Special Article

Methods to assess vitamin B_{12} bioavailability and technologies to enhance its absorption

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Vitamin B_{12} (B-12) deficiency is still relatively common in low-, medium-, and high-income countries, mainly because of dietary inadequacy and, to a lesser extent, malabsorption. This narrative review is based on a systematic search of evidence on methods to assess B-12 bioavailability and technologies to enhance its absorption. A total of 2523 scientific articles identified in PubMed and 1572 patents identified in Orbit Intelligence were prescreened. Among the reviewed methods, Schilling's test and/or its food-based version (using cobalamin-labeled egg yolk) were used for decades but have been discontinued, largely because they required radioactive cobalt. The qualitative CobaSorb test, based on changes in circulating holo-transcobalamin before and after B-12 administration, and the ^{14}C-labeled B-12 test for quantitative measurement of absorption of a low-dose radioactive tracer are currently the best available methods. Various forms of B-12 co-formulated with chemical enhancers (ie, salcaprozate sodium, 8-amino caprylate) or supplied via biotechnological methods (ie, microbiological techniques, plant cells expressing cobalamin binding proteins), encapsulation techniques (ie, emulsions, use of chitosan particles), and alternative routes of administration (ie, intranasal, transdermal administration) were identified as potential technologies to enhance B-12 absorption in humans. However, in most cases the evidence of absorption enhancement is limited.

INTRODUCTION

Vitamin B_{12} (B-12) is an essential vitamin that humans must ingest through dietary sources, including supplements or fortified food. In nature, B-12 is only present in food from animal sources.[1] Biochemical data indicate that the global prevalence of B-12 deficiency (based on combined low [<148 pmol/L] and marginal [148–221 pmol/L] serum/plasma B-12 values) varies widely, with some countries having rates that exceed 40%.[2] Infants, young children, women of reproductive age, pregnant and lactating women, vegetarians, and the elderly are groups at higher risk.[2] Maternal B-12 depletion or low B-12 intake during pregnancy and/or lactation increases the risk of delayed development in the offspring. This problem is exacerbated when depleted mothers exclusively breastfeed because of the low concentration of B-12 in their milk.[3] Older adults are at higher risk of deficiency due to physiologically impaired absorption associated with aging.[4]

B-12 is essential for normal erythropoiesis and neurological functions. In the cytoplasm, B-12 participates in the conversion of homocysteine to methionine, a precursor of the universal methyl group donor,

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S-adenosylmethionine.[4] Thus, B-12 is required for methyl group turnover during the synthesis of creatine, phospholipids, proteins, lipids, neurotransmitters, and deoxyribonucleic and ribonucleic acids.[4] B-12 is also a cofactor in the intramitochondrial conversion of methylmalonyl-CoA to succinyl-CoA catalyzed by methylmalonyl-CoA mutase.[4] Considering the key role of B-12 in these metabolic processes, the importance of optimal status is not limited to the hematological and neurological domains but also involves general health, including energy utilization and the prevention of neural tube defects.[2,3] Biochemically, B-12 deficiency is usually characterized by low concentrations of the circulating serum B-12 and holo-transcobalamin (holoTC), accompanied by elevated total homocysteine in plasma and elevated methylmalonic acid in serum and urine.[5] As with other micronutrient deficiencies, the best approach to reduce the prevalence of B-12 deficiency is to ensure consumption of a healthy balanced diet. Low intake of food from animal sources is common in most developing countries because of poor accessibility, high cost, or cultural-religious beliefs.[1] As a result, B-12 supplementation programs and fortified products that target vulnerable populations could substantially reduce the prevalence of this deficiency. However, effectiveness of these approaches is conditioned by several factors, including the vitamin dose, bioavailability from the food source or delivery vehicle, frequency of supplementation, and the health and social conditions of the targeted population.[6]

Several endogenous and exogenous factors have been shown to profoundly affect B-12 absorption,[7] including genetic or acquired diseases that result in decreased production of intrinsic factor (IF),[8] atrophic gastritis, and malabsorption due to long-term chronic infection with *Helicobacter pylori* and/or bacterial overgrowth in the small intestine.[9–13] Gastrointestinal malabsorption due to inflammatory bowel disease,[14] celiac disease, tropical sprue, bypass or extensive resection of the ileum,[15] total or partial gastrectomy[16]; and parasitic infestations (eg, *Diphyllobothrium latum*, *Giardia lamblia)* are also recognized factors affecting B-12 absorption. Zollinger-Ellison syndrome and exocrine pancreatic insufficiency are rare causes of B-12 malabsorption caused by low pH in the small intestine and impaired degradation of transcobalamin-I by pancreatic enzymes.[17] Finally, B-12 malabsorption may also be iatrogenic (eg, caused by the use of gastric acid suppression medications that impair the release of B-12 from food).[18,19]

B-12 deficiency may be accompanied by high folate status because of mandatory folic acid fortification of flour in many countries.[20] A negative biochemical interaction between high serum folate and biomarkers of B-12 status has been reported in the National Health and Nutrition Examination Survey and other studies that were mainly conducted in the elderly.[21–26] In addition, some studies have found a negative association between high folate/low B-12 status and cognitive impairment, anemia, and slower nerve conductivity.[21–26] Of particular concern are women during the perinatal period, when future mothers may be exposed to higher amounts of folic acid because of the potential combination of mandatory fortification and folic acid supplementation. For example, in India the prevalence of insulin resistance was higher in offspring from mothers with low serum B-12 (<160 pmol/L) and high red blood cell folate (>1144 vs 807 nmol/L) during pregnancy, as found in the Pune Maternal study.[27] Concerns about excessive folic-acid exposure have also been extended to associations with unmetabolized folic acid, which increases when folic-acid intakes are high.[28] These interactions remain controversial, and randomized controlled trials are needed to substantiate any adverse effects of high folic-acid intake on B-12 status and function. Meanwhile, it is sensible to ensure adequate B-12 intake, absorption, and status, particularly in countries with mandatory folic-acid fortification, where supplementation of folic acid is also common practice across the population and in individuals at risk of B-12 deficiency.

B-12 bioavailability has been studied for decades, and some technologies to enhance the absorption of B-12 have been developed. However, there is uncertainty about the efficacy of different methods, and there has been no critical discussion of existing technologies for measuring and enhancing absorption. This work provides a critical review of the available literature on methodologies to study B-12 bioavailability and technologies to enhance its absorption.

Mechanisms of B-12 absorption: key for methodological and technological development

Active absorption starts when B-12 is released from food and is bound by salivary transcobalamin-I (haptocorrin) (Figure 1).[29] Simultaneously, gastric parietal cells produce hydrochloric acid and IF stimulated by histamine, gastrin, and acetylcholine.[30] In the stomach, haptocorrin protects B-12 from acid degradation, but once in the duodenum, the protein is partially degraded by pancreatic trypsin favoring transfer to IF, which is more resistant to proteolysis. The distal ileum is the primary absorption site of the B-12–IF complex, which is taken up via receptor-mediated calcium-dependent active transport. The complex binds to the cubam receptor (consisting of cubilin and a receptor-associated

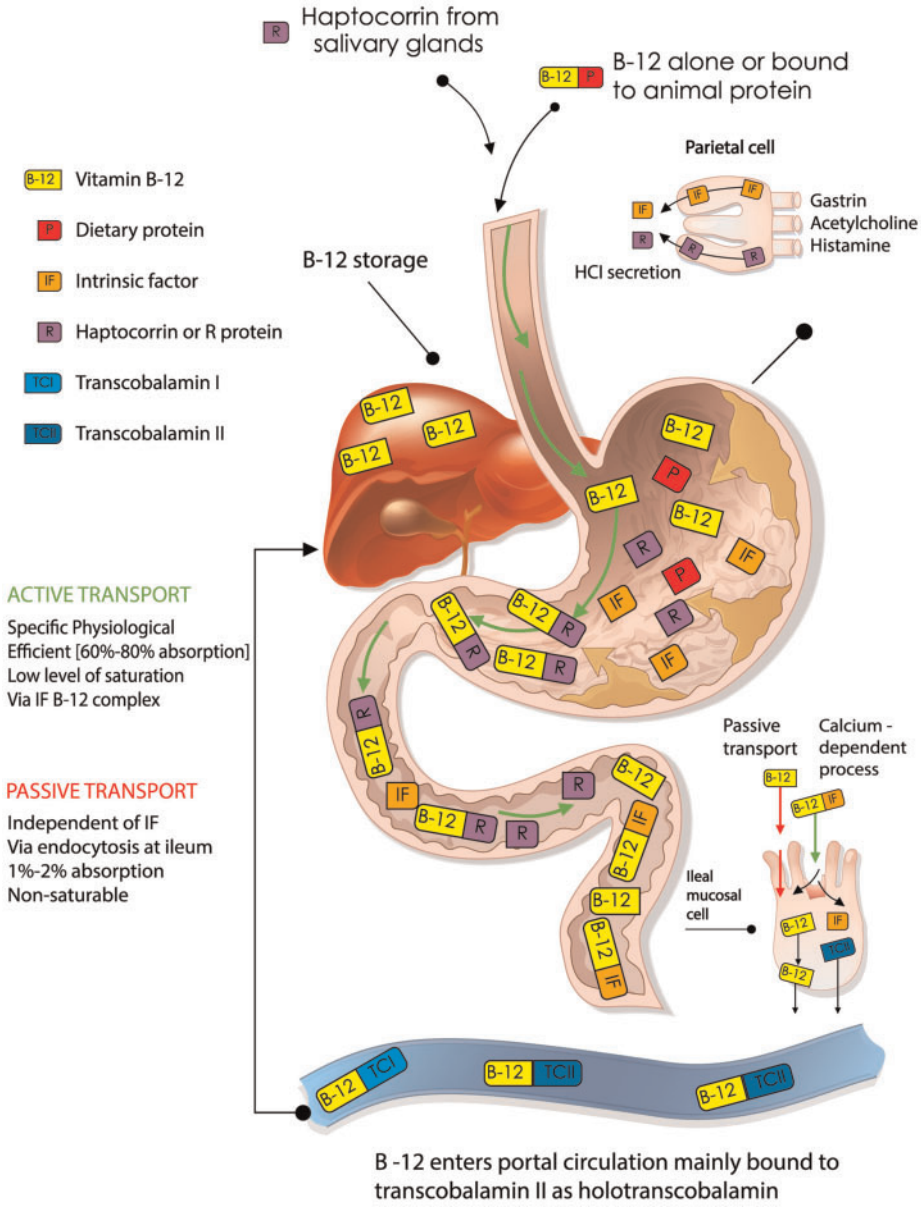

Figure 1 **Vitamin B_{12} absorption.** Active absorption of dietary B-12 in humans is a complex, multistep process. First, B-12 is released from food proteins by proteolysis in the acidic stomach and bound to salivary haptocorrin, which protects B-12 from acid degradation. Second, the B-12–haptocorrin complex is degraded due to the action of pancreatic trypsin and then binds to intrinsic factor (IF), a glycoprotein produced by the gastric parietal cells (that secreted hydrochloric acid) upon stimulus by histamine, gastrin, and acetylcholine. Third, the B-12–IF complex enters the mucosal cells in the distal ileum by cubam receptor–mediated endocytosis. Fourth, after internalization within the ileal enterocyte, the IF–B-12 complex enters lysosomes in which IF is degraded. Then, the B-12 is either metabolized to its active cofactor forms for use within the enterocyte or processed for release into the portal circulation. Abbreviations: HCl, hydrochloric acid; IF, intrinsic factor.

protein).[18,31,32] Upon its internalization within the ileal enterocyte, IF is degraded in lysosomes, releasing the free B-12. Subsequently, B-12 is either metabolized to its active cofactor forms for use within the enterocyte or transferred into the portal circulation by the ABC drug transport protein ABCC1 (also known as multidrug resistance protein 1) The free cobalamin binds to unsaturated transcobalamin II (TC) and is transported as holoTC in serum, from which it is taken up by cells.[33] Uptake of the TC–B-12 complex into tissues occurs by receptor-mediated endocytosis. HoloTC is then degraded in lysosomes, releasing B-12 to be further converted to its cofactor forms. Between 0.5 and 5.0 µg of B-12 is excreted in bile per day. This biliary B-12 is readily reabsorbed across the ileal enterocyte, and thus enterohepatic circulation represents a mechanism by which B-12 is recycled in the body.[2] A small fraction of overall B-12 absorption (~1%–2% of an oral dose)

occurs by passive diffusion.[2] This pathway is inefficient but important, especially in populations with low or zero IF (eg, gastrectomized patients). Passive absorption of B-12 also occurs in mucous membranes when it is administered into the nose.[34]

METHODOLOGY

Identification and selection of studies and patents

A systematic search of scientific articles complemented by an exhaustive search of published patents was conducted in June 2017 and revised in July 2017. Evidence regarding B-12 absorption, B-12 bioavailability, and technologies to enhance the absorption of B-12 was considered. Combinations of the terms ("vitamin B-12" OR "vitamin B12" OR "cobalamin") AND ("bioavailability" OR "absorption") were used in PubMed. In addition, commercial websites were assessed to identify potential sources of information. The exhaustive search of patents was done in Orbit Intelligence (Paris, France), an integrative database for intellectual and industrial property. Patents were selected using a search engine based on the combination of the following keywords: "vitamin B-12" OR "vitamin B12" OR "cobalamin"; "absorbed" OR "absorption" OR "digestion" OR "bioavailability" OR "uptake"; "microorganism" OR "probiotic" OR "lactobacilli" OR "reuteri" OR "gasseri" OR "bifidobacter" OR "longum" OR "bifidus." Patent classes A23L1–C12N were considered for identification of food particles and the use of microorganisms or enzymes in the formulation. The figure was plotted using Adobe Illustrator version 11.0 (Adobe System, San Jose, CA, USA).[35]

Eligibility criteria and study selection

The majority of the evidence reported in this review was based on intestinal absorption of B-12, with a limited number of publications focused on absorption by nonintestinal routes. This review considered any evidence relevant to the development of methodologies to assess B-12 bioavailability and technologies to enhance B-12 absorption regardless of absorption route. In vivo and in vitro studies were included; studies conducted in humans, animals, or cells were also considered. In the case of clinical human studies, those that focused of application of methods to assess B-12 absorption in pathologies were excluded, unless the information was relevant to the context of methodological or technological development. Studies were not excluded based on language or year of publication.

RESULTS AND DISCUSSION

A total of 2523 studies were identified in PubMed. After exclusion of 2225 studies because the information was not eligible or relevant for the present review, 298 studies were initially selected. After in-depth review of the titles and abstracts, an additional 188 studies were excluded, leaving 75 studies focused on methods and 35 studies focused on technologies. Finally, 72 studies for methods and 26 for technology were included in the review after further exclusions as well as inclusions of references recommended by experts in the field and 1 patent found for methods (Figure 2*A*). A total of 1572 patents were identified in Orbit Intelligence, 1113 of which were excluded after checking eligibility criteria. From this total of 459 early selected patents, 42 were selected for final inclusion, after exclusion of those not relevant (Figure 2*B*).

Methods to measure vitamin B_{12} absorption

Schilling's test, the egg-yolk cobalamin absorption test, the CobaSorb test, and the use of ^{14}C-labeled B-12 are the main methods used to assess B-12 absorption. Table 1 summarizes these methods.

Assessment of bioavailability using colabeled B-12 and Schilling's test for malabsorption. The first attempts to quantify B-12 absorption were conducted several decades ago.[36] Pioneering work used B-12 radioactively labeled with ^{57}Co, ^{58}Co, or ^{60}Co with 1–100 nanocuries (nCi).[37–47] The most common approach consisted of giving individuals a dose of labeled B-12 and calculating absorption based on recovery of the unabsorbed isotope in urine and/or feces.[48–57] An alternative third approach that involves performing serum counting of the isotope has been proposed.[42,58–61] Among these different methods, the Schilling's test, which is described in detail below, has received the most attention and application.

The Schilling's test was the gold-standard test used for decades to detect defective B-12 absorption, especially in patients with B-12 deficiency, and to diagnose malabsorption in the elderly. The multiple-stage method was developed by Professor Robert F. Schilling[62–66] and tested in both humans and animals.[67] In the first stage of the method, oral administration of radiolabeled B-12, usually as ^{57}Co or ^{58}Co, was followed an hour later by a 1-mg intramuscular dose of unlabeled B-12 that aimed to saturate blood transport of B-12 and ensure that a measurable amount of isotope appears in the urine during the first 24 hours.[62] If $< 10\%$ of the oral dose appeared in urine, the diagnosis was malabsorption. In the second stage, if abnormal absorption was found, the test was repeated with the addition of a

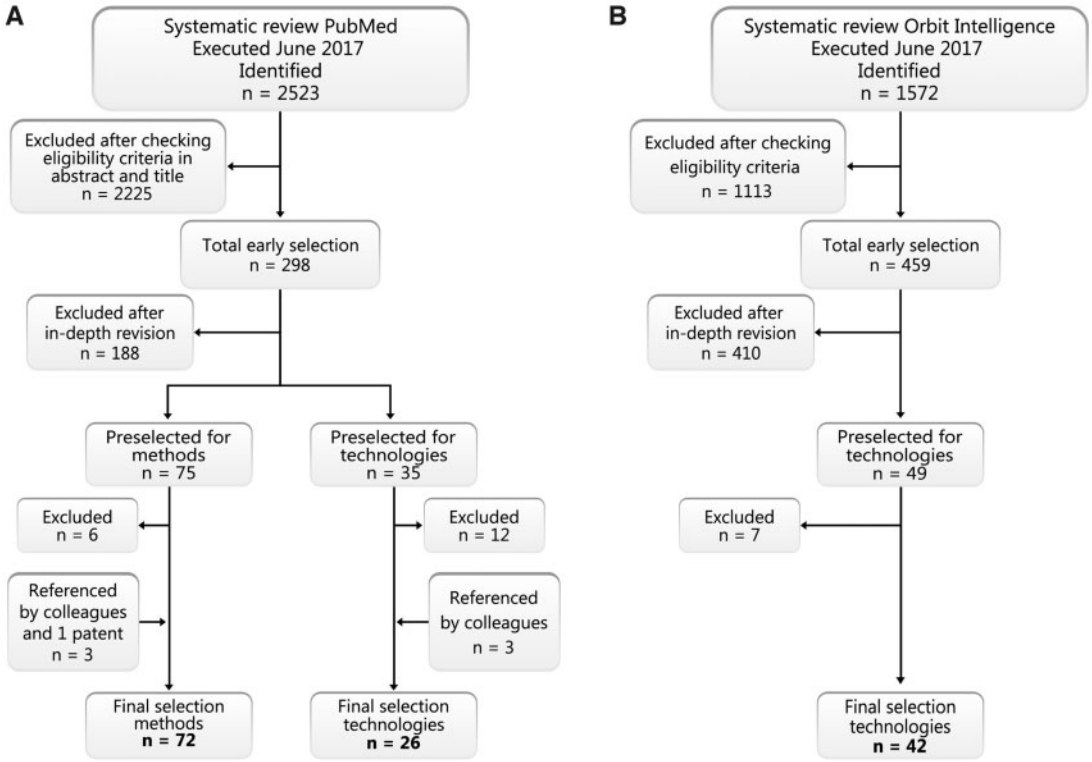

Figure 2 **Systematic search. A**, Systematic search of methods and technologies in PubMed. **B**, Systematic search of patents for technologies in Orbit Intelligence.

Table 1 **Methodologies to measure vitamin B_{12} absorption**

Method	General description	Advantages	Limitations
Schilling's test	Tests based on oral administration of radioactive B-12 labeled mainly with ^{57}Co and/or ^{58}Co. An intramuscular dose (1 mg) of unlabeled B-12 is given 1 h later, and urine radioactivity is measured during the first 24 h. If $<$10% of the oral dose appears in urine, this is defined as diagnosing malabsorption. Repetition of the test is suggested with IF to diagnose pernicious anemia	Mainly used to diagnose malabsorption in the elderly or in pernicious anemia Widely used in the past in clinical practice	Provides a qualitative estimate of B-12 absorption Requires exposure to radioactivity In its simplest form does not assess absorption from B-12 in food Need to collect a complete urine sample for 24 h (often difficult in elderly people) Not currently used due to lack of availability of radiolabeled B-12 required for the test, high cost, difficulty for patients
The egg-yolk cobalamin absorption test	Modification of Schilling's test, by mixing the labeled B-12 with albumin, egg yolk, or chicken	Most often used in clinical investigations	Same limitations as Schilling's test except that it can detect malabsorption of the vitamin bound to food
CobaSorb test	By giving 3 9-μg doses of crystalline B-12 in water over the period of 24 h and measuring the increase in serum holoTC on the following day, malabsorbers can be detected	Detection of B-12 malabsorption Useful for determining if patients will respond to low-dose B-12 supplements or require treatment with pharmacological doses Readily available for diagnostic or research use	Qualitative assay. It measures relative absorption but does not provide a quantitative estimate of bioavailability The test cannot be used once the patient is treated with B-12
^{14}C-labeled B-12	Biotechnological production of ^{14}C–B-12. Measurement of the isotope excreted in urine and feces by accelerator mass spectrometry	Very low dose of radioactivity Quantitative estimate of the percentage absorbed Test for food-bound absorption or crystalline form	Not yet been developed into a clinical test

Abbreviations: C, carbon; CO, cobalt; holoTC, holo-transcobalamin; IF, intrinsic factor.

dose of oral IF mixed with water.[68] If the second urine collection contained > 10% of the oral dose, this was a sign of poor IF production and led to the identification of probable pernicious anemia.[69] For confirmation of pernicious anemia, an augmented Schilling's test was proposed by which patients who failed to respond to the standard test received 8 times the conventional dose.[70] Many modifications and alternatives have been proposed through the years, including giving different cobalt radioisotopes, usually ^{57}Co and ^{58}Co, at the same time and thus only requiring a single radioactive urine collection (a double-isotope technique)[71–76]; using labeled B-12 co-ingested with antibiotics to identify patients with bacterial overgrowth; and providing labeled B-12 with pancreatic enzymes to identify patients with pancreatitis.[77] Validation of Schilling's test was a topic of interest,[78–80] often used for comparative purposes against other methods of assessing absorption. For example, an effort to introduce an unlabeled B-12 technique failed after comparison with Schilling's test.[81]

An alternative to Schilling's test was the fractional B-12 absorption test "FAB12," a double-isotope technique that included the addition of carmine powder, ^{51}Cr-chromic chloride, and a single stool sample test, as well as a complete stool collection.[82,83] The fractional B-12 absorption test based on a single stool sample that had the highest content of ^{51}Cr (corresponding to the most carmine-colored stool) correlated closely with the fractional B-12 absorption test based on complete stool collection.[82,83] Nowadays, Schilling's test and alternatives methodologies, such as the double-isotope Schilling's test for measuring absorption of food-bound and free B-12 simultaneously,[84] have virtually disappeared from standard clinical practice. The main reasons for this are 1) difficulties implementing the tests, including failure of the simpler standard Schilling test to detect food-bound B-12 malabsorption; 2) lack of availability of radiolabeled B-12; 3) the need to dose with radioactivity and to accurately collect patient's urine for 24 hours; and 4) high cost.

Egg-yolk cobalamin absorption test. Schilling's test was usually performed with B-12 in its crystalline form without considering the food matrix effect. However, to detect food cobalamin malabsorption, the test was modified by mixing labeled B-12 with egg yolk (the egg-yolk cobalamin absorption test). Other proteins such as albumin,[85] chicken serum, or chicken meat were also used, but egg yolk was most predominant. This approach was used frequently in clinical research studies.[86,87]

CobaSorb test for cobalamin malabsorption. The CobaSorb is one of the most advanced and reliable tests available for diagnosis of B-12 malabsorption. This qualitative assay is based on the analysis of cyanocobalamin carried as holoTC (the active form of B-12) in serum before and after oral intake of the vitamin.[88] This test is the most readily available for diagnostic or research use.[88,89]

Lindgren and coworkers[90] demonstrated that holoTC is a sensitive marker of cobalamin malabsorption by showing a high correlation between cobalamin malabsorption and holotTC in patients with low levels of holoTC (< 35 pmol/L). Later, Bor and collaborators[91] also showed that holoTC concentrations (and TC saturation) reflected B-12 absorption better than serum B-12 levels because holoTC concentrations and TC saturation produced a higher increase than serum B-12 after oral administration of 3 high physiological doses (9 µg) of B-12.

The CobaSorb test was further performed in patients with inherited malabsorption of B-12 attributable to Imerslund-Gräsbeck syndrome or with IF deficiency, their heterozygous biological parents, and healthy controls.[92] Three 9-µg doses of B-12 were given orally at 6-hour intervals, and serum B-12 and holoTC were measured 24 hours after the last dose was given. In the patients, there were no changes in B-12 or holoTC after the B-12 load, whereas in controls there were increases in B-12 and holoTC.[92] Thus, the CobaSorb test was able to confirm that holoTC was the best marker for reflecting B-12 absorption.[93] The performance of the test was evaluated using a cutpoint for holoTC or B-12 of < 75% percentile of healthy absorbers. Changes were well-reflected by an increase in holoTC after 2 days of administration of oral B-12.[93] Further assessments included the optimal timeline for B-12 administration and the magnitude and patterns of change in the postabsorption response of holoTC to oral B-12.[94] Healthy adults with normal B-12 status were given the 3 9-µg doses of B-12 at 6-hour intervals beginning early in the morning (baseline) on day 1. Blood was taken at 17 timed intervals over the course of 3 days for analyses of holoTC and other indicators of B-12 status.[94] The timeframe for this methodology is based on the observation that the greatest increase in holoTC was observed 24 hours after B-12 ingestion.[94] By measuring the different forms of cobalamin isolated by high-performance liquid chromatography and quantified with an enzyme-linked immunosorbent essay,[88] the CobaSorb test showed that cyanocobalamin is absorbed unchanged in healthy individuals, indicating that cyanocobalamin bound to TC reflects B-12 absorption better than the total B-12 bound to TC.[88] Increases in serum holoTC have also been used to reliably assess B-12 absorption in individuals with low serum B-12.[95] For example, in the Pune Maternal Study in India, a population for which the main cause of B-12 deficiency

is presumably low intake of food from animal sources, participants had a rise in serum holoTC concentrations (a proxy for B-12 absorption) after they were given a standard dose of oral B-12.[95]

The CobaSorb test has also been used to investigate whether treatments with intramuscular B-12 injections were effective in patients with B-12 deficiency and to identify individuals able to absorb B-12 orally versus the parenteral route.[89] The study highlighted the importance of considering the capacity of an individual to absorb B-12 prior to treatment.[89] The CobaSorb test has also served biochemical validation purposes—for example, to validate the commercial enzyme immunoassay kit for measurement of holoTC.[96]

Currently, the CobaSorb test is based on giving 9-μg doses of crystalline B-12 in water 3 times over a 24-hour period and measuring the increase in serum holoTC on the following day to identify malabsorbers. To detect an increase in holoTC, the test has to be performed before participants are treated for B-12 deficiency. Detection of B-12 malabsorption using the CobaSorb test can help to determine whether patients will respond to low-dose B-12 supplements or require treatment with pharmacological doses of the vitamin, either oral or parenteral. The CobaSorb test measures relative absorption, and it is important to recognize that it does not provide a quantitative estimate of bioavailability.[97]

Assessment of bioavailability using ^{14}C-labeled B-12. Accelerator mass spectrometry has been proposed for assessing absorption and kinetics of ^{14}C-labeled substances after oral ingestion and can measure levels of isotopic carbon in tiny volumes (microliters) of biological samples with negligible exposure to radioactivity. The radioactivity from this tracer can be measured at doses as low as approximately 30 nCi, which makes it an excellent alternative for human feeding trials that require detection of absorbed radioactivity in plasma or urine for a long period of time. The ^{14}C-labeled B-12 is produced by growing *Salmonella enterica* in a medium containing ^{14}C dimethylbenzimidazole, which results in cobalamin labeled in the dimethylbenzimidazole ring rather than cobalt in the corrin ring.[98] The test has been used with doses of B-12 in the range of normal dietary intake (1.4–2.6 μg). In humans, a physiological dose (1.5 μg, 2.2 kilobecquerel/59 nCi) of purified ^{14}C-labeled B-12 was administered, and it showed plasma appearance and clearance curves consistent with the predicted behavior of the natural vitamin. ^{14}C-labeled B-12 has been used to measure bioavailability from chicken eggs labeled in vivo by injecting hens once a day with the labeled vitamin over a 4-day period (unpublished data). A high sensitivity of the test was observed compared with the use of labeled cobalt (as in the Schilling's test) in an intervention where a single dose of labeled eggs given to humans showed a several-fold increase of radioactive cobalamin in urine after 8 days of urine and feces collection. Because the ^{14}C was incorporated into the dimethylbenzimidazole ring, the outcome of this study suggested that the moiety is detached from the unabsorbed vitamin in the intestine, probably by bacteria.[98] The ^{14}C-labeling method confirmed that the relative bioavailability was inversely proportional to the dose consumed due to saturation of the active absorption receptor, even within the range of usual intake from foods.[1] This method has not yet been developed into a clinical test, but it opens new avenues for studying B-12 assimilation and kinetics in humans.[98]

Efficiency of absorption of a single oral dose of vitamin B_{12} across a range of intakes

The first studies based on radioactive isotopes showed that the efficiency of absorption of B-12 was inversely proportional to the dose. Indeed, 50% absorption was observed for a 1-μg oral dose, 20% for a 5-μg dose, and approximately 5% for a 25-μg dose.[99,100] After 4–6 hours there was no inhibitory effect of the first dose on the absorption of subsequent doses.[49] At doses $\geq$ 500 μg, only 1% was absorbed.[101] The reason for this is saturation of the B-12–IF receptors in the ileum at higher doses. Although, the B-12–IF complex receptor is saturated at relatively low doses, 1%–2% of an oral dose is passively absorbed from any level of intake that exceeds the specific binding capacity of IF in the digestive tract.[36]

Vitamin B_{12} bioavailability from foods

In general, an absorption of 50% of the vitamin from food is assumed when estimating, for example, B-12 requirements.[100,102] Studies assessing bioavailability of B-12 from different food sources in healthy participants showed that absorption ranges 36%–24% for egg products (dose 0.3–0.94 μg, respectively), 83%–52% for lean meat (dose 0.54–5.11 μg, respectively), 42%–30% for fish (dose 2.1–13.1 μg, respectively) and 49%–4.5% for liver products (dose 0.5–38 μg, respectively). The turnover of the vitamin in these studies was measured as loss in body radioactivity after administration of a dose of radioactive co-labeled B-12 or as B-12 excretion in bile corrected for estimated reabsorption.[103] In the case of B-12 bound to food, the proteins present in the meal increase the intragastric pH,[104] a situation that is sensed by antral G cells, triggering the release of gastrin, which increases the secretion of hydrochloric acid and IF by

the parietal cells.[105] In such a situation, the higher secretion of IF is hypothesized to play a key role in increasing the efficiency of B-12 absorption from protein-bound B-12.

Technologies to enhance B-12 absorption

Information on technologies to enhance B-12 absorption that was found in the scientific articles and patents included in this review was organized and classified into 4 main categories: 1) different forms of B-12 coformulated with enhancers; 2) alternative biotechnological solutions; 3) encapsulation technologies; and 4) alternative routes of administration (Table 2).[106–174] Technological solutions have focused on both passive and active absorption mechanisms. An example of a technological solution that aims to enhance B-12 absorption using the regular B-12 active absorption process is the use of B-12 bound to transport proteins, including IF or conjugates of B-12 proteins or peptides. Other technologies, such as the use of salcaprozate sodium (SNAC), have been proposed to enhance B-12 absorption via passive diffusion. Among the different technological solutions proposed in the literature, only B-12 formulated with SNAC and B-12 formulated with recombinant human IF were identified as having fairly good evidence of their enhancing effect on the absorption of B-12; strong scientific evidence is lacking for other proposed technologies.

Different forms of B-12 coformulated with enhancers. Ding et al[106] reported the enhanced absorption of B-12 coformulated with SNAC via the formation of noncovalent complexes capable of transcellular passage without altering tight junctions. Later, Castelli and colleagues[107] reported improved B-12 bioavailability of an oral formulation of cyanocobalamin with SNAC (4.7%) versus commercially available cyanocobalamin (2.2%) when given as a single 5-mg dose in healthy individuals. The efficacy and tolerability of the B-12 formulation with SNAC was further investigated in individuals with serum B-12 concentrations < 350 pg/mL[108]. A dose of 1000 µg/day of B-12 in this oral formulation was provided to 22 patients for 90 days. The effects on B-12 levels were compared with that of 26 patients who were administered 1000 µg of intramuscular B-12 on 9 occasions for 90 days. Both the oral and intramuscular formulations were effective in restoring normal B-12 status, and no differences or adverse effects were attributed to the use of SNAC. At those levels of B-12 provision and at those frequencies, B-12 status would be expected to improve with or without inclusion of SNAC in the oral formulation. Therefore, this study was unable to prove the absorption-enhancing property of SNAC because B-12 status was improved by B-12 provision, rather than by addition of the potential enhancer.

B-12 bound to transport proteins, IF, or conjugates of B-12 proteins or peptides has been proposed as a way to administer bioavailable B-12. Cobalamin transport proteins are not limited to IF, but also include transcobalamin-I, transcobalamin–II, and transcobalamin-III.[109–115] A TC–B-12 complex derived from bovine milk has been highlighted for its capacity to stimulate the uptake of B-12 in cultured bovine, mouse, and human cell lines.[116] The complex stimulated the uptake of B-12 via the apical surface of differentiated Caco-2 human intestinal epithelial cells.[116] In rats, however, cobalamin bound to bovine milk proteins was absorbed as efficiently as free cobalamin, and comparable amounts of hydroxocobalamin (the main form in animal products, including milk) and cyanocobalamin were absorbed. Interestingly, hydroxocobalamin was found to accumulate at 3-fold higher levels in the liver than cyanocobalamin, while less remained in the kidney and plasma.[117] The efficacy and metabolism of these forms of B-12 should be examined in clinical studies, as well as in isolated intestinal tissue and whole animals, to confirm in vitro data.

Oral combinations of 8-amino caprylate or amino derivatives,[118,119] mixtures of B-12 chemical forms,[120–123] B-12 formulations with D-sorbitol,[124–126] poly(acrylic acid)–cysteine,[127] diterpene glycoside and triterpene glycoside,[128–130] B-12 bound by a glycoprotein matrix,[131] and resin adsorbate have been proposed for the administration of B-12.[132] However, it is unclear whether there is a formulation effect on B-12 bioavailability and what mechanisms are involved, as well as to what extent better absorption should be expected.

Alternative biotechnological solutions. The evidence of biotechnological solutions for enhancement of absorption is limited except for evidence for binding B-12 to recombinant human IF. Fedosov and colleagues[133] suggested the use of a recombinant human IF expressed in the plant *Arabidopsis thaliana* as an approach to enhance B-12 absorption. The IF produced by this method bound B-12 and interacted with the cubilin receptor with good or better affinity than human IF.[133] Using the CobaSorb test, Hvas and colleagues demonstrated that the recombinant human IF promoted B-12 absorption among patients with evident B-12 deficiency.[134] There are several commercial sources of recombinant human IF, but to the authors' knowledge it is not being used clinically. Many commercial B-12 supplements now contain porcine-derived IF, but the amount of B-12 is also very large, and it is unclear whether the IF has additional benefit, especially considering that the supplements would usually be consumed only once a day.

Table 2 **Technologies identified as potential enhancers of vitamin B_{12} absorption**

Technology	Description	References
Different forms of B12 coformulated with enhancers		
B-12 formulation with salcaprozate sodium as absorption enhancer	Salcaprozate sodium has been proposed to enhance B-12 absorption by forming a noncovalent complex that enables transcellular absorption without altering tight junctions	Ding et al (2004)[106], Castelli et al (2011)[107], Castelli et al (2011)[108]
B-12 bound to transport proteins, IF, or conjugates of B-12 proteins or peptides	B-12 transport proteins are administered in combination with B-12. Cobalamin transport proteins include, but are not limited to IF, transcobalamin-I, transcobalamin–II, and transcobalamin-III. Formulations include proteins conjugated to B-12	Ellenbogen et al (1962)[109], Oertli R (1957)[110], Collins (2003)[111], Russell-Jones et al (1994)[112], Seetharam and Bose (2001)[113], Serfontein (1994)[114], Habberfield et al (1996)[115], Hine et al (2014)[116], Williams and Spray (1968)[117]
B-12 formulation with 8-amino caprylate or amino derivates as absorption enhancers	Oral preparations containing combinations of 8-amino caprylate or amino derivatives	Yuhong (2012)[118], Castelli and Kragie (2009)[119]
B-12 formulations containing mixtures of B-12 chemical forms to enhance absorption	Mixtures of chemical forms, including cyanocobalamin, adenosylcobalamin, hydroxocobalamin, methylcobalamin, in substantially equivalent ratios and with pharmaceutically acceptable carriers	Brown (2013)[120], Brown (2007)[121,122], Brown (2006)[123]
B-12 formulation with D-sorbitol as absorption enhancers	This polyhydric alcohol received attention as an enhancer of B-12 absorption with studies in humans and animals	Okuda (1961)[124], Okuda et al (1960)[125], Authors Unknown (1959)[126]
Poly(acrylic acid)-cysteine–based formulations	The oral administration of B-12 with poly(acrylic acid)-cysteine improved B-12 absolute bioavailability compared with B-12 with buffer, verapamil, and glutathione	Sarti et al (2013)[127]
Use of diterpene glycoside and triterpene glycoside	Formulations for immediate release comprising B-vitamins developed with the addition of diterpene glycoside and triterpene glycoside	Burge et al (2016)[128], Copp (2016)[129], Koch et al (2015)[130]
B-12 bound by a glycoprotein matrix	Formulation comprising B-12 bound by a glycoprotein matrix	Chokshi (2002)[131]
Use of resin	Resin adsorbate proposed for the administration of B-12	Davis et al (1982)[132]
Alternative biotechnological solutions		
Plant cells for expression of cobalamin binding proteins	Plant cells are transformed with nucleotide sequences adapted for expression and secretion of B-12 binding proteins	Fedosov et al (2003)[133], Hvas et al (2006)[134]
Microbiological technologies	Microbiological technologies include natural enrichment of beverages with multiple vitamins by fermentation or the use of probiotics	Hugenholtz and Strachotta (2014)[135], Kelemen et al (1987)[136], Johan et al (1976)[137], Szemler et al (1971)[138], Mogna et al (2013)[139], Mogna et al (2012)[140], Adams and Huang (2003)[141], Hugenschmidt et al (2011)[142], Madhu et al (2010)[143], Zelder et al (2008)[144], Bijl and Sardjoepersad (2004)[145], Bijl (1998)[146], Berglund et al (2003)[147]
Encapsulation techniques		
Emulsions	Method for delivering B-12 via a suspension or emulsion of water-soluble vitamins and/or minerals in edible oils	Schramm et al (2008)[148], Schramm and McGrath (2005)[149]
Chitosan nanoparticles as delivery agent	A chitosan derivative capable of being specifically absorbed by the small intestine accompanied by a preparation method and a drug-carrying nanoparticle of the derivative and B-12	Yang et al (2017)[150], Goto et al (2015)[151]
Hydrogels and hydrophilic polymers for sustained release dosage	Oral administration of a sustained release nutritional supplement containing B-12, which elevates B-12 in plasma for a period of at least 12 h after ingestion	Smidt et al (2009)[152]

(continued)

Table 2 **Continued**

Technology	Description	References
Use of soy protein isolate nanoparticles	Soy protein isolate nanoparticles have been proposed as a promising carrier to facilitate the oral delivery of B-12	Zhang et al (2015)[153]
Alternative routes of administration		
Intranasal administration of B-12	Compositions have been designed for nasal administration	Wenig (1986)[154], Quay et al (2016)[155], Riepma (2014)[156], Patel et al (2012)[157], De Casteele and Gerike (2006)[158], Berenguer Huertas (1995)[159], Wenig (1986)[160], Van den Berg et al (2003)[161], Pisal et al (2004)[162], Garcia-Arieta et al (2001)[163], van Asselt et al (1998)[164], Slot et al (1997)[165], Wenig (1987)[166]
Transdermal administration of B-12	Formulations and devices (transdermal patch). Main principle based on encapsulation in nano-reservoirs to release the vitamin in small quantities and in a sustained manner	Zeltman (2008)[167], Madhaiyan et al (2013)[168], Yang et al (2011)[169]
Transmucosal administration of B-12	Solid compositions comprising B-12 permeation enhancers (ie, isopropyl myristate), a mucoadhesive agent, and a penetration enhancer (chitosan)	Daud et al (2017)[170], Gerike an De Casteele (2007)[171]
Sublingual or buccal administration of B-12	B-12 formulations including propylene glycol, a solid adsorbent, and a solid water-soluble excipient	McCarty (2014)[172]
Buccal bioadhesive devices	B-12–loaded buccoadhesive devices and films have been proposed	Tiwari et al (1999)[173], Mohamad et al (2017)[174]

Abbreviation: IF, intrinsic factor.

Microbiological technologies include the natural enrichment of beverages with multiple vitamins by fermentation[135–138] or probiotics. Some formulations include at least 1 probiotic bacterial strain belonging to the species *Lactobacillus reuteri*,[139,140] *Propionibacterium jensenii*,[141] *Propionibacterium freudenreichii*,[142] and probiotic lactic acid bacterium from kanjika,[143] which are B-12 producers. Genetically modified microorganisms have been described.[144–147] However, there is uncertainty about how useful and effective these technologies are for improving B-12 bioavailability.

Encapsulation techniques. Methods for delivering B-12 via a suspension or emulsion of water-soluble vitamins and/or minerals in edible oils have been developed.[148,149] Particles containing the water-soluble fraction are coated with edible oils as a means to improve absorption through an increased resistance to degradation in the acidic environment of the stomach. A chitosan derivative capable of being specifically absorbed by the small intestine, as well as a preparation method and a drug-carrying nanoparticle of the derivative, has been proposed.[150] In vivo application of chitosan to improve bioavailability of cyanocobalamin has been demonstrated in rats.[151] Goto et al[151] showed that the bioavailability of B-12 was $0.6 \pm 0.2\%$ when the chitosan-free B-12 solution was administered, whereas it increased to $10.5 \pm 3.3\%$ when chitosan was dissolved in the B-12 solution at a concentration of 1%. A method exists for orally administering a sustained-release nutritional supplement containing B-12 based on hydrogels and hydrophilic polymers whereby the encapsulated vitamin is slowly released starting in the stomach and continuing into the upper intestinal tract, elevating B-12 in plasma for at least 12 hours after ingestion.[152] Finally, soy protein isolate nanoparticles have been proposed as a promising carrier to facilitate the oral delivery of B-12 by improving its intestinal transport and absorption. B-12 transport across Caco-2 cell monolayers was increased 2- to 3-fold after nanoencapsulation, depending on particle size, with smaller particles better absorbed ($30 > 100 > 180$ nm soy protein isolate nanoparticles).[153] However, clinical substantiation of this technology in humans has not yet been demonstrated.

Alternative routes of administration. To avoid the barriers existing in oral administration, the following technologies were used: intranasal administration of B-12[154–166]; transdermal administration of B-12 through formulations and devices (transdermal patch)[167–169]; transmucosal administration of B-12[170,171]; sublingual or buccal administration of a B-12 formulation[172]; and buccal bioadhesive devices.[173,174] There is uncertainty about how many of these have

been clinically tested, and evidence for improvements of absorption or status in humans is limited.

Strengths and limitations of this review

This narrative review focused on a broad topic. The systematic search was based on specified comprehensive sources, with an explicit search approach and a criterion-based selection, uniformly applied. The synthesis of the information was qualitative, and the inferences were based on evidence. A unique aspect of this review was the incorporation of an exhaustive search of patents. Furthermore, the review was not restricted to specific interventions or exposures, comparators, outcomes, or settings, which allowed for identification of a high spectrum of available evidence and activity in the field of B-12 bioavailability and technologies to enhance it.

It was not posible to synthesize the information quantitatively as recommended for systematic reviews (Table S1 in the Supporting Information online).[175] We acknowledge that it is possible that some available evidence was not identified in the search, and there may be risk of publication bias.

Future perspectives

Technologies are highly desired for improving B-12 absorption in elderly individuals with B-12 absorption problems and for those with malabsorption associated with reduced secretion of IF and low dietary intake of B-12. In the case of advanced gastric problems, a technology such as SNAC may be effective as a single oral solution. Questions remain about the methods and technologies that would be most effective for increasing absorption in subgroups of apparently healthy populations, such as infants, young children, nonpregnant women, and pregnant women. Intrinsic factor deserves special attention in the context of both methods and technologies. Ideally, technologies to enhance B-12 absorption must take into consideration the role of B-12–specific binding glycoproteins with high affinity for B-12 in the alkaline medium of the duodenum.[176]

Several approaches have considered individual or mixtures of chemical forms of B-12, including cyanocobalamin, aquacobalamin, 5-deoxyadenosylcobalamin, adenosylcobalamin, methylcobalamin, and hydroxocobalamin.[111,112,120–123,128–130,143,151–166] There is not yet clarity about differences in absorption and metabolism of these chemical forms of B-12 and their capacity to accumulate in tissues and improve status.[177–179] Development or optimization of existing strategies are needed to improve basic physiological knowledge (eg, to understand how B-12 absorption in young infants is related to haptocorrin [present in high amounts in human milk][180] and/or TC [which binds cow milk B-12] and to confirm the low percentage of absorption of B-12 from human milk and mechanisms involved in passive diffusion).[181,182] Advances are needed to study appropriate interventions to mitigate B-12 deficiency (eg, to measure the extent to which the limited absorption capacity of B-12 in healthy individuals might explain some of the high prevalence of B-12 deficiency worldwide); to study differences between B-12 analogs[183]; to further improve methods to detect and quantify food-bound malabsorption; to investigate the role of the microbiota; to assess and understand bioavailability values; and to interpret relationships between intake and status.[184]

Improvement of clinical study designs to assess the efficacy of new technologies to enhance B-12 absorption (eg, assessments of clinical or functional outcomes in well-designed, double-blind, randomized controlled clinical trials) and validation of new technologies that attempt to enhance absorption of crystalline B-12 are needed. Also needed are appropriate assessment tests to set more accurate dietary requirements. Recommended dietary intakes of B-12 are approximately 2.0–2.5 μg/day for adults, assuming approximately 50% bioavailability from the diet.[185] Is this 50% independent of the dietary source and method of delivery, such as frequent smaller doses versus daily larger doses? Improving the accuracy of the recommended dietary intakes for B-12, as well as knowledge of the bioavailability of current strategies for B-12 delivery, could benefit current intervention programs.[186,187] A recent national wheat flour fortification program that included B-12[188] showed surprisingly large increases in plasma and breast milk B-12 at the end of 1 year, suggesting that the recommended levels of addition were higher than predicted or necessary. In flour fortification programs, usual intake is likely to be 1–2 μg/day, so absorption from fortified flour is usually estimated at around 50%. However, absorption from repeated small amounts of fortified food appears to be >50%. This requires verification.

CONCLUSION

This work provides a critical review of the available literature on methods to measure the absorption of B-12 and of technologies to enhance its absorption. Absorption tests are important for assessing and interpreting bioavailability values, setting dietary requirements, and interpreting relationships between intake and status of the vitamin. Advances in the development of formulations, technologies, and discoveries are needed to confirm the utility of agents whose function

is to enhance gastrointestinal absorption and therefore improve B-12 status and protect health.

Supporting Information

Table S1 **Characteristics of narrative and systematic literature reviews versus present review**

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Author contributions. A.B. participated in the concept and design of the study, executed the systematic search of scientific articles, interpreted data, and wrote the manuscript. E.H. participated in the concept and design of the study, supervised the execution of the systematic search of patents, interpreted data, and provided input to the drafts of the manuscript. I.S.-Z., N.G., and L.H.A. participated in the concept of the study, interpreted data, and provided input to the drafts of the manuscript. All authors read and approved the final version of the manuscript. A.B. has final responsibility for all parts of the manuscript.

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