymorphism occurs in the gene that encodes for transcobalamin (*TCN2*). It is a C-to-G substitution at base position 776 (776C>G) that results in an arginine in place of a proline in amino acid position 259 (186, 187). The 776G allele is most prevalent in Asian populations, with a lower prevalence in whites, and still lower prevalence in blacks, Hispanics, and Native Americans (188, 189). The 776C>G polymorphism is most consistently associated with reduced apotranscobalamin and holoTC in serum (190–199). In some studies, it has been associated with increased serum MMA and a lower percentage of total B12 bound to transcobalamin (holoTC:serum total B12 ratio) (195). Cerebrospinal fluid holoTC also is lower in 776G homozygotes (200). Total B12 and homocysteine typically are not significantly affected, although the transcobalamin genotype modifies the association between measures of B12 status (total B12, holoTC, and tHcy) (193). These observations suggest that the 776G allele may

affect the affinity of transcobalamin for B12. Clinically, the 776C>G polymorphism may affect the risk of several birth outcomes, including spontaneous abortion and cleft lip or palate (201–203). It is associated with peripheral neuropathy in elderly individuals with high folate intake (38).

Single nucleotide polymorphisms have also been identified in the genes encoding the enzymes methionine synthase and methionine synthase reductase, and in IF. The 2756A>G polymorphism in methionine synthase has been associated with various birth defects, including NTDs, orofacial clefts, and Down syndrome (201, 204–207). The 66A>G polymorphism in methionine synthase reductase has been associated with NTDs and Down syndrome (204, 206–209). Recently, a polymorphism has been identified in *IF*, 68A>G; 1 study associated this polymorphism with congenital IF deficiency, but this finding has not been confirmed (210).

Prevention and Treatment of B12 Deficiency

Treatment of symptomatic cobalamin deficiency, resulting from pernicious anemia or other malabsorptive conditions, requires a different strategy from treating dietary deficiency, with repeated follow-ups to confirm the efficacy of treatment (112). Remission of clinical symptoms of deficiency associated with malabsorptive disorders requires a higher dose than treatment of dietary deficiency, and is less effective with increasing time between the onset of symptoms and the start of treatment. Specific considerations regarding treatment of B12 deficiency under these conditions are outlined in **Text Box 17**.

Text Box 17 Considerations for the treatment of B12 owing to malabsorption disorders

- The first step is to confirm the diagnosis of the cause of the deficiency is correct, including biomarker assessment, because clinical values will change markedly once treatment is started.
- The usual treatment is an intramuscular injection of 1000 μg cyanocobalamin (or hydroxocobalamin) of which ~150 μg is retained. Response to treatment:
  - Corrects the anemia, increases reticulocyte count within 48 h, and results in a peak reticulocyte count 1 wk after treatment.
  - Normal MCV within 2 mo, and begins to rebuild liver stores.
  - MMA and tHcy should start to correct within 1 wk.
  - Neurologic symptoms should also improve within 1 wk and be resolved in 6–12 wk unless the deficiency is untreated for >6 mo or 1 y in which case neurologic symptoms may not be corrected because of irreversible damage to the nervous system.
- Repeated 1000-μg injections should be given weekly during the initial treatment phase, after which monthly injections are routine for maintaining adequate status.
- High-dose oral supplements (≥500 μg/d) can also be considered in conditions of physiologic malabsorption because ~1% of oral doses is absorbed by passive diffusion.

Dietary B12 deficiency

Vegan or vegetarian diets, or diets limited in animal source foods, are likely to cause biochemical signs of B12 depletion, or even deficiency. Clinical symptoms, including anemia, are less likely than SCCD, but there are still risks attached to poor B12 status. Such diets are especially ill-advised during pregnancy unless supplements or fortified foods providing the RDA are consumed, because fetal liver stores will not be built, breast-milk B12 concentrations will be low, and there is risk of developmental delays in the infant (94).

Vegetarians and vegans can obtain sufficient amounts of B12 from fortified cereals and special B12-fortified vegetarian products, or from supplements. The Vegan Society in the United Kingdom recommends that vegans consume B12-fortified foods such as breakfast cereals, soya drinks, or yeast extracts several times a day or take a daily supplement.

In a randomized placebo-controlled trial, the benefits of supplementation with 2 or 10 μg of B12/d for 4 and 12 mo were tested in 300 individuals from Indian families, of whom 48% were B12 deficient at baseline (211). After 12 mo plasma B12 increased by 64% with 2 μg/d and 119% after 10 μg/d, an increase similar to that at 4 mo. At 12 mo tHcy fell by 5.0 μmol/L in the 2 μg/d group and 7.1 μmol/L in the 10 μg/d group. Plasma B12 was higher in the 10 μg/d group, but few participants in either group remained deficient. Adding folic acid made no difference to the change in tHcy. In a follow-up study, recovery in response to a high dose of 500 μg every other day for 6 wk was evaluated in vegetarian Indian women (212). Within 2 wk, tHcy fell from 18.0 to 13.0 μmol/L where it remained for the next 4 wk at a concentration achieved within 4 mo after the 2 μg/d dosage. These studies support the observation that the efficiency of B12 absorption from doses between 1 and 2 μg is ~50%, but falls to 1% with high doses.

Public health interventions to prevent B12 deficiency

B12 is a constituent of micronutrient powders, lipid-based nutrient supplements (LNSs), and multiple micronutrient supplements distributed in many LMICs to prevent micronutrient deficiencies. Randomized controlled trials show that providing recommended amounts of B12 in such supplements increases adult and infant serum, and human milk concentrations of the vitamin, although not by a substantial amount, as highlighted in the studies listed in **Text Box 18** that have tested the efficacy of B12 supplements.

Text Box 18 Studies of B12 supplementation for dietary deficiency

Nepal (213): in pregnant women provided with B12 in multiple micronutrient supplements from early pregnancy, serum B12 in late pregnancy was 30 pmol/L higher and the prevalence of deficiency (serum B12 <150 pmol/L) was reduced by 35% compared with controls. Importantly, however, 45% of the supplemented women still had concentrations <150 pmol/L.

Bangladesh (214): 2.6 μg/d B12 included in a supplement for pregnant women increased serum B12 by ~30 pmol/L in infants at 6 mo of age.

Malawi (215, 216): LNS containing B12 provided to exclusively breastfeeding, HIV-infected women for 6 mo increased concentrations of maternal serum and breast-milk B12. Antiretroviral treatment of the mother

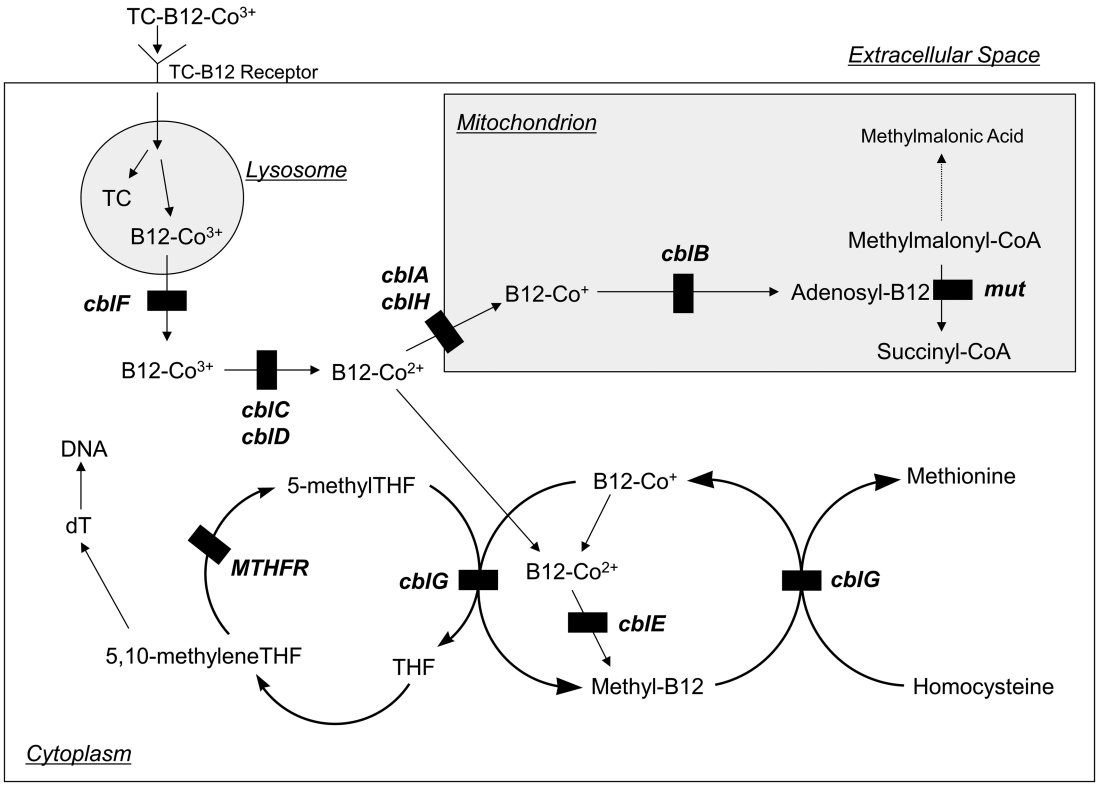

FIGURE 6 Sites of genetic inborn errors of B12 metabolism. Reproduced with permission from reference 20. B12, vitamin B-12; cbl, prefix for genetic mutations affecting B12; *MTHFR*, methylenetetrahydrofolate reductase; TC, transcobalamin; THF, tetrahydrofolate.

eliminated these effects of LNS. Several studies provided higher doses of B12 where B12 status is poor.

Bangladesh (97): 250 μg/d from 11 to 14 wk of pregnancy increased B12 in cord blood, colostrum, and breast milk, and infant and maternal plasma at 3 mo postpartum. It also lowered plasma MMA in mothers and neonates at 3 mo postpartum and increased H1N1-specific antibodies in mothers who were given H1N1 vaccine.

India (96): 50 μg/d provided during pregnancy increased plasma concentrations by ~100 pmol/L in the second and third trimesters, and by 60 pmol/L in infants at age 6 wk. Breast-milk B12 at 6 wk postpartum was 87 pmol/L in the placebo group compared with 136 pmol/L in the supplemented group.

Food-based interventions

There are few studies of the effect of food fortification on B12 status. In general, status is better in people consuming B12-fortified cereals, especially those who do not consume animal source foods. Interest is increasing in B12 fortification of staple commodities such as flour, especially in LMICs with a high prevalence of deficiency.

Guidelines for the fortification of flour with B12 have been developed by the Food Fortification Initiative (217, 218), and approved by the WHO. Reasons to fortify flour with B12 include the high prevalence of depletion and deficiency of the vitamin that occurs in all age groups in LMICs owing to low intake, and in elderly worldwide who need a source of the crystalline vitamin. Moreover, because flour in most countries is now fortified with folic acid, cofortification with B12 would eliminate any of the still-controversial concerns about high folate status exacerbating B12 deficiency (31, 37).

The recommendation is to add 20 μg/kg flour assuming consumption of 75–100 g flour/d. This would provide 75–100% of the EAR. Adding more than this amount may add too much to the cost of the flour. The vitamin should be added to the vitamin-mineral premix in a carrier to ensure that the very small amounts can be distributed evenly through the flour. The efficacy and effectiveness of this strategy have not been widely tested. Interestingly, when the recommended amount was added to wheat flour in Cameroon, where the prevalence of B12 deficiency is high, there was a very pronounced impact on both serum and breast-milk B12, greater than is seen with high-dose supplements (219). It is possible that small doses throughout the day in a staple food are absorbed more efficiently than larger doses as a supplement. More studies are needed to establish the appropriate amount of fortification.

Currently Available Biomarkers: Overview

Dietary assessment of B12 intake can be a useful indicator of the risk of insufficiency, but biomarkers are needed to assess actual status. A description of dietary assessment issues is followed by further characteristics and analytic details of each of the 4 priority biomarkers selected by the BOND Vitamin B-12 Expert Panel:

- Serum (or plasma) B12 concentration
- Serum holoTC concentration
- Serum MMA concentration
- Plasma tHcy concentration

A summary of the relative strengths and weaknesses of these biomarkers is provided in Table 5.

TABLE 5 Relative strengths and weaknesses of B12 biomarkers[1]

Biomarker	Usefulness for purpose	Advantages	Disadvantages	Analytic considerations
Serum or plasma B12	Provides information on the long-term B12 status of the individual, and liver stores. Is a reasonable indicator of B12 status of a population group and response to interventions. Correlates with B12 intake. Definition of status based on usual cutoffs often disagrees with that based on other B12 biomarkers.	Assay is widely available and inexpensive. Not greatly affected by recent intake. Concentrations within an individual are relatively constant within and across days. No need for fasting before sample collection. Not influenced by age or infection. Can be used for animal studies.	Takes months to increase in response to dietary or low-dose supplement interventions, but responds to high-dose interventions. i.m. or oral doses within days. Not very sensitive or specific resulting in false positive and negative diagnosis. Not an indicator of adequacy for metabolic function. Concentrations are 25–30% lower mid-pregnancy but do not indicate depletion. Increased in conditions where haptocorrin production is elevated.	Measurement is not possible in the field. Results are reasonably comparable across methods and laboratories. Samples should be protected from light during collection and handling but are relatively stable under refrigeration and freezing.
Serum holoTC	Provides information on serum B12 available to cells. Is correlated with B12 intake.	Most sensitive to recent intake, responds to intake within hours. Reaches a plateau when status adequate (MMA <0.25 μmol/L). Is a slightly more sensitive indicator of deficiency than serum B12 but estimated population prevalence of deficiency often similar. Not affected by infection.	Concentrations can be increased by recent intake even if stores are low. No consensus on cutoffs, and assay in clinical and research settings is limited compared with serum B12. Not a functional indicator of status. May be best test of status in late pregnancy. Postdose change can be used to test for malabsorption. More expensive than serum B12 and less widely available.	
Serum MMA	Reflects adequacy of B12 for metabolic function i.e., activity of methylmalonyl CoA mutase. Concentrations increase when serum B12 <287 pmol/L.	The most sensitive marker of B12 status. Reflects liver stores. Responds more quickly to interventions than serum B12 but not to recent intake. Not affected by folate, riboflavin, or vitamin B-6 status.	Analysis requires expensive equipment. Increases with aging, not entirely explained by poor renal function. Low diagnostic specificity of marginally elevated values. Increases with renal dysfunction so need to measure serum creatinine especially in elderly. Tends to be higher in low-income populations independently of B12 status; increased with bacterial overgrowth.	Stable under refrigeration and freezing.
Plasma tHcy	Reflects adequacy of B12 for metabolic function i.e., activity of methionine synthase. Concentrations increase when serum B12 <300 pmol/L.	tHcy responds rapidly to improvement in status of deficient individuals.	Not specific for B12 status—increased in folate, riboflavin, and vitamin B-6 deficiency, as well as renal insufficiency and hypothyroidism.	Stable under refrigeration and freezing.

[1] B12, vitamin B-12; holoTC, holotranscobalamin; MMA, methylmalonic acid; tHcy, total homocysteine.

Dietary/supplement intake assessment

Methods available for the quantitative assessment of B12 intake are the same as those used for other nutrients, namely repeated 24-h recalls and food records (113, 220, 221). Computer-based methods are now available for data collection and entry, including self-administration by the participant (113, 221). A web-based Automated Self-Administered 24-h recall method has been developed by the National Cancer Institute in the United States, based on the Automated Multiple-Pass Method developed by the USDA for use in NHANES (222). It provides online assistance and images of portion sizes and is feasible for low-literacy populations. The intake of B12 may be very variable from day to day and average intakes will be greatly affected if foods rich in the vitamin, such as liver, are consumed during the assessment period. Thus it is recommended that several days of intake data should be collected on each individual and that intakes should be expressed as medians, and correction be applied for day-to-day variation in intake among population groups (223, 224). The distribution of B12 intakes in a population is usually highly skewed so data need to be

normalized before statistical analysis of the relation between intake and biomarkers of status, for example.

Consumption of the vitamin in fortified foods and in supplements should be captured when assessing usual intakes. Intermittent consumption of these sources will require assessment over more days, or their estimation from an FFQ that refers to intake over the past week, for example. The actual content of nutrients in supplements may be different from the amount on the label, a situation that is addressed by the federal Dietary Supplement Ingredient Database. The most common amount of B12 in a supplement is ~6 μg and the difference between the labeled and analyzed content is only 2.9%. The B12 status of users of supplements or fortified foods is higher than that of nonusers. Because the absorption of crystalline B12 is only slightly greater than that of the same amount of food B12, there is typically no correction made for differential bioavailability of the 2 forms.

Because dietary sources of B12 are limited to animal source foods (except for fortified foods), estimates of usual consumption of animal products, for example times per day, week, or month, can provide useful information on the risk of inadequate intakes among individuals in households and among population groups. In many surveys and studies, animal source food intake is significantly associated with B12 status (150, 225). Importantly, when faced with an individual with biochemical evidence of poor B12 status, clinicians should question whether a low intake of animal source foods might be responsible.

The effectiveness of different biomarkers of B12 status to detect a change in B12 intake was assessed through a systematic review of 8 published randomized controlled trials of oral B12 supplementation (226). All studies measured serum and plasma total B12, 3 studies measured MMA, and 6 measured total homocysteine response. All 3 indicators were responsive to a change in B12 intake.

Relation between B12 intake and status

In many studies, including those from the United States and Europe, dietary intake and plasma concentrations of B12 are quite strongly and positively correlated. For example, in Massachusetts plasma B12 and dietary intakes were assessed in 2999 adult participants. Mean intake of B12 was 9 μg/d and individuals with higher plasma B12 (>185 pmol/L compared with <148 pmol/L) had higher intakes of the vitamin from all food sources (144). However, plasma B12 reached a plateau at intakes >10 μg/d. Overall, for each doubling of B12 intake there was a 45-pmol/L increase in plasma B12 with no difference among supplements, fortified cereals, or other foods. In those not taking supplements a doubling of B12 intake from all sources combined was associated with 34 pmol/L higher plasma B12, but the increment per unit intake was higher for dairy products (39 pmol/L), less for fortified cereals (24 pmol/L), and only 12 pmol/L for meat, fish, and poultry. This suggests superior absorption of B12 from dairy products, in which it is bound to transcobalamin (227). However, in a randomized controlled trial in which meat and milk supplements were compared as a source of the vitamin for Kenyan schoolchildren, there was no difference in the effect on serum B12 at the end of 1 y (150).

A Norwegian study also investigated the association of dietary intake of different food items with plasma B12 concentrations in the general population (228). Plasma B12 was associated with increasing amounts of B12 from dairy products or fish (P for trend <0.001 for both) but not with intakes of the vitamin from meat or eggs. For the same content of B12, intake from dairy products was associated with the greatest increment in plasma B12. These findings are based on a cross-sectional, population-based study of 5937 subjects in 2 age groups (47–49 and 71–74 y) from the Hordaland Homocysteine Study in Norway, with intake measured by an FFQ. In a similar study of Dutch elderly, higher serum B12 concentrations were associated with higher intakes of dairy, meat, fish, and shellfish, with meat and milk—predominantly milk—being the most potent sources (229).

The relation between B12 intake, serum B12, and the other biomarkers of status was examined in a US study (230). As seen in **Figure 7**, serum concentrations of B12, MMA, and tHcy all tended to reach a plateau at an intake of ~4–7 μg/d in persons aged 18–50 y who had normal absorption of the vitamin. The same group had reported similar results from Danish postmenopausal women where an intake of 6 μg/d normalized B12-related biomarkers (231). In the systematic review of daily losses compared with intake of B12, estimated intakes needed to compensate for losses of 1.4–5.1 μg/d were 3.8–20.7 μg/d (118). These estimates agree with both the US (144) and Norwegian (228) studies in which maximum plasma B12 concentrations were found at a B12 intake of 6–10 μg/d.

Together these studies raise the possibility that recommended intakes of the vitamin should be higher. However, the functional benefits of long-term intakes above the current RDAs for persons with normal absorption of the vitamin remain to be determined, especially because in B12-deficient Indians supplemented with either 2 μg/d or 10 μg/d of the vitamin, there were no differences in the effect of the dosages on reductions in tHcy at 4–6 or 12 mo after supplementation (211). Moreover, in a large (n = 2919) study of the relation between B12 intake and biomarkers in Dutch elderly, saturation of the biomarkers occurred with intakes >5 μg B12 (232).

The strong relation between B12 intake and status is further supported by studies of different types of vegetarians. Data on usual diet and serum B12 were analyzed from the European Prospective Investigation into Cancer and Nutrition (EPIC)-Oxford study in the United Kingdom, categorizing 689 men as omnivores (3 servings of meat/wk, n = 226), lacto-ovo vegetarians (n = 231), and vegans (n = 232) (233).

Serum B12 (mean and 95% CI) was 281 pmol/L (270, 292 pmol/L) in omnivores, 182 pmol/L (175, 189 pmol/L) in vegetarians, and 122 pmol/L (117, 127 pmol/L) in vegans. In Germany, B12 status (including plasma B12) was better in omnivores than in lacto- or lacto-ovo vegetarians, who in turn had better status than ovo-vegetarians, with strict vegetarians (vegans) having the poorest status (234). Notably, tHcy and MMA increased consistently as animal source food intake fell and were elevated in >60% of vegetarians. Thus, not only vegans can become depleted in B12; there is a higher risk of B12 deficiency even in lacto- or lacto-ovo vegetarians than in omnivores.

Serum/plasma B12

Total B12 concentration in serum or plasma is most commonly used as the biomarker of B12 status. Total B12 includes both the metabolically active cobalamin bound to transcobalamin (holoTC), which delivers B12 to body cells, and cobalamin bound to haptocorrin. Serum B12 reflects the general amount of B12 stores and is a useful indicator of B12 status although it lacks sensitivity and specificity for diagnosing B12 deficiency (235, 236). Concentrations are expressed as pmol/L or as pg/mL with the latter most often used in clinical practice (1.0 pg/mL = 0.7378 pmol/L). Serum/plasma B12 is useful for assessing long-term status (body stores) and is relatively

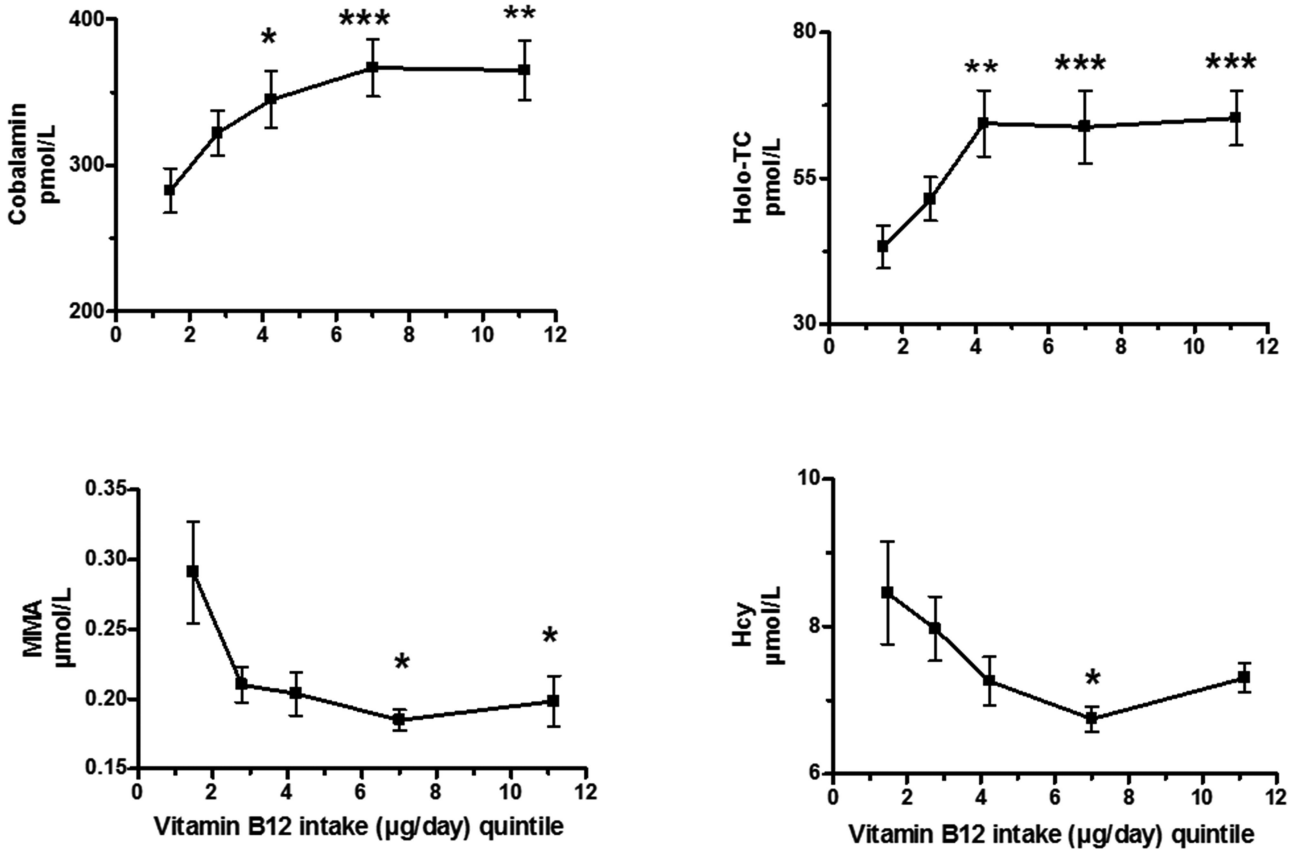

FIGURE 7 Relation between B12 intake and serum biomarker concentrations. Reproduced with permission from reference 230. B12, vitamin B-12; Hcy, homocysteine; holo-TC, holotranscobalamin; MMA, methylmalonic acid. $^{*},^{**},^{***}$Significantly different from lowest quintile of B12 intake: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

unaffected by recent intake. Several observational studies show that serum B12 increases with intake but reaches a plateau at 350–400 pmol/L when intake is 7–10 μg/d (228, 230, 231), although taking supplements results in higher concentrations. Concentrations are increased in conditions that produce elevated levels of haptocorrin, including chronic granulocytic leukemia, autoimmune lymphoproliferative syndrome, alcoholism, liver disease, and cancer (237).

Serum holoTC

There has been considerable interest in the potential for use of serum holoTC as a B12 status marker. HoloTC constitutes 20–30% of total serum B12. It is the component that delivers the vitamin to cells and is therefore a potentially better marker of the adequacy of B12 for its metabolic functions, and sometimes referred to as "active vitamin B-12." However, in general, studies comparing the specificity and sensitivity of holoTC against serum MMA have found only slightly better performance of holoTC. In 5 studies comparing holoTC and serum B12 for the diagnosis of B12 deficiency defined by elevated MMA, holoTC performed slightly better (238). But in a group of vegan men, holoTC and serum B12 had similar specificity and sensitivity for predicting B12 deficiency (defined as MMA >0.75 µmol/L and tHcy >15 µmol/L) (239). The analysis suggested that when holoTC is <25 pmol/L B12 deficiency is likely, but if >50 pmol/L, deficiency is unlikely. Values in the intermediate range need follow-up and further testing.

Concentrations in serum increase by 6 h after a dose or meal so holoTC is a better marker of recent intake than serum B12, MMA, or tHcy. This has led to the development of the CobaSorb and C-Cobasorb tests for B12 absorption which measure holoTC or cyanocobalamin-transcobalamin response to 3 doses of B12 administered over 1–2 d (162, 163). A daily oral dose of 500 μg produces a maximal increase in holoTC after 3 d, and no further increase after 84 d (240). In population studies there is little difference in the prevalence of deficiency using serum B12 or holoTC (37, 241), although different individuals may be classified differently by the 2 biomarkers (241). As is true for the other biomarkers, low holoTC concentrations are more prevalent in populations consuming low amounts of B12 (231) and less animal source foods (242). There remains uncertainty about the cutoff that should be used to diagnose deficiency. Although cutoffs around 35–45 pmol/L are often used in clinical practice and in research, in a large clinical study there was no association between holoTC and MMA in the range of 25–50 pmol/L (243). HoloTC is elevated in individuals with impaired renal function (244) but unaffected by pregnancy (245, 246), so it may be a better biomarker of B12 status in pregnancy (247).

Serum MMA

Serum MMA is considered to be a relatively specific and sensitive biomarker of B12 status. Serum MMA concentrations reflect the adequacy of B12 status for the biochemical function of the enzyme methylmalonyl CoA mutase which is required for the conversion of methylmalonyl CoA to succinyl CoA; MMA, usually a side-reaction product of methylmalonyl CoA metabolism, increases with B12 depletion. The metabolite is not affected by the status of folate or other B vitamins. It is a good indicator of B12 stores but not of recent B12 intake. Higher

concentrations of serum MMA occur with impaired renal function so that should also be assessed, e.g., by measurement of serum creatinine, especially in the elderly. The gut microbiota produce propionic acid which can be converted to MMA, and thus serum MMA can increase with intestinal bacterial overgrowth, and be lowered by antibiotics in this condition (248). Differences in the type or quantity of bacteria in the intestine may explain the generally higher MMA concentrations in lower-income populations relative to serum B12 (249), which persist even after B12 supplementation (241, 250). MMA increases with aging, especially after age 70 y; neither lower intake nor impaired renal function entirely explain why this occurs. There is an inflection point for serum MMA which increases substantially around serum B12 concentrations of 150 nmol/L (251). However, models based on NHANES data and controlling for known covariates revealed 2 inflection points, 281 nmol/L at serum B12 <126 pmol/L which was determined to indicate true deficiency, and 120 nmol/L at serum B12 >287 pmol/L where status was likely adequate (252). The status of the intermediate group was indeterminate.

Urinary MMA is also increased in B12 deficiency and avoids the need for blood collection. Values should be expressed per mg or mmol creatinine to correct for variability in urine concentration, thus 2 analyses are required. Total 24-h excretion avoids this problem, but accurate 24-h collection may be difficult to achieve. A limitation of urinary MMA is that it increases after meals.

Plasma total homocysteine

tHcy is increased as a result of B12 deficiency owing to insufficient amounts of the vitamin for the activity of methionine synthase, which converts homocysteine to methionine. Concentrations are relatively unaffected by mild B12 deficiency, but rise rapidly when plasma B12 falls below 300 pmol/L (251). It can be considered a test of the adequacy of the vitamin for enzyme function. However, because folate, riboflavin, and vitamin B-6 deficiencies result in elevations of tHcy, as do renal insufficiency and hypothyroidism, it is not a specific biomarker for B12 status especially in populations with poor micronutrient status. In contrast, in generally well-nourished populations where flour is fortified with folic acid (which is now a widespread global practice), B12 deficiency may be the main cause of elevated tHcy (253). In US elderly the population attributable risk that elevated tHcy is due to B12 deficiency is ~30%. A fall in tHcy can confirm an improvement in B12 status due to supplementation or fortification programs. More details of the measurement and interpretation of tHcy are provided in the companion BOND report on folate (22).

Data Interpretation and Diagnostic Cutoffs

Diagnostic cutoffs and ranges have been proposed for all 4 biomarkers, and are summarized in **Table 6**. Cutoffs are needed for estimating the prevalence of deficiency in population groups, but in clinical practice it is often more useful to refer to information on the reference range of values. The cutoffs and ranges for B12 status all are somewhat controversial owing to the high prevalence of subclinical B12 deficiency and limited data linking these to functional outcomes. The primary "gold standard" for setting cutoffs has been serum MMA because it is the most sensitive and specific marker of status. Even then, there remains debate about the MMA concentrations to use when setting cutoffs for the other biomarkers. It is clear that cutoffs and ranges for all 4 biomarkers should be different for infants, based on longitudinal data from well-nourished Norwegian infants (131, 254). There are marked changes during the first year of life. Serum B12 and holoTC are generally lower than in adults, and MMA is especially elevated at ~6 mo of age, at a time when breast-milk B12 concentrations are lowest. Status of all 4 biomarkers is consistently better in formula-fed than in breastfed infants, although whether this is functionally important is not clear and this situation occurs even when mothers are B12 sufficient. MMA concentrations increase in the elderly independently of the effects of chronic kidney disease (133) for reasons that are not understood.

The diagnosis of B12 deficiency in individuals and especially the estimated prevalence of B12 deficiency in population groups is often very different depending on which indicator is selected, and may be contradictory (9, 241, 255). This is not surprising in that each marker responds differently to stage of deficiency and age. Clinicians usually use serum B12 as the initial indicator to screen for deficiency, and follow up with additional markers if deficiency is suspected (235, 256, 257). Ratios of 2 biomarkers have also been used: the ratio of holoTC to serum B12 was a stronger predictor of cognitive function in depressed elderly than was either indicator individually (56).

Serum or plasma B12

For infants and young children the ranges are values for healthy breastfeeding Norwegian infants and children to 2 y of age (131). Concentrations fall between birth and 6 mo, after which they increase. Values are higher in nonbreastfed infants (131, 254) accompanied by lower MMA and tHcy, but whether this difference is functionally significant is still unknown. Formulas and cow milk contain more B12 than does breast milk.

Below 75 pmol/L (severe deficiency), ~50% of people will have clinically diagnosable symptoms (258). Serum B12 cutoffs for deficiency typically range from 120 to 180 pmol/L (depending on the assay used), with the most common cutoff being 148 pmol/L (15).

The criteria for setting the cutoff for deficiency at <150 pmol/L in adults in Table 6 are that 98% of a group will have elevated MMA (>376 nmol/L which is 3 SD above normal values in NHANES) (252), and 40–50% in the 75–150 pmol/L range will have clinically diagnosed symptoms (258). With concentrations of 150–250 pmol/L adults are assumed to be depleted in the vitamin, with some individuals probably deficient, and an increased risk of clinical and metabolic dysfunction across this range. In NHANES 2.5–5% of individuals were diagnosed as B12 deficient (serum B12 <148 pmol/L and MMA >271 nmol/L). Adequacy is likely >250 pmol/L based on normal MMA concentrations. However, it has been suggested that the risk of cognitive impairment and white matter damage in the elderly is elevated even when serum B12 is within 250–350 pmol/L (50).

In pregnancy there is a 25–30% fall in concentration between 20 and 30 weeks of gestation (245), caused by altered B12 binding to haptocorrin rather than hemodilution (246, 259). The fall is unlikely to indicate true depletion because holoTC does not change and serum B12 concentrations recover by 14 wk postpartum (245). Reference values are not different for older adults because unlike MMA, serum B12 does not change substantially with age in healthy, well-nourished individuals (Figure 4).

TABLE 6 Cutoffs for biomarkers of B12[1]

Age group	Serum B12			Serum holotranscobalamin		Serum methylmalonic acid	
	Deficient pmol/L	Depleted pmol/L	Adequate/normal pmol/L	Deficient pmol/L	Adequate/normal pmol/L	Deficient nmol/L	Adequate/normal nmol/L
Newborn (cord blood)			120–690		33–240		170–500
6 mo			121–520		12–90		140–220
12 mo			165–580		19–100		120–830
24 mo			183–260		29–110		120–300
Adults	Severe <75, deficient 75–<150	150–221	>221	<35–40	40–150 or 40–200	>376 >271	

[1]Values are ranges. B12, vitamin B-12.

Serum holoTC

For infants and young children the ranges in Table 6 are values for healthy breastfeeding Norwegian infants and young children (131). Similarly to serum B12, concentrations fall between birth and 6 mo and are higher in nonbreastfed infants and young children (131, 132, 254) accompanied by lower MMA and tHcy. The adult cutoffs are based on the relation of holoTC to serum MMA. The ranges are based on data from 1613 participants in 6 studies (238).

MMA

Values for MMA ranges for infants and young children (Table 6) are also from the Norwegian sample of well-nourished breastfed infants (131). The >376 nmol/L cutoff for adults is a concentration found in only 2.4% of US elderly with normal renal function, whereas >271 pmol/L is seen in 5.9% of the same group (260). Renal insufficiency greatly increases serum MMA, so renal function must be evaluated based on serum creatinine, for example, especially in the elderly who are at high risk of chronic kidney disease. Normal serum creatinine concentrations are generally accepted to be 0.6–1.1 mg/dL for women and 0.7–1.3 mg/dL for men. Serum MMA concentration increases with age even in those with normal renal function, and much more so than serum B12 (Figure 4).

Plasma homocysteine

Cutoffs for plasma homocysteine concentrations are provided in the BOND Folate Review (22).

Multiple analyte testing

As reviewed above, no single blood biomarker provides sufficient sensitivity and specificity for diagnosing B12 deficiency in all people. In recent years, there has been movement toward multiple analyte testing to address this problem (239, 244, 261–263). Some strategies use sequential algorithms, whereby B12 status is initially assessed by serum B12 or holoTC. If these analytes are low (e.g., B12 <148 pmol/L or holoTC <35 pmol/L), then B12 deficiency is diagnosed. If these analytes are in an indeterminate range (e.g., B12 between 148 and 250 pmol/L or holoTC between 35 and 50 pmol/L), then a functional indicator of status, MMA or tHcy, is measured and if elevated then B12 deficiency is diagnosed. An alternative strategy is to measure ≥2 of these analytes simultaneously. These strategies have the putative advantage of increased specificity and sensitivity, but also increase the expense of screening and diagnosis.

Most recently a model has been developed that enables calculation of a "combined B12" indicator (cB12) of B12 status from values for all 4 status biomarkers (serum B12, MMA, holoTC, and tHcy) (255). It is based on a pooled database of 5211 participants in 9 studies with a range of B12 and folate status, and the cutoffs for cB12 are validated against hemoglobin concentrations and cognitive status (the Mini Mental State Examination). It has now been adapted for use with 3 or 2 of these biomarkers because measurement of 4 biomarkers can be prohibitively expensive in some situations, and for application when folate status is poor resulting in increased tHcy (9). The algorithm is also age-adjusted recognizing that MMA increases in the elderly. The general 4-component algorithm is expressed as cB12 = log10[(holoTC·B12)/(MMA·Hcy)] – (age factor), and the formulas that allow for 1 or 2 missing biomarkers are provided in the article. Suggested interpretations and cutoffs for cB12 are presented in **Table 7**. These need to be validated in additional studies against not only hemoglobin and the Mini Mental State Examination, but using other cognitive and neurologic outcomes. The combined indicator detected increased risk of cognitive impairment in UK elderly (62), biochemical and neurologic responses to B12 supplementation of deficient Chilean elderly, and a negative interaction between neurologic response and folate status which was not detected through the use of any of the biomarkers individually (37).

Analytic Methods

The most commonly used valid assays for serum or plasma B12, holoTC, and MMA are summarized in **Table 8**. Analytic methods used for the measurement of tHcy are provided in detail in the BOND Folate Report (22).

Earlier radioisotopic dilution assays for serum and plasma B12 measured some of the nonbiologically active analogs of cobalamin and therefore overestimated actual concentrations. This situation was avoided by the inclusion of IF as a reagent, which only binds to cobalamin. Radioassays have largely been displaced by immunoenzymatic assays, although these should not be used to measure serum B12 in patients with pernicious anemia because IF antibodies in their serum interfere with the binding of B12 to the IF used in the assay (264). The National Institute for Standards and Technology (NIST) has developed serum-based standard reference materials (SRMs) for use as control materials. These improve the accuracy and reliability of analyses because they enable calibration of each laboratory's methods and values against the reference value. SRM 3951 Vitamin B-12 in Human Serum consists of 3 sera with different B12 concentrations, 100 pg/mL, 200 pg/mL, and 450 pg/mL (74, 148, and 332 pmol/L). SRM 1955 is available for tHcy. There are no National Institute of Standards and Technology reference materials for MMA or holoTC.

TABLE 7 Suggested cutoffs and interpretation of values of the combined indicator cB12[1]

For researchers and clinicians			For epidemiologic purposes	
Classification	Biological interpretation	Guidelines	Classification	Guidelines
Elevated B12 >1.5	The pathogenesis of high B12 is not fully understood	Consider potential causes of high concentrations such as liver disease or current or recent supplementation or treatment	B12 adequacy >–0.5	No action
B12 adequacy –0.5 to 1.5	Expected to accomplish all B12 status–dependent functions	No action advised		
Low B12 –1.5 to –0.5	Potential subclinical manifestations of B12 deficiency, i.e., absence of hematologic changes, but subclinical neurologic impairment	Consider recommending oral supplements	Transitional B12 status –0.5 to –2.5	Need fortification
Possible B12 deficiency <–2.5	Potential manifestations of B12 deficiency	Potentially prescribe oral supplements, assess again in 3–6 mo	Low B12 status <–2.5	Need supplementation, rechecking, monitoring of status over time
Probable B12 deficiency <–2.5	It is possible to observe clinical manifestations of B12 deficiency. Clinical outcomes are needed to confirm potential clinical deficiency	Consider immediate treatment with i.m. injections, determine cause with primary consideration for the possibility of pernicious anemia		

[1] From Fedosov et al. (9). cB12 = log10[(holoTC · B12)/(MMA · Hcy)] – (age factor). B12, vitamin B-12; Hcy, homocysteine; holoTC, holotranscobalamin; MMA, methylmalonic acid.

Preanalytic effects on biomarker concentrations

Valuable information on the effects of fasting, inflammation, renal function, and pregnancy on serum B12, MMA, and tHcy was obtained from NHANES, based on a representative sample of the US adult population (Table 4) (178). Data were available from 1400–9000 participants and additional covariates were controlled for in multiple regression analyses. HoloTC was not analyzed. Fasting is generally not required, but if possible should be required for MMA analyses.

Estimates of biological variability were also obtained from NHANES (182). The imprecision of the selected laboratory method should be less than half of the CV. Based on the within-person CVs in Table 4, this is quite achievable. All 4 B12 biomarkers can be analyzed on relatively small sample volumes and are quite stable during handling and storage. When whole blood is stored at 32°C for 1 or 2 d there is a 10 pmol/L increase in serum B12, and a 4 pmol/L increase after 3 d, compared with serum separated within 2 h and immediately frozen (265). For tHcy analysis, plasma should be separated rapidly to avoid leakage from RBCs, and serum is usually not recommended because clotting at room temperature will cause an increase in tHcy. However, tHcy is stable if whole blood in a vacutainer is kept on ice for ≤6 h before centrifugation. For B12 and MMA, there is no influence of the type of anticoagulant (serum, serum separator, plasma EDTA, plasma Na heparin, or plasma citrate), but lithium heparin can sometimes produce gelatinous serum and elevated B12 concentrations.

It is usually recommended that samples are protected from light during collection and separation; a study of this question found the mean fall in serum B12 was ∼7.5% in both light-protected and unprotected tubes when stored at room temperature or at 2–8°C for 1, 2, or 7 d (266).

Other Biomarkers

Other markers of B12 adequacy sometimes used for research purposes can include DNA methylation, uracil misincorporation into DNA, and measurement of micronuclei. These are not specific to B12 deficiency, and are described in detail in the BOND Folate Report. B12 concentrations in breast milk might be useful for assessing maternal B12 status and the risk of inadequate intake of the vitamin by breastfed infants, and for monitoring and evaluating B12 intervention programs, but this requires further confirmation including establishing appropriate times postpartum for milk collection.

New Directions and Technologies

The BOND Folate Report provides a description of the concepts underlying the use of "omics" (genomics, transcriptomics, proteomics, and metabolomics) to improve our understanding of the functional impacts of vitamin deficiency, and the effects of interventions to improve status. Although this is a promising area for future research, these methods are still under development and validation in the context of B12 status biomarkers (267).

Research Gaps and Needs

The consultation identified the following information gaps and research needs:

- Development of practical, efficient methods for measuring multiple biomarkers in population studies, e.g., methods for dried blood spots
- Validation of biomarker cutoffs and reference ranges by age and gender, including testing recently proposed cB12 cutoffs against functional outcomes
- Further evaluation of whether holoTC is the best biomarker of B12 status in pregnancy
- Assessment of the functional significance of low B12 status in general, and especially in pregnancy, lactation, infancy, and young children

TABLE 8 Main analytic methods for the measurement of biomarkers for B12 status

Biomarker	Method	Requirements	Advantages	Disadvantages
Serum or plasma B12	Chemiluminescence immunoassay	Clinical chemistry analyzer	Rapid, fully automated, inexpensive. Can measure 22–2000 pmol/L. Folate can be analyzed simultaneously.	Requires expensive equipment. Between-run imprecision >12%. Requires regular flow cell cleaning to lower the imprecision. Plasma can clot if collected in green top mineral-free tubes. Samples and calibrators need protection from light.
	Microbiological assay	*Euglena gracilis* or *Lactobacillus leichmanii* or colistin sulfate–resistant *L. leichmanii*. Plate reader	96-well plate method inexpensive and can eliminate interference from antibiotics.	Can be affected by antibiotics in serum. Requires establishing bacterial cultures and expertise. Dilution and other pipetting steps laborious but can be automated with a liquid handler.
	Competitive protein binding radioassay, dual radioisotope dilution with ^{125}I and ^{57}Co	Gamma counter	Can measure B12 and folate simultaneously, and process many samples at once. Inexpensive.	Requires radioactivity and a gamma counter. The assay tends to have a high variability and is falling into disuse with possible discontinuation of kits.
Serum holotranscobalamin	Monoclonal antibody specific assay	Clinical chemistry analyzer using an AxSYM platform (Abbott Diagnostics)	Rapid, fully automated, inexpensive reagents. Total imprecision 6–9%. Range 3–100 pmol/L.	Expensive equipment.
	ELISA	Plate reader	Rapid, inexpensive.	Serum only. Few manufacturers of kits.
Serum or urine methylmalonic acid	MS	LC-MS/MS or GC-MS/MS	Only small sample volumes needed; high throughput, good precision. Uses stable-isotope-labeled internal standard.	Requires derivatization of analyte. Equipment and internal standard expensive. Needs expertise and high maintenance.

[1] B12, vitamin B-12; MS/MS, tandem mass spectrometry.

- Availability of practical methods to detect and quantify food-bound malabsorption
- Validation of equations or more simple approaches for estimating bioavailability from diets
- Confirmation of the safety of repeated consumption of very high doses of the vitamin
- Further assessment of the functional consequences of high folic acid intakes in B12-deficient population groups
- Improved understanding of why MMA increases with age, the extent and cause of malabsorption in the elderly, and the best advice to give the elderly about need for supplementation or intramuscular injections of B12
- Understanding of the extent to which the intestinal microbiome affects B12 degradation and synthesis
- Assessment of why some patients with pernicious anemia appear to need much higher doses of B12 to relieve their symptoms
- Investigation into why high serum B12 is associated with cancer diagnosis

Acknowledgments

We thank our partner at the Academy for Nutrition and Dietetics (AND), Alison Steiber. Special thanks go to Constantina (Tina) Papoutsakis for her leadership and technical skills in preparing and coordinating the successful submission of this manuscript. Additional thanks go to AND staff, Rosa Hand and Kathryn Kelley, for their support throughout this process. This review is the result of deliberations and contributions of the BOND Vitamin B-12 Expert Panel [LHA (Chair), JWM, IHR, and LdG] and its scientific consultants (Alex Brito, USDA, Agricultural Research Service Western Human Nutrition Research Center, Davis; Christine M Pfeiffer, Division of Laboratory Science, CDC/National Center for Environmental Health; Anne Molloy, Department of Clinical Medicine, Trinity College, Dublin; Ebba Nexo, Department of Clinical Chemistry, Aarhus University Hospital, Aarhus, Denmark; Regan Bailey, NIH/Office of Dietary Supplements; Lisa Rogers, Nutrition for Health and Development, WHO, Geneva). We acknowledge Kamilla Eriksen, Sophie Moore, Ryan Wessells, and Sonja Hess for providing unpublished B12 prevalence data to construct Figure 5. Each panel member contributed significant sections of the manuscript. The authors' responsibilities were as follows—LHA, JWM, and DJR: oversaw the compilation and revisions of the review with input of all panel members throughout the process, edited all sections of the manuscript, and had primary responsibility for the final content; and all authors: read and approved the final manuscript.

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