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Vitamin B₁₂ status in health and disease: a critical review. Diagnosis of deficiency and insufficiency – clinical and laboratory pitfalls

Agata Sobczyńska-Malefora^{a,b} , Edgard Delvin^{c,d}, Andrew McCaddon^e, Kourosh R. Ahmadi^f and Dominic J. Harrington^{a,b} 

^aThe Nutristasis Unit, Viapath, St. Thomas' Hospital, London, UK; ^bFaculty of Life Sciences and Medicine, King's College London, London, UK; ^cSainte-Justine UHC Research Centre, Montreal, Canada; ^dDepartment of Biochemistry and Molecular Medicine, University of Montreal, Montreal, Canada; ^eChester Medical School, University of Chester, Chester, UK; ^fDepartment of Nutrition & Metabolism, School of Biosciences and Medicine, University of Surrey, Guildford, UK

ABSTRACT

Vitamin B₁₂ (cobalamin) is an essential cofactor for two metabolic pathways. It is obtained principally from food of animal origin. Cobalamin becomes bioavailable through a series of steps pertaining to its release from dietary protein, intrinsic factor-mediated absorption, haptocorrin or transcobalamin-mediated transport, cellular uptake, and two enzymatic conversions (*via* methionine synthase and methylmalonyl-CoA-mutase) into cofactor forms: methylcobalamin and adenosylcobalamin. Vitamin B₁₂ deficiency can masquerade as a multitude of illnesses, presenting different perspectives from the point of view of the hematologist, neurologist, gastroenterologist, general physician, or dietician. Increased physician vigilance and heightened patient awareness often account for its early presentation, and testing sometimes occurs during a phase of vitamin B₁₂ insufficiency before the main onset of the disease. The chosen test often depends on its availability rather than on the diagnostic performance and sensitivity to irrelevant factors interfering with vitamin B₁₂ markers. Although serum B₁₂ is still the most commonly used and widely available test, diagnostics by holotranscobalamin, serum methylmalonic acid, and plasma homocysteine measurements have grown in the last several years in routine practice. The lack of a robust absorption test, coupled with compromised sensitivity and specificity of other tests (intrinsic factor and gastric parietal cell antibodies), hinders determination of the cause for depleted B₁₂ status. This can lead to incorrect supplementation regimes and uncertainty regarding later treatment. This review discusses currently available knowledge on vitamin B₁₂, informs the reader about the pitfalls of tests for assessing its deficiency, reviews B₁₂ status in various populations at different disease stages, and provides recommendations for interpretation, treatment, and associated risks. Future directions for diagnostics of B₁₂ status and health interventions are also discussed.

Abbreviations: AI: adequate intake; AD: Alzheimer's disease; AIG: autoimmune gastritis; CSF: cerebrospinal fluid; Cbl: cobalamin; Co: cobalt; cB₁₂: combined indicator of vitamin B₁₂ status; CBLA: competitive binding chemiluminescence assays; CN-Cbl: cyanocobalamin; Ado-Cbl: 5'-deoxyadenosylcobalamin; ELISA: enzyme-linked immunosorbent assay; GWAS: genome-wide association studies; HC: haptocorrin; holoHC: holohaptocorrin; holoTC: holotranscobalamin; OH-Cbl: hydroxocobalamin; IGS: Imerslund-Gräsbeck; IM: intramuscular; IF: intrinsic factor; LC-MS/MS: liquid chromatography-tandem mass spectrometry; MS: methionine synthase; Me-Cbl: methylcobalamin; MMA: methylmalonic acid; MCM: methylmalonyl-CoA mutase; 5-MTHF: 5-methyltetrahydrofolate; MAF: minor allele frequency; MRP1: multidrug resistance protein; MS: multiple sclerosis; NHANES: National Health and Nutrition Examination Survey; N₂O: nitrous oxide; OC: oral contraceptives; PCA: parietal cell antibodies; PA: pernicious anemia; PPIs: proton pump inhibitors; RR: risk ratio; RYGB: Roux-en-Y gastric bypass; SAM: S-adenosylmethionine; SG: sleeve gastrectomy; SCDC: subacute combined degeneration of the spinal cord; THF: tetrahydrofolate; tHcy: total plasma homocysteine; TC: transcobalamin; T2D: type 2 diabetes; UK: United Kingdom; US: United States; uMMA/C: urinary MMA/creatinine ratio; B₁₂: vitamin B₁₂

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CONTACT Agata Sobczyńska-Malefora  agata.malefora@viapath.co.uk  The Nutristasis Unit, Viapath, St. Thomas' Hospital, London, SE1 7EH, UK
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Introduction

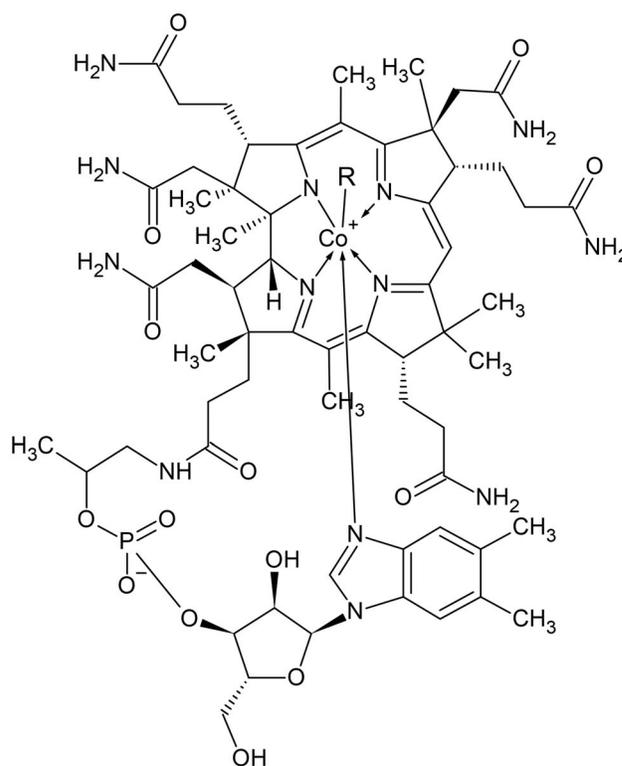
Historical background

As early as the nineteenth century, James Combe [1], Thomas Addison [2], and Anthony Biermer [3] wrote about a fatal diathesis now recognized as vitamin B₁₂ (B₁₂, synonym cobalamin [Cbl]) deficiency caused by pernicious anemia (PA). William Osler and William Gardner described “progressive pernicious anemia” (a term used five years earlier by Biermer) in a 52-year-old Englishman with numbness of the fingers, hands, and forearms, as well as large red blood cells [4]. Increased marrow cellularity had already been reported by Julius Cohnhein [5]. The pathophysiological consequences of PA were extended to spinal cord lesions reported by Lichtheim [6], with Russell et al. coining the term “subacute combined degeneration of the spinal cord” (SCDC). In 1900, Russell noted characteristics of B₁₂ deficiency that are overlooked with surprising frequency today: “some of the most typical cases presented no anemia throughout the course, others only late in the disease, while in other cases anemia was an obtrusive symptom ... and preceded the nervous symptoms by many months” [7].

Whipple and Robscheit-Robbins discovered that feeding liver to anemic dogs promoted the regeneration of hemoglobin, leading George Minot and William Murphy to the first effective treatment for PA when they fed raw and later cooked liver to patients [8,9]. In 1934, Whipple, Minot, and Murphy received the Nobel Prize for their work. While searching for the remission-inducing component, Minot used an early method for counting reticulocytes that enabled him to address the hematological response to different liver fractions. Other than this “human bioassay” (i.e. testing liver fractions on patients with PA) no reliable laboratory diagnostics were available to support the search for the extrinsic antipernicious factor(s) over the next 20 years. A breakthrough came in 1946 when Lester Smith noticed that the most potent liver fractions were reddish. We now know that B₁₂ contains a system of conjugated double bonds, which confer the red color and make B₁₂ easily detectable at high concentrations. Several teams worked on isolating the colored material in quick succession, providing Dorothy Hodgkin with the opportunity to undertake her remarkable X-ray crystallographic studies for which a second Nobel Prize in the vitamin B₁₂ field was awarded in 1964 [10].

Vitamin B₁₂ structure

Vitamin B₁₂ is one of the largest and most structurally complex nonpolymeric biomolecules described in



R = 5'-deoxyadenosyl, OH, CN, CH₃

Figure 1. The chemical structure of vitamin B₁₂.

nature, with a molecular weight of 1355 daltons for its frequently used cyano- form. The generic term cobalamin refers to a set of structures named corrinoids, consisting of one central cobalt (Co) atom coordinated with 4 equatorial nitrogen atoms contributed by pyrrole residues (Figure 1). The 5th coordination site (α) in B₁₂, below the planar structure, is occupied by a 5',6'-dimethyl benzimidazole residue linked to a α -ribosyl-3-phosphate. The 6th coordination site (β), above the corrin ring, is occupied by a methyl- (methylcobalamin [Me-Cbl]), 5'-deoxyadenosyl (5'-deoxyadenosylcobalamin [Ado-Cbl]), aquo- (hydroxocobalamin [OH-Cbl]), or a cyano-group (cyanocobalamin [CN-Cbl]). The metabolic utility of B₁₂ in humans is conferred by an upper ligand consisting of either a methyl or adenosyl moiety. Cyanocobalamin is the most chemically stable form, though it has no vitamin activity per se. Cyanocobalamin undergoes enzymatic conversions during intracellular processing in human cells, as described below, and it is eventually converted to the two cofactors, Me-Cbl and Ado-Cbl [11]. Me-Cbl and Ado-Cbl regularly undergo catalytic reductions and oxidations and occasionally generate OH-Cbl, which returns to the catalysis *via* a reactivation cycle [12].

B₁₂ sources and de novo biosynthesis

Vitamin B₁₂ is synthesized exclusively by micro-organisms, sometimes inhabiting higher plants that cannot produce B₁₂. Fermentation in the gastrointestinal tract of herbivorous animals supports the growth of micro-organisms that synthesize B₁₂, which is then absorbed by the animal and incorporated into its tissues [13]. Omnivores and carnivores, including humans, acquire B₁₂ from animal tissues or animal products such as milk, cheese, and eggs. Liver in particular is a very rich source of cobalamin, followed by kidney and heart. Although the B₁₂ content of various types of milk is not high, regular intake of milk is associated with higher serum B₁₂ concentration in healthy elderly adults [14]. Egg intake does not significantly contribute to higher serum B₁₂ concentration [14]. Vitamin B₁₂ exists in foods in several forms; meat and fish contain mostly Ado-Cbl and OH-Cbl, OH-Cbl predominates in milk, and Me-Cbl with Ado-Cbl and OH-Cbl are found in nearly all dairy products [15–17]. Animals also procure B₁₂ through coprophagia and as a consequence of bacterial contamination of their feed.

Biosynthesis of B₁₂ is restricted to certain bacterial strains, such as *Lactobacillus rossiae* and *Lactobacillus reuteri* [18,19], and involves one of two alternative routes, either the aerobic or anaerobic pathway in bacteria and archaea, respectively. Approximately 30 enzyme-catalyzed reactions are responsible for the synthesis of full Cbl. The process starts with condensation leading to uroporphyrinogen III (the first macrocyclic intermediate in tetrapyrrole synthesis), continues *via* the transformation of uroporphyrinogen III into cobinamide, and ends with nucleotide loop assembly and attachment to the corrin ring [20]. To conserve energy, many gram-negative bacteria salvage B₁₂ or other corrinoids and transport them into the cell [21].

Dietary intake and bioavailability

Dietary intake of B₁₂ exceeds the metabolic requirement for the majority of people and leads to the accumulation of substantial hepatic stores of ~1–5 mg. The typical Western diet provides ~4–6 µg/d of B₁₂, from which 1–5 µg is absorbed [22]. The bioavailability of B₁₂ from food is assumed to be ~50% for healthy adults with normal absorption, while the absorption of crystalline B₁₂, present in supplements and fortified food products, is between 55 and 74%. However, the absorption from specific foods can vary greatly, for example, 24 to 36% for egg products (B₁₂ dose 0.3–0.94 µg), 42% for fish (dose 1.95–2.18 µg), and 65% for lean meat (dose 0.95 µg) [23]. Data from the 1999–2000 National

Health and Nutrition Examination Survey (NHANES) indicates that the median intake of B₁₂ for the United States (US) population is 3.4 µg/d [24]. It should be noted that intakes are often reported collectively for adults from different populations [25], despite the fact that dietary intake of B₁₂ varies by sex and age. For example, the evaluation of micronutrient consumption across United Kingdom (UK) adults in their twenties, thirties, forties, and fifties showed the daily B₁₂ intake for females to be statistically lower amongst females aged 20–59 years when compared with males of the same age ($p < .001$) [25].

Recommended intakes

The recommended intakes were set to maintain normal hematological values and serum B₁₂ within normal concentrations. They also assume that 50% of vitamin B₁₂ is absorbed from the diet. Intakes of 1.5 µg/d of B₁₂ are recommended by the UK government [26] and 4 µg/d (adequate intake [AI], covers the needs of all in the group) by the European Union for adults [27]. The US recommends an intake of 2.4 µg/d [28]. Higher intakes are recommended for pregnancy and lactation in the US (2.6 and 2.8 µg/d, respectively) and lactation in the UK (2.0 µg/d). The recommended intakes for children vary from 0.3 µg/d for 0–6 month olds to 1.0 µg/d for 9–13 year olds in the UK and 0.4 µg/d to 1.8 µg/d for the same age groups in the US [26,28].

It remains debatable whether the above intakes are sufficient to achieve optimum vitamin B₁₂ status. Using a factorial approach, which includes a summation of daily losses that need to be compensated for by dietary intake of vitamin B₁₂, Doets et al. [23] estimated that vitamin B₁₂ intakes ranging from 3.8 µg/d to 20.7 µg/d are needed to prevent deficiency in apparently healthy adults and elderly people.

In addition to the above, due to the high prevalence of food-bound malabsorption (inability to release vitamin B₁₂ from its binding proteins) in people >60 years of age, many European countries recommend food products fortified with crystalline vitamin B₁₂, as its absorption is not affected in this condition [29].

Vitamin B₁₂ absorption

There are two mechanisms whereby B₁₂ is absorbed: (i) passive diffusion and (ii) a receptor-mediated process. Only about 1–2% of an oral dose can be absorbed passively *via* mucous membranes and the surface of the gastrointestinal tract [30]; hence, high doses of oral B₁₂

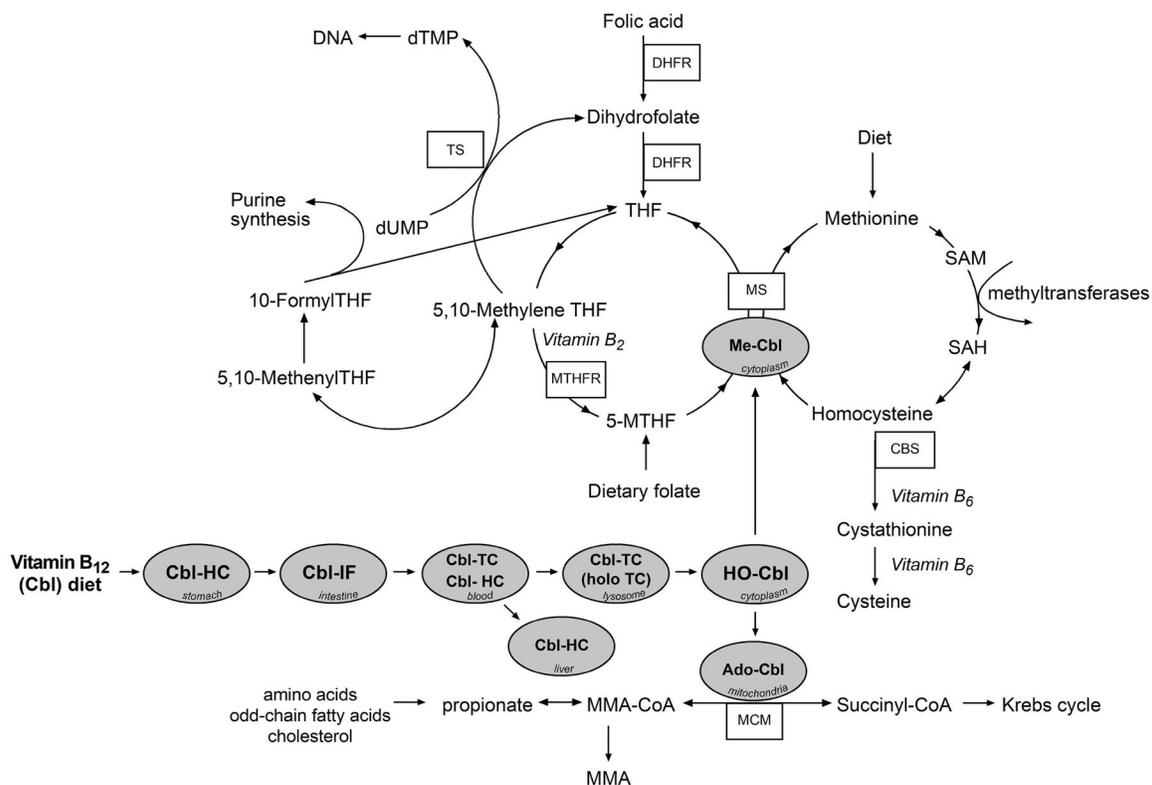


Figure 2. Vitamin B₁₂ absorption and intracellular processing *via* two enzymatic pathways. In the absence of vitamin B₁₂, 5-MTHF becomes metabolically trapped in this form producing a *pseudo* folate-deficient state (methyl-trap) and cannot be utilized for regeneration of THF. Cbl: cobalamin; CBS: cystathionine beta-synthase; dTMP: deoxythymidine monophosphate; dUMP: deoxyuridine monophosphate; DHFR: dihydrofolate reductase; HC: haptocorrin; holoTC: holotranscobalamin; HO-Cbl: hydroxocobalamin; IF: intrinsic factor; MS: methionine synthase; Me-Cbl: methylcobalamin; MTHFR: methylene tetrahydrofolate reductase; MMA: methylmalonic acid; MCM: methylmalonyl-CoA mutase; 5-MTHF: 5-methyltetrahydrofolate; SAH: S-Adenosyl homocysteine; SAM: S-Adenosyl methionine; THF: tetrahydrofolate; TS: thymidylate synthase; TC: transcobalamin.

(e.g. 1 mg daily) are required to provide an “adequate” intake of B₁₂ if the specific transport does not function.

Absorption *via* receptors is initiated after release of cobalamin from the dietary matrix. This begins in the stomach due to the action of gastric acid and by the digestion of food by pepsin. The stomach contains B₁₂-specific proteins as well as intrinsic factor (IF) and haptocorrin (HC, also known as R-binder), which are synthesized by gastric parietal cells and the salivary glands, respectively. HC and IF compete for the liberated vitamin but, at an acidic pH, HC has a higher affinity for B₁₂ (Figure 2). When gastric contents enter the first part of the duodenum, with an alkaline environment, R-binders become partly digested by pancreatic proteases, freeing up the vitamin, which subsequently attaches to IF.

In the terminal ileum, the vitamin B₁₂-IF complex binds to cubam receptors comprised of amnionless and cubilin protein moieties on intestinal mucosal enterocytes [31]. The complex is then taken up by endocytosis into the ileal cell. This process is highly specific and only takes place at a neutral pH in the presence of

calcium ions. The receptors only take up the vitamin B₁₂ bound to IF [32]. This step is rate-limiting since ileal receptors have a finite capacity for B₁₂ amounting to 1.5–2.5 µg from a single meal. Based on early studies with isotopes, it was shown that 50% of a 1 µg oral dose was absorbed, 20% of a 5 µg dose, and 5% of a 25 µg/d dose (Figure 3) [33,34]. A recent study by Devi et al. [35], which used [¹³C]-cyanocobalamin, achieved similar results (Figure 3). The receptors are recycled to the surface and become available again within 4–6 h [36]. After internalization, IF is degraded by lysosomal proteases, releasing the vitamin B₁₂ for transport by the lysosomal transmembrane protein, LMBD1 [37]. From the cytosol, B₁₂ is exported to blood circulation by the ATP-driven exporter, multidrug resistance protein (MRP1) [38]. In the blood, free B₁₂ immediately binds two proteins: transcobalamin (TC) and HC (Figure 2). Transcobalamin is apparently synthesized by all tissues and circulates in a predominately unsaturated form [39]. Most newly-absorbed B₁₂ binds to TC, forming holotranscobalamin (holoTC). HoloTC has a much faster turnover than the B₁₂-HC complex, holohaptocorrin

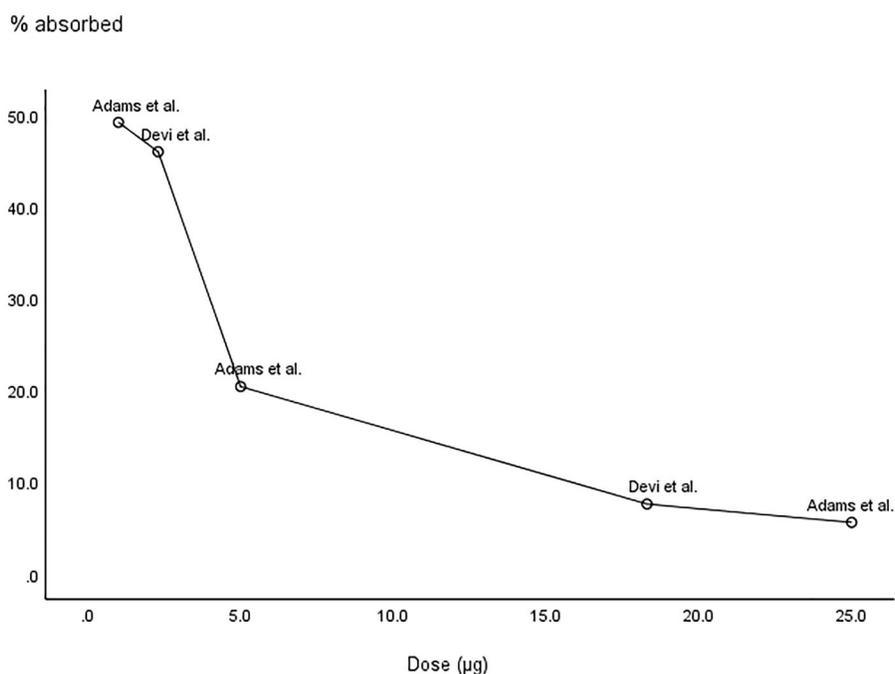


Figure 3. Relationship between the intake and percentage of absorbed B₁₂ from a dose of [⁵⁸Co]-cyanocobalamin (Adams et al. [34]) and [¹³C]-cyanocobalamin (Devi et al. [35]) in *n* = 10–12 healthy volunteers.

(holoHC), but controversies exist over its rate of clearance, which is likely to be attributed to methodologies used for this estimation. This is best exemplified by Hom et al., where authors first estimated half-life ($t_{1/2}$) = 1.5 h of labeled B₁₂ bound to TC [40], and 15 h [41] in their later study, based on the assumption that radioactive B₁₂ does not reappear due to reutilization once cleared from plasma. This latter approach calculated the plasma disappearance half-life obtained from the final slope of the plasma curve, which probably included both clearance and reappearance of B₁₂ [41]. A half-life of 6 min was reported by others and, more recently, 7 min for clearance of a holoTC-conjugate in patients with malignancies [42].

Only ~10% of total TC is normally bound to B₁₂ and this fraction is responsible for the transport of ~4 nmol/d of B₁₂ into cells [41]. If B₁₂ is no longer absorbed, circulating holoTC is not formed. Because the half-life of holoTC is much shorter than that of holoHC (10 days) [41], low holoTC is believed to be the earliest indicator that a patient is no longer absorbing vitamin B₁₂ from food [43].

Conversely, HC in blood is almost fully saturated with B₁₂ and inactive B₁₂ analogs, and although this protein carries the majority of the vitamin in the circulation (~80%), only few sites are available for absorption of HC-B₁₂. Haptocorrin attaches to cell surface receptors on the liver, with a turnover of 0.1 nmol/d of B₁₂ [44]. There is no evidence that HC has any role in the cellular uptake of vitamin B₁₂, however, its role is still poorly

understood. One suggestion for its function is the transportation of potentially harmful cobalamin analogs (other corrinoids) to the liver for secretion into the bile, thus ensuring that only holoTC is available for cellular uptake [45].

Vitamin B₁₂ excreted in the bile is effectively reabsorbed through enterohepatic circulation. The amount of B₁₂ excreted in bile varies from 1 to 10 µg/d. Biliary B₁₂ is reabsorbed across the ileal enterocytes and requires IF, in the absence of which nearly all cobalamin is excreted with the feces. People with a low dietary intake of B₁₂ increase their efficiency of reabsorption of B₁₂. Efficient reabsorption is the reason why it can take some time for deficiency to develop, even in those people whose diets are very low in B₁₂. In their systematic review, Doets et al. [23] estimated that 0.13% of the total body store is lost daily, while the mean store values were between 1.1 and 3.9 mg. Based on these values, total losses of B₁₂ would range from 1.4 to 5.1 µg/d. Assuming a normal initial store (1.1–3.9 mg) and a loss of 0.13% of B₁₂ per day, we can expect that 10% of the whole store remains after approximately 692 days. In his study, Paul Golding [46] experimentally addressed the time scale for development of B₁₂ deficiency and assessed it as 500–700 days based on vitamin B₁₂ markers being in deficiency ranges following a period of lower or nil B₁₂ intake. There was a 71% decrease in serum B₁₂ after 728 days of B₁₂ intake depletion and a 70% (609 days) and 81% (658 days) increase in plasma homocysteine and MMA concentrations, respectively.

The subject in this study was diagnosed with B₁₂ deficiency in the past, was B₁₂ replete at baseline, and gradually decreased his B₁₂ intake to 0 µg on day 371 of this experiment; he then took 100 µg of vitamin B₁₂ for 4 weeks, but from day 497 to 751 his B₁₂ intake was 0 µg [46]. Despite the variations in B₁₂ intake, the results produced in this work suggest that the liver's stores may not maintain adequate vitamin B₁₂ status for a long time. This was also recently suggested by Kornerup et al. [47] in their study on patients following bariatric surgery, where increased methylmalonic acid and decreased holoTC were found as early as 2 months after surgery.

For factors that can affect B₁₂ absorption, the reader is referred to the malabsorption section.

Metabolic processing and function

There are only two intracellular enzymes for which vitamin B₁₂ is required, namely, methionine synthase (MS) in the cytosol and methylmalonyl-CoA mutase (MCM) in the mitochondria. Cellular processing leading to formation of methylcobalamin and adenosylcobalamin, the active vitamin forms supporting enzyme activity, starts with the transport of holoTC into cells. There are receptors for holoTC on the surface of all DNA-synthesizing cells. Following endocytosis of the holoTC complex by the CD320 receptor [48], the complex is degraded in lysosomes and B₁₂ is released into the cytoplasm via two proteins, LMBD1 (cbIF) [37] and ABCD4 (cbIJ) [49]. In the cytosol, the cbIC protein dealkylates methylcobalamin and adenosylcobalamin as well as decyanates cyanocobalamin to cob(II)alamin, which, under aerobic conditions, is oxidized to cob(III)alamin and hydroxycob(III)alamin (cob(III)alamin) [12,50,51]. Kolhouse et al. [52] provided evidence that cob(II)alamin was the form most active in binding to apo-methionine synthase (cbIG). CbIC combines with the cytosolic form of cbID to direct B₁₂, probably as cob(II)alamin, to methionine synthase (cbIG) for the formation of methylcobalamin (Me-Cbl) in the presence of methionine synthase reductase (cbIE) [53–55]. The mitochondrial form of cbID in combination with cbIC directs B₁₂ to mitochondria where it is converted to adenosylcobalamin (Ado-Cbl) in the presence of the gene products of cbIA, cbIB, and methylmalonyl-CoA mutase (MCM) (Figure 2) [11].

Methylcobalamin is essential for the remethylation of homocysteine to methionine by MS; 5-methyltetrahydrofolate (5-MTHF) supplies the methyl group and is converted to tetrahydrofolate (Figure 2). Methionine is subsequently adenosylated to S-adenosylmethionine

(SAM) to supply methyl groups that are critical for the methylation of proteins, phospholipids, neurotransmitters, RNA, and DNA.

Conversely, Ado-Cbl is a cofactor for MCM, catalyzing the conversion of methylmalonyl-CoA to succinyl-CoA. Methylmalonyl-CoA is a degradation product of propionate. In most mammals, propionate arises from the utilization of some amino acids (isoleucine, valine, methionine, threonine, thymine) or cholesterol as well as after β-oxidation of odd-chain fatty acids.

Inborn errors of vitamin B₁₂ absorption and intracellular metabolism

A number of genes that encode for proteins involved in B₁₂ absorption and transport harbor highly penetrant genetic mutations that cause inborn errors of vitamin B₁₂ absorption and metabolism. All of these diseases are characterized by a deficiency of the coenzyme forms of B₁₂. They can be successfully diagnosed and treated with regular high doses of B₁₂ injections, B₁₂ supplements, and other therapies [56].

Inborn errors of B₁₂ absorption

Congenital PA is caused by mutations in a gene (*GIF*, *CBLIF*) and defects in IF synthesis. The condition usually presents during the first five years of life, but it can manifest later if a partial defect is present [57]. This disease exhibits many features resembling non-dietary adult PA, an autoimmune disorder associated with autoantibodies to gastric parietal cells or gastric IF, as well as Immerslund-Gräsbeck (IGS) disease [58].

IGS is a rare, potentially life-threatening, autosomal recessive disorder caused by mutations in either the *CUBN* or *AMN* gene, responsible for the synthesis of the cubam receptors. This disorder is clinically highly heterogeneous. It is accompanied by a mild proteinuria in ~50% of cases, owing to cubam receptor expression also in kidney cells. In terms of treatment, life-long B₁₂ injections lead to resolution of PA and IGS-anemia [58,59].

Genetically predetermined haptocorrin deficiency leads to a benign condition not requiring treatment [45]. Interestingly, some animals such as rodents do not produce HC and survive with just TC and IF [60]. Diagnosis is often suspected following a low serum B₁₂ concentration in the absence of any symptoms related to deficiency. Conversely, genes that cause transcobalamin deficiency will often result in a severe phenotype [61] if not treated with high doses of vitamin B₁₂ from an early age.

Inborn errors of intracellular metabolism

To rationalize the etiological heterogeneity of these disorders, they have been catalogued into a total of eight defects of Cbl metabolism [57], designated cblA-cblG and mut, defined by means of *in vitro* somatic complementation analysis. Defects in genes encoding for cbl proteins result in a failure to utilize B₁₂ by the target cells irrespective of its normal supply. In the cblC, cblD, cblF, and cblJ group of disorders, the synthesis of both Ado-Cbl and Me-Cbl are affected, leading to combined methylmalonic aciduria and homocystinuria. In cblA and cblB disorders, the synthesis of Ado-Cbl is impaired, resulting in methylmalonic aciduria but normal homocysteine concentrations, whilst cblE and cblG disorders result in homocystinuria without methylmalonic aciduria [57]. Finally, the mut defect describes mutations (>200 identified) in the *MUT* gene (encodes for MCM) that partially (class mut⁻) or totally (class mut⁰) abolish MCM-mediated conversion of methylmalonyl-CoA to succinyl-CoA and so perturb substrate (odd-chain fatty acids, branched-chain amino acids, and cholesterol) utilization and energy metabolism in the mitochondria [62].

Of the eight defects, the cblC defect is the most common, although still rare with an incidence ~1/200,000 births. Clinical manifestations often appear in infancy but can also occur later in life. Typical symptoms associated with early-onset disease are feeding difficulties, failure to thrive, hypotonia, seizures, pigmentary retinopathy, developmental delay, and macrocytic anemia. Late-onset disease is frequently accompanied by neurological dysfunction, including cognitive decline, confusion, psychosis, or dementia. In comparison with early-onset patients, late-onset patients have better survival and response to therapy [57,63]. For a detailed description of other intracellular disorders and response to treatment the reader is referred to detailed reviews on this subject [57,62,64].

Genetic polymorphisms associated with poor B₁₂ status

Developments in genomic technology have allowed us to move beyond single-gene disorders and to study the polygenic basis of vitamin B₁₂ status. This has led to the discovery of a myriad of new genes/pathways that harbor genetic polymorphisms associated with variable B₁₂ status among different population groups. The most extreme consequences of genetic variation have already been described in the case of rare inborn errors of B₁₂ metabolism. More common effects of genetic variation in B₁₂ levels can be described by its heritability

(h^2) in different populations as well as associations between specific polymorphisms in genes and the various indicators of B₁₂ status. Dib et al. [65] have provided robust h^2 estimates for all markers of vitamin B₁₂ status for UK-based Caucasian populations that range from ~15% in the case of MMA right up to ~80% for holoTC, highlighting that B₁₂ status is a complex and multifactorial trait, whereby several polymorphisms in multiple genes interact with the environment to cause variable B₁₂ status. So far, candidate gene and genome-wide association studies (GWAS) have identified ~59 SNPs from 19 genes involved in cobalamin metabolism and various indicators of its status [66]. We refer the reader to Surendran et al. [66], who provide a very useful guide for each gene and polymorphism associated with B₁₂ status, but would like to highlight that most, if not all, of the findings thus far refer to “associations” identified mainly through observational studies with currently unclear clinical and pathophysiological significance. Therefore, “causal” genetic determinants of vitamin B₁₂ status, functional consequences, and context dependency remain poorly understood [65,67].

Corrinoids and microbiome

As stated before, cobalamin belongs to a family of compounds referred to as corrinoids. Corrinoids with a cobalt ion in the corrin ring are called cobamides. Vitamin B₁₂ is differentiated from other cobamides (analogues), which usually cannot provide cofactor activity in mammalian cells, by having a 5',6'-dimethyl benzimidazole group attached to the lower nucleotide loop. There are also other corrinoids (analogues) that differ in the tetrapyrrole ring or have a central atom other than cobalt, such as nickel, copper, or zinc. Corrinoids are synthesized exclusively by bacteria and archaea, and are abundant in the large intestine due to the activity of the gut microbiota [68]. Corrinoids play key roles in shaping the gut microbial community composition and diversity [69]. A majority of human gut microbial species either directly require access to the host's dietary-derived B₁₂ or are dependent on analogues [70] produced by other bacteria [71]. In humans, bacterial synthesis of vitamin B₁₂ and analogues takes place only in the large intestine and the cecum, and there is evidence that humans cannot utilize bacterial B₁₂/analogues [32] as part of the orchestrated steps that ensure adequate absorption and reabsorption of dietary-only Cbl. However, there is some evidence to show that certain diseases or age-related changes to the gastrointestinal tract can lead to protein-bound vitamin B₁₂

malabsorption and that bacterial-derived analogs may play a role [72–74].

Analogues act as cofactors for corrinoid-dependent enzymes for gut microbes, hence the constituents of the microbiome compete with the host, as well as other bacteria, for B₁₂ or corrinoid analogs, in order to ensure viability and stability of the microbiome ecology. Previous studies have predicted that ~86% of human gut bacteria species are dependent on corrinoids as cofactors but <25% have the capacity to synthesize them *de novo* [75]. Although many bacteria cannot synthesize B₁₂, they can remodel it by removing the original nucleotide and adding a new one. About 50% of total dietary Cbl is thought to be converted to other corrinoids by the gut bacteria [76]. Therefore, a majority of gut bacteria either rely on Cbl-uptake mechanisms from either the host's diet or are dependent on related corrinoids [77] produced by other bacteria [78]. This is likely associated with the individual variability in dietary Cbl exposure as well as the specificity of the individual gut microbial community composition. For example, Visconti et al. [79] have shown that only 43% of bacterial species are shared by any two randomly selected individuals from the population. Estimates suggest high dietary deficiency rates in the population, especially in an exponentially increasing number of individuals who, for ethical and health reasons and also encouraged by scientific observations, have been shifting toward a higher consumption of plant-based food [80]. Although Cbl deficiency is a modifiable risk factor, which itself is associated with a number of rare/common non-communicable (neurological/vascular) diseases [81], evidence of the specific role of Cbl and other corrinoids, in shaping the gut bacterial community and as mediators of human-microbiome symbiosis, and their impact on human health, remains largely unknown [76,82,83]. Analogues were found to delay growth in chicks [84] or induce severe demyelinating disease in baboons [85]. Mechanisms that prevent absorption and tissue dissemination of analogues have evolved, including the poor affinity for IF. Of those that are bound by IF, most appear to be retained by the ileum. Analogues that do reach plasma are largely bound to haptocorrin and are transported to the liver and excreted *via* the bile and feces. Analogues may also be bound by transcobalamin and transported to tissue [83], but the amount absorbed might be insignificant [86].

Diagnosis of deficiency and insufficiency – clinical and laboratory pitfalls

One might suppose that diagnosing B₁₂ deficiency should be relatively simple. The laboratory provides a

diagnostic “cutoff” point, the patient is proclaimed to be deficient, and the vitamin should be replenished. The truth can be far more complicated, especially if insufficiency of B₁₂ (subclinical deficiency) is also considered. Clinical vitamin B₁₂ deficiency is often the result of prolonged and severe malabsorption and patients are symptomatic, while in insufficiency, symptoms associated with B₁₂ deficiency are not well expressed but biochemical markers, most notably total plasma homocysteine (tHcy) and methylmalonic acid (MMA), are elevated [87]. In such cases, serum B₁₂ concentration is often normal. However, relatively high holoTC, low serum B₁₂, and high MMA may also be seen in some patient cohorts with B₁₂ insufficiency [88].

Therefore, in the course of B₁₂ status assessment, the physician can encounter pitfalls at each step of the process including: (A) consideration of B₁₂ deficiency as a major cause for presenting illness; (B) testing for B₁₂ deficiency/insufficiency and interpreting results; (C) investigating the potential causes of deficiency/insufficiency; (D) treatment; and (E) considering the associated risks.

Consideration of B₁₂ deficiency as a major cause of presenting illness

B₁₂ deficiency can masquerade as a multitude of illnesses; therefore, the physician requires considerable clinical acumen. The disease presents different perspectives from the point of view of the hematologist, neurologist, gastroenterologist, general physician, dietician, and psychiatrist, and is a prime example of “elephanomics” [89]. A high index of suspicion is required. Importantly, the absence of anemia does not preclude a positive diagnosis, a trap easily fallen into since many physicians erroneously equate B₁₂ deficiency with PA, though the latter is only one potential cause of the former [90].

Classical features of deficiency include those relating to anemia (weakness, tiredness, dyspnea on exertion), gastrointestinal symptoms (appetite loss, sore tongue and mouth, epigastric discomfort, nausea, vomiting, heartburn), neurological symptoms (numbness of extremities, pins and needles, impaired fine finger movements, gait ataxia, positive Romberg's sign, impaired vibration and position sense, orthostatic dizziness, loss of taste or smell), and those relating to psychological and psychiatric disturbances (irritability, personality change, memory and intellectual impairment, disorientation, depression, psychomotor slowing, delirium, dementia). The deficiency predominantly affects hematological and neurological parameters, but

neuropsychiatric symptoms are often the first clinical manifestation [91]. Gynecological and urological symptomatology (infertility, cystitis, and pyelonephritis), though rare, should also be considered. The full spectrum of clinical features associated with deficiency is perhaps broader. In a survey of individuals with PA, symptomatology also included brittle nails, flushing, fever, hair loss and graying, and nominal aphasia [92]. Occasionally, the deficiency is entirely asymptomatic, its discovery arising from the alert physician pursuing abnormal results from a routine full blood count.

Megaloblastic anemia describes the morphological features of hematopoietic tissue caused by a deficiency of B₁₂ (and/or folate). It includes ineffective erythropoiesis, moderate hemolysis, and inefficient leukopoiesis and thrombopoiesis [93]. Abnormal morphology of blood elements include normochromic and macrocytic erythrocytes, lower reticulocyte count, hypersegmented neutrophils, abnormal morphology of bone marrow cells, and megaloblastic changes in granulocytes and platelet precursors [93]. Megaloblastic cells are in a state of unbalanced growth with impairment of DNA synthesis and an increase in the RNA/DNA ratio. Morphological mimics include myelodysplastic syndromes and sideroblastic anemia [94]. Conversely, B₁₂ deficiency can co-exist with iron deficiency or thalassemia, thus masking macrocytosis.

It was generally believed that hematological features preceded any neuropsychiatric abnormality, but in the last few decades it has been noted that these manifestations might be considered as two major, and sometimes entirely separate (or even excluding each other), clinical syndromes [95,96]. It remains unclear why some B₁₂-deficient patients present with neurological disorders in the absence of hematological changes and why some develop a predominantly cerebral picture, while others a spinal or peripheral nerve disorder. This may be partially related to folate status, as folate deficiency is primarily responsible for hematological changes due to defective DNA synthesis. A good supply of folates may delay the hematological symptoms of B₁₂ deficiency, even though tetrahydrofolate (THF) is not regenerated from 5-MTHF (Figure 2). In the absence of vitamin B₁₂, 5-MTHF becomes metabolically trapped in this form, producing a *pseudo* folate-deficient state (methyl-trap) [97]. The methyl-trap hypothesis explains why vitamin B₁₂ deficiency produces apparently identical megaloblastic anemia to that seen in folate deficiency. In both cases (folate and vitamin B₁₂ deficiency), the cells would be deficient in folate cofactors required for DNA and RNA synthesis. It also explains the clinical observation that treating a patient with megaloblastic

anemia caused by vitamin B₁₂ deficiency with a pharmacological dose of folic acid allows the cells of the bone marrow to start to divide again. It is thought that the new folate molecules would carry out several such cycles before they are trapped as 5-MTHF (Figure 2). If folic acid continued to be administered to a patient with megaloblastic anemia, which would occur if the wrong clinical diagnosis had been made, hematological remission would be maintained [97].

Our “preconception” of B₁₂ deficiency still associates it with anemia, and its diagnosis usually proceeds in three steps: the recognition of anemia, the observation that it is macrocytic, and finally, the confirmation of an underlying deficiency. However, if suspicion of the deficiency were based on the presence of macrocytic anemia alone, a substantial proportion of B₁₂-deficient individuals would escape detection [90,98], a point elegantly expressed by Carmel, who notes that “The proscription that vitamin B₁₂ deficiency should not be diagnosed unless megaloblastic changes are found is akin to requiring the presence of jaundice to diagnose liver disease” [99].

Early presentation, due to increased physician vigilance and heightened patient awareness, can result in tests during a phase of the illness when laboratory findings of a “full-blown” deficiency are not yet apparent. Before patients become clinically deficient, they traverse earlier stages, beginning with insufficiency. The concept of B₁₂ deficiency as a gradually progressive disorder [99] is an important development over the last 30 years [100]. The five stages of vitamin B₁₂ status/deficiency described by Victor Herbert in 1987 [100] are likely to be applicable to the majority of cases today: stage I – normal vitamin B₁₂ status; stage II – negative vitamin B₁₂ balance; stage III – vitamin B₁₂ depletion accompanied by possible clinical signs and symptoms such as fatigue, dizziness, nausea or poor appetite, diarrhea, palpitations and rapid heartbeat, bleeding gums, and mouth sores; stage IV – deficient erythropoiesis with potentially reversible neurological symptoms such as numbness and tingling in the hands and feet, ataxia, muscle weakness, depression and psychosis; and stage V – related anemia with possible irreversible neurological symptoms.

Testing for B₁₂ deficiency/insufficiency and interpretation of laboratory results

“We sanguinely hope that the laboratory will answer our questions with a dogmatic ‘yes’ or ‘no’; its usual answer is perhaps” (Anon).

There are four biological markers of B₁₂ deficiency: total serum B₁₂, holoTC (also known as “Active B₁₂”), and

measures of the related metabolites tHcy and MMA. Each has its limitations, which the physician should be alert to.

Serum B₁₂ test

Serum B₁₂ is the most frequently used laboratory marker of B₁₂ status, measuring the circulatory concentration of B₁₂ bound to both vitamin binding proteins, TC, and HC. As is the case for a number of other vitamins, serum B₁₂ was initially measured by microbiological methods, for example, using *Lactobacillus leichmannii*, for which cobalamin is an obligate nutrient [101]. Microbiological assays for B₁₂ suffered from inter-laboratory variability, interference by antibiotics, and an extended turnaround time inherent to the assay principle. The main disadvantage however is its low specificity when compared to IF-based assays [102]. These assays were challenged by nonspecific R-protein-binding assays due to availability of R-proteins from saliva, for example. However, the values obtained with these were markedly higher than those obtained with the microbiological assays, as they measured a broad variety of cobalamin analogs that are not necessarily biologically active [83].

Since the 1990s, clinical laboratories have opted for high throughput automated competitive binding chemiluminescence assays (CBLA), using purified IF as a reagent, to measure total cobalamin after its release from endogenous binding proteins [103]. Caution should however be observed when interpreting results, since some of these assays could be influenced by the presence of interfering anti-IF antibodies, particularly in patients with PA, thereby providing spuriously elevated serum cobalamin concentrations in otherwise B₁₂-deficient patients [104–106].

Serum B₁₂ can also be determined using B₁₂-antibodies and B₁₂ enzyme-linked immunosorbent assay (ELISA) kits. Various kits are available. Although these could potentially provide an alternative to IF-based assays, they have not been completely verified [107–109].

Functional indicators of B₁₂ status, tHcy and MMA, are based on the rationale that deficiency leads to the deactivation of two key enzymes of the one-carbon cycle, namely MS and mitochondrial MCM, causing the accumulation of tHcy and MMA, respectively [110]. While providing a better insight into the B₁₂ status of different groups of individuals, the lack of consensus in choosing cutoff values for each of the biomarkers remains problematic in diagnosing B₁₂ deficiency. Reported cutoffs for B₁₂ vary from as low as 100 pmol/L (Immulate 2000) when associated with plasma MMA

concentrations ≥ 260 nmol/L [111], to as high as 258 pmol/L (IF radioassay, Quantaphase) on the basis of increased MMA [112]. These two reports exemplify the uncertainties in the reported cutoffs for individual biomarkers of B₁₂ status. Shelub et al. [113], in a study based on NHANES III 1991–1994 and NHANES 1999–2002 involving ~5000 middle-aged participants in both cohorts, used <148 pmol/L as the conventional cutoff for B₁₂ deficiency. Using this cutoff, they reported a prevalence of 1.6 to 2.2% of the deficiency in NHANES III and NHANES, respectively, and showed significant differences in circulating tHcy and MMA between subjects with B₁₂ deficiency vs normal status. In a 2001 epidemiological study based on combined NHANES 1999–2000, 2001–2002 and 2003–2004 data totaling 12,612 middle-aged adult participants, Bailey et al. [114] reported a steady increase in the prevalence of B₁₂ deficiency, from 2.9% to 25.7% by moving decision points from <148 pmol/L to >296 pmol/L of total serum B₁₂. They observed a similar trend but of much less importance ($2.3 \pm 0.2\%$ to $5.8 \pm 0.3\%$) when the cutoff point for MMA was decreased from >376 to >271 nmol/L. Concerned by the impact of different B₁₂ and MMA cutoffs in the diagnosis of clinical outcomes and using data collected from the 1999–2004 surveys and different statistical models, Bailey et al. [115] attempted to identify a single change point at which the relation between plasma MMA and serum B₁₂ changes slope to differentiate between inadequate and adequate B₁₂ status, but the results were not as simple as expected. They reported three slopes resulting in two change points for three subgroups. The first subgroup had serum B₁₂ <126 pmol/L and the highest median plasma MMA of 281 nmol/L. This group, representing 1.2% of the population studied, was considered at high risk for severe deficiency because of the frequently accompanying elevation of MMA and tHcy. The second group, representing 67.6% of the population studied, in which serum B₁₂ was >287 pmol/L, likely had adequate B₁₂ status with a median MMA concentration of 120 nmol/L. The third group (33%) was classified as indeterminate and difficult to interpret because of intermediate serum concentrations of B₁₂ (126–287 pmol/L). Although none of these studies provide a clear cutoff, they all call for the measurement of associated metabolites when assessing B₁₂ status.

Holotranscobalamin testing

HoloTC, the bioactive form of B₁₂, is also routinely used for evaluating B₁₂ status, often in combination with an MMA test [116,117]. It remains debatable whether holoTC “alone” serves any better than the measurement

of serum B₁₂. It has particularly been criticized by Golding [118], who provides an extensive review and argumentation concluding that “the holoTC immunoassay cannot be used to measure B₁₂ status any more reliably than total B₁₂, or to predict the onset of a metabolic deficiency, because it is based on an erroneous hypothesis and a flawed model for the staged development of B₁₂ deficiency.” This conclusion is challenged by Kornerup et al. [47], who provide evidence that holoTC is superior to total B₁₂ in detecting early changes in the vitamin’s status following bariatric surgery. Jarquin Campos et al. [119] also demonstrated from a cohort study of 11,833 samples that holoTC had the highest diagnostic accuracy for recognizing B₁₂ insufficiency, with significantly higher diagnostic accuracy than B₁₂ and tHcy. The sensitivity of holoTC was found to be between 0.55–0.87 vs 0.33–0.76 for total B₁₂, whilst the specificity ranged from 0.50–0.96 and 0.41–0.98 for holoTC and B₁₂, respectively, in various studies summarized by Golding [118]. The variations in sensitivity and specificity values largely depended on the MMA cutoffs used, which served as the proxy gold standard marker representative of metabolic vitamin B₁₂ deficiency in these studies. Most of these studies demonstrated only a slightly higher sensitivity and specificity for holoTC than total B₁₂. Conversely, the correlation coefficient (*r*), which reflects the relationship between holoTC and B₁₂, ranged from 0.42 to 0.882 in twenty-two studies summarized by Golding in his review [118]. A high correlation over a wide range of values may imply that holoTC could not detect the onset of B₁₂ deficiency earlier than total B₁₂ [118].

However, separate *r* values for low and high B₁₂ were not provided in most of the studies. Herrmann and Obeid [120] additionally reported *r* = 0.524 for low total B₁₂ <300 pmol/L and 0.403 for values above ≥300 pmol/L. The authors concluded that total B₁₂ was a poor predictor of holoTC, and low holoTC occurred at higher total vitamin B₁₂ concentrations [120]. The *r* value (Spearman’s) based on randomly selected, consecutive diagnostic results from the authors’ laboratory (AS-M and DJH) was 0.741 (*N* = 298) across the whole holoTC and B₁₂ range (unpublished data). Separate correlation analysis of holoTC <25 pmol/L (deficiency cutoff) with total B₁₂ gave *r* = 0.404 (*N* = 175) and for holoTC ≥25 pmol/L, *r* = 0.627 (*N* = 123) (Figures 4 and 5). Assuming B₁₂ deficiency based on holoTC <25 pmol/L, 56.0% of patients would have been classified as sufficient if the serum total B₁₂ test with a cutoff of 138 pmol/L had been used (Figure 3).

In addition, holoTC has been shown to be unaffected by assay interference from high-titer IF antibody levels [121]. Furthermore, holoTC is not subject to the ~50% fall as seen for total B₁₂ in pregnancy [122]. The diagnostic utility of holoTC in other cohorts requires further study and evaluation.

The holoTC assay is useful when transcobalamin and haptocorrin deficiencies are suspected (Table 1). In haptocorrin deficiency, holoTC concentration is normal/high while total serum B₁₂ (the sum of holohaptocorrin and holoTC) can indicate severe deficiency. In haptocorrin deficiency, the serum B₁₂ test measures the holoTC fraction only. Without the availability of holoTC testing, a clinician may wrongly diagnose a patient and

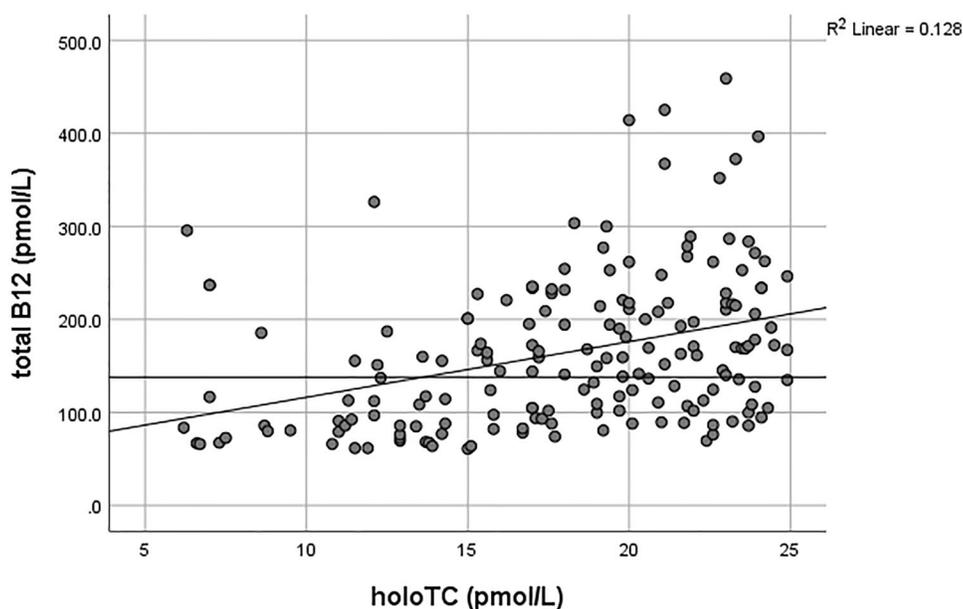


Figure 4. The correlation between holoTC and serum B₁₂ in the low holoTC range, <25 pmol/L. The horizontal line on the y-axis represents the serum B₁₂ deficiency cutoff of 138 pmol/L used in the authors’ laboratory (AS-M and DJH).

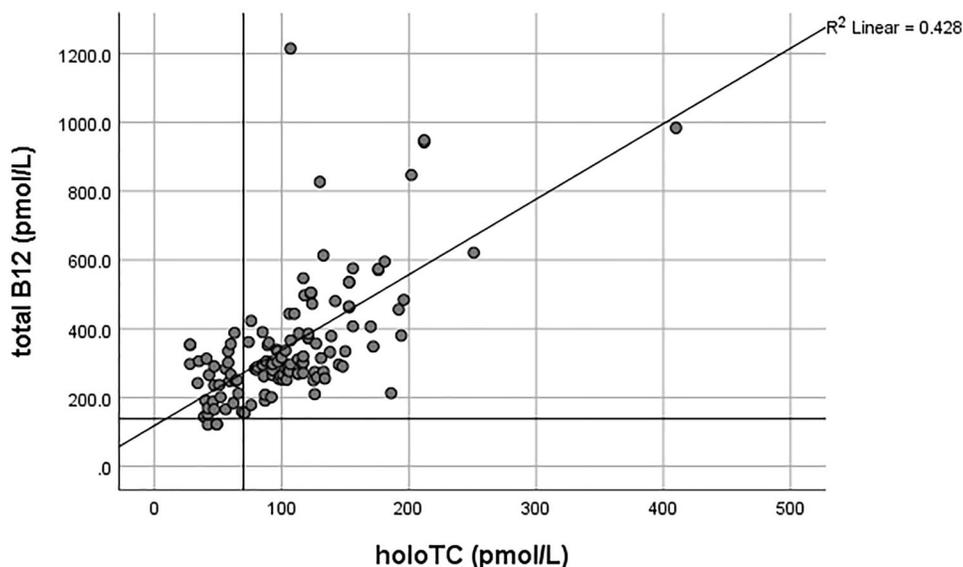


Figure 5. The correlation between holoTC and serum B₁₂ in the holoTC range ≥ 25 pmol/L. The horizontal line on the y-axis represents the serum B₁₂ deficiency cutoff of 138 pmol/L and the vertical line represents the holoTC cutoff of 70 pmol/L for B₁₂ replete patients as used in authors' laboratory (AS-M and DJH). Samples with holoTC results between 25–70 pmol/L are referred for additional testing.

Table 1. The commonly seen vitamin B₁₂ marker patterns in selected clinical scenarios.

Serum holoTC	Serum total B12	Plasma MMA	Plasma tHcy	Possible diagnosis
N	N	↑	↑	Suboptimal B ₁₂ status
↓	↓	N or ↑	↑	Mild B ₁₂ deficiency, on antibiotics
N	N	↑	N	Bacterial overgrowth, B ₁₂ replete
↓↓↓	↓↓↓	↑↑	↑↑	Pernicious anemia
N or ↓	↓	N or ↑	N	Pregnancy, B ₁₂ replete
↓	↓	↑	N or ↑	Pregnancy, B ₁₂ deficiency
N	N	N	↑ - ↑↑↑	Mild to severe folate deficiency, B ₁₂ replete
N or ↑	↓	N	N	Haptocorrin deficiency
↓↓↓	N or ↓ or ↑	↑↑	↑↑	Transcobalamin deficiency
N	N	↑↑↑	↑↑↑	CblC, D, F, J disorder
N	N	↑ ↑ ↑	N	CblA, B disorder
N	N	N	↑↑↑	CblE, G disorder
N or ↓	N or ↓	N or ↑	↑↑↑	Nitrous oxide abuse
↑↑	↑↑	N	N	On vitamin B ₁₂ injections
↑↑↑	↑↑↑	N	N	Chronic myeloid leukemia

Cbl: cobalamin; holoTC: holotranscobalamin; MMA: methylmalonic acid; tHcy: total plasma homocysteine. ↓↓↓: very low/undetectable concentration; ↓↓: low; ↓: decreased; N: within reference range; ↑: elevated; ↑↑: highly elevated; ↑↑↑: very highly elevated.

incorrectly prescribe B₁₂ replacement for an otherwise benign condition. In transcobalamin deficiency however, early treatment can lead to a good clinical outcome. In this condition, holoTC is unmeasurable since deficiency of the transcobalamin protein does not allow for B₁₂ binding and the formation of holoTC. Ultimately, genetics analysis can confirm the diagnosis of both conditions.

Conversely, unmeasurable holoTC may rarely imply variants in the transcobalamin gene that interfere with assays such as “Active B₁₂” [123,124]. For instance, the minor allele rs35838082 (p.R215W), which is rare in Caucasians with a minor allele frequency (MAF) of <0.01 but more common in South Asians (MAF ~0.02)

and those of African origin (MAF ~0.25), was found to interfere with the “Active B₁₂” test [124]. HoloTC results in these patients are erroneously low (<5 pmol/L), despite all other B₁₂ laboratory markers being normal and an absence of clinical symptoms [124].

Transcobalamin receptor (TCBIR/CD320) polymorphisms may also have an impact on holoTC concentrations. In an elderly population, 5% were found to have a heterozygous codon 88 GAG deletion [125]. This is associated with proportionately more serum B₁₂ bound to holoTC. Hence, holoTC might not be a marker of “true” intracellular B₁₂ status in some individuals; an elevated holoTC may reflect decreased cellular uptake due to the GAG deletion.

Table 2. Selected serum/plasma results from longitudinal studies of uncomplicated pregnancies: pre-conception, pregnancy, labor, post-delivery and cord samples.

Geo region	Variable (unit)	Methodology	Sampling time Pre-conception/weeks pregnancy/labor/cord/ weeks postD (n)	Concentration (range)	Concentration/ range definition	Reference		
Germany	Vitamin B ₁₂ (pmol/L)	FP IF Ligand assay	9–12 weeks (31)	257 (226–292)	Geometric Mean (95% CI)	Koebnick <i>et al.</i> 2002 [111]		
			20–22 weeks (39)	239 (212–268)				
	tHcy (μmol/L)	HPLC	36–38 weeks (38)	178 (161–198)	Mean (95 th CI)			
			9–12 weeks (31)	6.9 (6.2–7.6)				
Spain	Vitamin B ₁₂ (pmol/L)	Microbiological assay <i>L. leichmannii</i>	20–22 weeks (39)	5.9 (5.4–6.3)	Geometric Mean (10 th –90 th percentiles)	Murphy <i>et al.</i> 2007 [109]		
			36–38 weeks (38)	6.6 (5.9–7.2)				
			holoTC (pmol/L)	MEIA			Pre-conception (89)	293 (155–535)
							8 weeks (88)	267 (144–449)
							20 weeks (90)	230 (123–432)
							32 weeks (90)	198 (107–339)
	Labor (84)	224 (117–444)						
	Cord (82)	325 (146–641)						
	MMA (nmol/L)	GC-MS	Pre-conception (56)	63 (38–98)				
			8 weeks (58)	47 (31–74)				
			20 weeks (61)	48 (34–78)				
			32 weeks (60)	45 (26–82)				
Labor (49)			40 (23–79)					
Cord (23)			92 (45–214)					
Denmark	Vitamin B ₁₂ (pmol/L)	MEIA	Pre-conception (88)	120 (90–170)	Mean (±1.96SD)	Milman <i>et al.</i> 2007 [112]		
			8 weeks (87)	110 (90–170)				
			20 weeks (89)	110 (80–150)				
			32 weeks (90)	140 (90–200)				
	MMA (nmol/L)	SID-MS	Labor (83)	140 (90–210)				
			Cord (72)	240 (130–400)				
			18 weeks (441)	216 (96–484)				
			32 weeks (310)	169 (73–388)				
	Hcy (μmol/L)	GC-MS	39 weeks (266)	154 (71–333)				
			8 weeks PostD (170)	315 (148–672)				
			18 weeks (413)	110 (40–290)				
			32 weeks (390)	130 (50–340)				
Canada	Vitamin B ₁₂ (pmol/L)	Competitive-binding immunoenzymatic assay	39 weeks (250)	150 (60–360)	Mean (95 th CI)	Visentin <i>et al.</i> 2016 [113]		
			8 weeks PostD (160)	160 (80–350)				
			12–16 weeks (188–342)	219 (210–229)				
			Labor (188–342)	169 (162–176)				
	MMA (nmol/L)	LC-MS/MS	Cord (188–342)	321 (300–344)				
			12–16 weeks (188–342)	109 (105–114)				
			Labor (188–342)	136 (129–142)				
			Cord (188–342)	313 (297–331)				
	tHcy (μmol/L)	Enzymatic assay	12–16 weeks (188–342)	5.0 (4.9–5.1)				
			Labor (188–342)	6.0 (5.8–6.2)				
			Cord (188–342)	4.9 (4.7–5.1)				
			12–30 weeks (124)	343 (238–401)				
USA	Vitamin B ₁₂ (pmol/L)	Electrochemiluminescent immunoassay	Labor (75)	216 (173–312)	Median (IQR)	Finkelstein <i>et al.</i> 2019 [114]		
			Cord (58)	569 (479–844)				
			8.3–13.9 weeks (596)	216 (89.9, 519)				
			14.9–20.9 weeks (640)	198 (84.0, 473)				
	holoTC (pmol/L)	MEIA	8.3–13.9 weeks (587)	83.6 (29.5, NA ^a)	G-Mean (lower and upper limits [90% CI] of central 95% RI)			
			14.9–20.9 weeks (567)	76.5 (26.0, NA ^a)				
			8.3–13.9 weeks (586)	130 (69.6, 371)				
			14.9–20.9 weeks (601)	120 (51.0, 374)				
	MMA (nmol/L)	LC-MS/MS						

CI: confidence interval; GC: gas chromatography; FP: fluorescence polarization; HPLC: high performance liquid chromatography; holoTC: holotranscobalamin; IQR: interquartile range; IF: intrinsic factor; LC-MS/MS: liquid chromatography-tandem mass spectrometry; MS: mass spectrometry; MMA: methylmalonic acid; MEIA: microparticle enzyme immunoassay; NA: not applicable; postD: post-delivery; SID: stable isotope dilution; n: study size; tHcy: total plasma homocysteine.

^aThe upper limit for holoTC is undefined.

Functional markers of B₁₂ status

Plasma tHcy and serum MMA are very sensitive markers, especially for B₁₂ insufficiency, but are also influenced by other conditions independently of B₁₂ status. Most

notably, renal impairment and hypothyroidism lead to elevations of tHcy and MMA, making these tests unreliable. MMA begins to accumulate from the early stages of renal impairment, leading to a decreased specificity

for B₁₂ deficiency. Urinary MMA/creatinine ratio (uMMA/C) may have diagnostic utility, due to the stable urinary excretion of MMA before severe renal failure [126]. With a threshold of 1.45 μmol/mmol, the uMMA/C ratio has been associated with good diagnostic performance for B₁₂ deficiency as a second line assay [127].

High tHcy is also a strong indicator of folate deficiency, therefore concomitant assessment of both vitamins is required if tHcy is being utilized as a marker of B₁₂ status. Postprandial and orthostatic variations, as well as the prompt separation of plasma following blood collection, are preanalytical factors that need to be addressed before a sample is collected for tHcy measurement [128]. Usage of special tubes may be considered if separation of plasma within 1 h of blood collection is not viable [128]. Bacterial overgrowth and hemoconcentration may additionally influence MMA concentrations. Both markers are age-dependent, and tHcy is higher in males.

All the above factors need to be considered when interpreting B₁₂ results (Table 1). A combination of tests for the assessment of B₁₂ status is preferable, but tests need to be carefully chosen while considering factors affecting B₁₂ markers independently of status. An understanding of the laboratory reference ranges, and using them as a guidance only rather than an indicator of “firm” B₁₂ status, will also help to avoid misdiagnosing patients [129].

Combined indicator of B₁₂ status

If markers of B₁₂ status do not show a “clear-cut” deficiency, the absence of a definitive diagnostic test can cause difficulty. Fedosov et al. [130] proposed a solution to this diagnostic dilemma using a “combined” indicator of B₁₂ status (cB₁₂ or 4cB₁₂). This is a rigorously derived mathematical model combining all four markers (total B₁₂, holoTC, tHcy, and MMA), which makes adjustments for age and folate status. It yields one of five diagnoses: elevated B₁₂, adequate B₁₂, decreased B₁₂, possibly B₁₂-deficient, and probably B₁₂-deficient [131]. The interpretive comments from individual B₁₂ markers combined vs the outcome from 4cB₁₂ calculations in $n = 44$ patient samples analyzed in the authors’ laboratory (AS-M and DJH) showed 100% agreement in B₁₂ status assessment (unpublished data).

The main drawbacks of 4cB₁₂ are cost and the relative lack of availability of all tests in routine practice. A refined model now provides “missing markers” [131], and although additional clinical research is required, this approach should be more widely appreciated. Our calculations using the 2cB₁₂ formula (holoTC and MMA

values only) for $n = 3290$ patients with holoTC 25–70 pmol/L showed that 1% of patients within this group were classified as possibly or probably B₁₂-deficient (“need immediate intervention with intramuscular (IM) injections”), while 14% were classified as having decreased B₁₂ (“start B₁₂ supplements”). Using the MMA test as a confirmatory marker for this holoTC range in the same cohort yielded 24% of patients with elevated MMA [116].

The cB₁₂ model has currently not been validated for use in pregnancy, however, our unpublished results suggest its potential use [132].

Considering groups at risk and potential causes for B₁₂ deficiency/insufficiency

Once a diagnosis of B₁₂ deficiency/insufficiency is made, it is incumbent on the physician to determine potential causes of deficiency/insufficiency, an important but often overlooked stage. Dietary and family history, pregnancy, age, concomitant nutrient deficiencies (folate and iron in particular), malabsorption, surgery, bacterial overgrowth, or pharmaceutical interactions should all be considered or investigated, as appropriate. This will determine the appropriate type, and duration, of treatment.

Restricted dietary intake (veganism/vegetarianism)

Vegetarian and vegan diets have become very popular in the last few decades, especially in developed countries, in view of their potential benefits to prevent coronary heart disease, cancer, and type 2 diabetes, as well as due to ethical and environmental issues [133]. However, these diets have restricted the intake of certain nutrients, including vitamin B₁₂. Also, non-vegetarians in the developing world can have low vitamin B₁₂ intake due to the high cost of meat in these areas [134]. In addition, in countries such as India or Bangladesh, vegetarianism has been practiced for centuries for religious and cultural reasons [135] and it is well documented that the prevalence of vitamin B₁₂ deficiency is high in these regions. Both vegans/vegetarians are unlikely to achieve recommended dietary allowances, since plant derived foods have no, or trace, B₁₂ contents [136,137]. A certain amount of B₁₂ may be provided by plants contaminated with B₁₂-producing bacteria through fertilization with manure [135], since feces are a good source of vitamin B₁₂ [76]. It was also observed that in largely vegetarian populations in places where hygiene standards might be low, poor people had a better B₁₂ status than the urban middle-class. This is possibly due to microbial vitamin B₁₂ from

ingestion of contaminated food and water. Using 150 pmol/L as threshold, 81% of urban middle-class men had low vitamin B₁₂-concentration compared to 51% of slum residents [138].

In a European study of 29 vegans, 66 lacto-vegetarians or lacto-ovo-vegetarians, and 79 omnivores who were not taking supplements, vegans had the lowest B₁₂ status; 92% had holoTC <35 pmol/L and 83% had MMA >271 nmol/L [139]. The prevalence of low holoTC and high MMA was also high in lacto-vegetarians and lacto-ovo-vegetarians (77% and 68%, respectively) compared to omnivores in this study (11% and 5%, respectively) [139]. Therefore, dietary supplements are essential to vegans and should be considered by those with poor intake of milk and milk products.

The choice of supplement should also be carefully considered, since the B₁₂ content of dietary supplements can vary greatly. When the B₁₂ content was determined in 19 dried *Chlorella* cells, which are used in dietary supplements, it varied from <0.1 µg/100 g to 415 µg/100 g of dried weight [140]; as *Chlorella* does not synthesize B₁₂ and is instead dependent on symbiotic microorganisms, their content is greatly variable [141]. *Chlorella* represents a group of eukaryotic green microalgae. Further analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS) showed the presence of inactive corrinoid compounds, a cobalt-free corrinoid, and 5-methoxybenzimidazolyl cyanocobamide in four and three high B₁₂-containing *Chlorella* tablets, respectively. In four *Chlorella* tablet types with high and moderate B₁₂ content, Ado-Cbl (~32%) and Me-Cbl (~8%) were present, whereas the unnaturally occurring corrinoid cyanocobalamin was present at the lowest concentrations. *Chlorella sorokiniana* is commonly used in dietary supplements and does not have a requirement of B₁₂ for growth despite B₁₂ uptake from the medium being observed. In further experiments, B₁₂-dependent MCM and MS activities were detected in cell homogenates. These results suggest that B₁₂ contents of *Chlorella* tablets reflect the presence of B₁₂-generating organic ingredients in the medium or the concomitant growth of B₁₂-synthesizing bacteria under open culture conditions [140].

Pregnancy state

Balanced nutrition during pregnancy is essential for the mother's and offspring's health status. Most importantly, pregnant women are at high risk of developing deficiency as the requirements for vitamin B₁₂ are exceptionally high during pregnancy as a result of increased metabolic rate and fetal demands. Studies conducted on maternal-fetal dyads demonstrate higher

cord blood B₁₂ concentrations than those of the respective mothers and a direct relationship between the two pools [142–145].

Moreover, maternal nutrition before and during pregnancy is the main determinant of the nutritional status of the offspring. Layden et al. [146] demonstrated that not only the mean cord blood B₁₂ concentration correlated to that of the mother at delivery, but it was almost tripled (maternal: 230.9 pmol/L, cord blood: 662.1 pmol/L, $p < .0001$), confirming fetal dependency on good maternal B₁₂ status. Low cobalamin intake prior to and during pregnancy has been related to increased risk of preterm delivery, low birth weight [147,148], and lower colostrum and early milk B₁₂ contents [149,150]. Importantly, the deficient stores, resulting from inadequate B₁₂ intake during pregnancy, have been shown to reflect on the newborn's hepatic B₁₂ stores [17]. Vitamin B₁₂ deficiency has also been suggested as a cause of recurrent spontaneous pregnancy loss [151].

Pregnancy is probably the most challenging state for the diagnosis of B₁₂ deficiency, since all B₁₂ markers are affected due to physiological and hormonal changes during pregnancy while B₁₂ status is also declining. Decades ago, observational studies of apparently healthy pregnant women with a normal hematological pattern and megaloblastic anemia showed a fall in serum B₁₂ concentration during pregnancy but that pre-pregnancy concentrations resumed soon after delivery [152–154]. In one study in the early 1960s, 33% of women had B₁₂ concentrations below 147 pmol/L compared with only 17% of non-pregnant controls [153]. In another study conducted five decades ago and involving 518 patients, 70% of pregnant women had serum B₁₂ concentrations below the normal value for non-pregnant subjects (110 pmol/L), including 20% of women with exceptionally low concentrations <37 pmol/L (*L. Leichmannii* assay was used) [152]. The question was raised whether this fall in circulating B₁₂ was the result of physiological processes or reflected true deficiency. To answer this question, Metz et al. [154] compared serum tHcy and MMA in pregnant women who had low B₁₂ (<150 pmol/L) vs women who had B₁₂ concentrations within the reference range for non-pregnant women. Interestingly, they found no correlation between these biomarkers and serum B₁₂, but they observed elevated MMA (>376 nmol/L) in one-third of women and elevated tHcy (>13.8 µmol/L) in only two women (3%). The authors concluded that there was a lack of evidence concerning the association of functional deficiency with low serum B₁₂ status. The same publication suggested homocysteine as a

valuable marker in assessment of B₁₂ status during pregnancy. Pardo et al. [155] later confirmed the above observations and conclusions. In short, these early studies indicated that using reference ranges for non-pregnant women during pregnancy is specious and warrants the use of separate reference ranges. We now know that serum B₁₂ concentrations decrease during pregnancy because of hemodilution and decreased synthesis of haptocorrin, making this test unreliable [122]. HoloTC is considered a superior diagnostic tool in pregnancy as it is less impacted by pregnancy-related changes [122,143]. Hemodilution and hormonal changes have likewise been proposed as modulators of MMA and tHcy concentrations during pregnancy [143]. In addition, folic acid supplementation, usually taken in the first trimester, influences tHcy concentration, making this marker less specific for B₁₂ deficiency [156]. Table 2 lists the values for B₁₂ markers measured in longitudinal studies in pregnant women, which were conducted more recently [142–144,157–159]. Although absolute values may differ, these studies unanimously show changes in B₁₂ markers during pregnancy, which are likely to be a result of both pregnancy-related factors and declining B₁₂ status. In addition, Milman et al. [158] and Schroder and Tan et al. [159] established pregnancy related reference intervals for some B₁₂ markers for all trimesters and the first two trimesters, respectively. Serum B₁₂ and MMA reference intervals were in good agreement between these studies. Schroeder and Tan et al. [159] also showed differences between women of European and South-Asian descent, reporting change points based on MMA in pregnancy and interestingly finding that these were not ethnicity-dependent. Accordingly, total B₁₂ <186 and <180 pmol/L and holoTC concentration <62.2 and <67.5 pmol/L in the 1st and 2nd trimesters, respectively, would indicate an increased risk of deficient B₁₂ status.

Newborns and infants

The mechanisms that determine B₁₂ status in newborns are not well defined, particularly during the early breastfeeding period. During the immediate postnatal period, the gastrointestinal tract undergoes profound colostrum-dependent growth, morphological changes, and functional maturation, including the capacity of acid secretion [160]. From binding studies of IF and of purified human milk HC using the immortalized enterocyte cell line Caco-2, and IF gastric secretion measured in terms of breastfed infant fecal extracts, Adkins et al. [161] have shown IF-dependent B₁₂ absorption mechanisms in breast-fed newborns. However, with IF concentrations appearing seemingly too low in this period of

life to participate effectively in B₁₂ absorption, they proposed that HC could take control until maturation of the IF component.

Importantly, the deficient stores resulting from inadequate B₁₂ intake during pregnancy have been shown to reflect on the newborn's hepatic B₁₂ stores [17]. Furthermore, exclusively breastfed infants are at risk of developing symptoms of deficiency, including anemia, irritability, failure to thrive, and developmental delays, often after five months of life if maternal B₁₂ stores, and hence breast milk, remain low [129,162,163]. Lower serum B₁₂ and holoTC were found in breastfed infants of mothers with poor vitamin B₁₂ status compared with breastfed infants receiving solid foods or solid foods/milk substitutes [164]. Chandyo et al. [165] reported that 11% and 24% of 6–11 month old breastfed infants ($n=316$) were B₁₂-deficient (<148 pmol/L) or marginally deficient (148–221 pmol/L), respectively. Interestingly, raised tHcy (>10 μmol/L) and MMA (>280 nmol/L) concentrations were observed in 53% and 75% of toddlers, respectively. They attributed these high frequencies to sub-optimal maternal nutritional status and the largely vegetarian additional feeding. Intervention clinical trials with 400 μg IM cobalamin showed a marked improvement in B₁₂ markers (serum B₁₂, tHcy, and MMA) of breastfed infants compared to controls, as well as an improvement in motor function, providing evidence that biochemical abnormalities seen in infants reflect vitamin B₁₂ status in addition to immature metabolism and that long-term exclusive breastfeeding does not provide adequate B₁₂ to the growing infant [166–168]. A plasma homocysteine value of 6.5 μmol/L was suggested as the cutoff for defining impaired B₁₂ function in infants [168]. Therefore, infants suspected of poor development and having feeding difficulties should be screened for vitamin B₁₂ deficiency before tests of the inborn errors of metabolism are carried out. Exclusive breastfeeding beyond 4–5 months of age should be another pointer for B₁₂ assessment.

Malabsorption

Malabsorption leading to B₁₂ deficiency is not only prevalent in the elderly; it can result from gastritis, intestinal diseases and infections, surgical resections, drugs, or it can have a genetic origin as described earlier. In B₁₂-deficient patients, for whom no “obvious” cause is apparent and who are IF antibody negative, the capacity to absorb the vitamin should be established. Unfortunately, the traditional Schilling tests used for this purpose have been discontinued due to the safety factors related to use of radioactive B₁₂. One

option is the CobaSorb test based on changes in circulating holoTC before and after administering oral B₁₂ [122]. Importantly, this test cannot be used once the patient is treated with B₁₂ [169]. Although the CobaSorb test has a potential diagnostic use, poor availability of holoTC measurements in many clinical settings, its dynamic design, as well as a lack of interpretative knowledge hinder its wider use.

Ageing. Malabsorption due to autoimmune disease or gastritis is the main cause of B₁₂ deficiency in the elderly, followed by inadequate dietary intake. For example, in a French study of 172 elderly patients with B₁₂ deficiency, 2% of the cases were a consequence of inadequate intake [170]. Atrophic gastritis is the main cause of dietary cobalamin malabsorption in the elderly and its prevalence increases with age [170,171] from as much as 24% in those aged 60–69 years to 37% in those aged >80 years [172].

The incidence of B₁₂ deficiency increases overall with age. In one longitudinal study, it was estimated that the mean annual decline in serum B₁₂ is 3.4 pmol/L for men and 3.2 pmol/L for women [173]. Estimates of B₁₂ deficiency of 1.6% were found in subjects over 51 years [174]. Five percent of deficiency was estimated in people 65–74 years and more than 10% in people 75 years or older (based on serum B₁₂ <150 pmol/L or B₁₂ <150 pmol/L and tHcy >20 μmol/L) [175]. In the same population-based cross-sectional study of people living in the UK, it was found that folate deficiency also increased with age, but only about 10% of people with low B₁₂ concentrations also had low folate concentrations [175]. This is relevant in view of the findings that elderly people who have high folate concentrations (>45.3 nmol/L) accompanied by low B₁₂ (<148 pmol/L) have higher metabolic evidence of B₁₂ deficiency than when folate is normal [176]. This has a potential implication for vitamin B₁₂ status, especially in the countries where mandatory folic acid has been implemented but not B₁₂ fortification.

The prevalence of vitamin B₁₂ deficiency in the elderly may be even higher in other countries. Using a serum B₁₂ cutoff of 220 pmol/L, an Iranian study found that 56% and 93% of people aged 65–74 and ≥ 75 years, respectively, were at high risk of B₁₂ deficiency [177].

The mechanisms behind decreasing B₁₂ status in the elderly are not known. However, decreases of B₁₂ with age were also found in total brain levels, particularly decreases in Me-Cbl that were observed in the frontal cortex [178]. The authors attributed these to changes in activity to one or more transport processes across the

lifespan, such as transport across the choroid plexus *via* megalin. Equally, cognitive deficits have been observed in elderly people who are well-nourished but have low B₁₂ status [179]. For more information on cognition, the reader is referred to the vitamin B₁₂, cognition, and dementia section below.

Autoimmune disease and gastritis. Some cases of pernicious anemia (PA) can be described as the end stage of autoimmune gastritis (AIG), in which parietal cell antibodies (PCAs) lead to the destruction of IF-producing parietal cells in the stomach, leading to B₁₂ malabsorption and, consequently, deficiency [180]. The prevalence of PA in the general population is 0.1%, increasing to 1.9% in those over the age of 60 [59,181]. Clinical features often include anemia, thrombocytopenia, neutropenia, glossitis, cardiomyopathy, jaundice, weight loss, and neurological symptoms [180]. PCAs are detected in more than 90% of patients with PA. However, these antibodies are not only specific to PA and are found in other autoimmune conditions that often occur concomitantly with PA, such as Graves' disease, hypothyroidism, thyroiditis, and Addison's disease [180]. Two types of antibodies related to IF have been detected in the sera of PA patients: type I blocks the binding of B₁₂ to IF (found in 70% of PA patients); type II prevents the attachment of IF or the IF-B₁₂ complex to ileal receptors (in ~40% of PA). Antibodies to vitamin B₁₂ binding proteins have also been reported [182]. These antibodies lead to high serum B₁₂ and holoTC concentrations, and they have not only been seen in cases of B₁₂ injections but also in patients not receiving B₁₂ supplements [183]. The presence of these antibodies does not appear to interfere with the binding of B₁₂ to TC, but they may impair the ability of cellular delivery. One study has shown that the ability to deliver B₁₂ to cells *in vitro* was impaired and clearance of B₁₂ *in vivo* of B₁₂ was abnormal in the presence of these TC antibody complexes [183]. Precipitation of these complexes with polyethylene glycol resulted in the normalization of serum B₁₂ concentrations in many patients with autoimmune diseases, hematological disorders, and plasma cell neoplasms [182]. Although the presence of these complexes is thought not to be pathological, one needs to be aware of them, especially in patients with normal/high B₁₂ concentrations and clinical signs of deficiency [180,182]. Development of diagnostic methods for the presence of immune complexes would help with the assessment of vitamin B₁₂ status.

Intestinal diseases and infections. Hypochlorhydria associated with atrophic gastritis hinders the release of B₁₂ from food and additionally causes intestinal bacterial overgrowth. The bacteria might compete for the available B₁₂, contributing further to B₁₂ deficiency [184,185].

Additionally, *Helicobacter pylori* infection in the intestines, which is highly prevalent in the general population, predisposes individuals to PA through inducing autoantibodies to antigens in the gastric mucosa. This effect is observed in half of infected patients, suggesting that gastric atrophy may be caused by a *Helicobacter pylori* driven autoimmune process [180,186].

Some patients develop B₁₂ deficiency as a result of conditions that slow the movement of food through the intestine, allowing intestinal bacteria to multiply and overgrow in the upper part of the small intestine. These bacteria utilize vitamin B₁₂ for their own metabolism, rather than allowing it to be absorbed by the body. Parasites, most notably the fish tapeworm, also lead to B₁₂ malabsorption since they consume B₁₂ for their own needs [187].

Patients with ileal Crohn's and Celiac disease will develop vitamin B₁₂ deficiency because of reduced absorption as a result of villous atrophy and mucosal impairment, respectively. One study has found B₁₂ deficiency in 33% of patients with Crohn's disease compared to 16% of patients with ulcerative colitis [188]. The study also showed that the serum B₁₂ test was insensitive during B₁₂ deficiency detection in these patients, as only 5% of the cases were deficient using this marker, compared to 32% using holoTC/MMA in 89 patients who underwent paired testing [188].

Patients suffering from chronic pancreatitis also have affected vitamin B₁₂ absorption since pancreatic enzymes are required for the release of cobalamin from R-binders [189]. Other intestinal disorders affecting vitamin B₁₂ include tropical sprue, intestinal lymphoma, amyloidosis, and short bowel syndrome [32].

In HIV infected patients, B₁₂ deficiency is also quite common. The causes of this deficiency can be ileal dysfunction, diminished IF secretion, or IF antibodies [190].

Surgery. Surgery involving any part of the gastrointestinal tract will lead to diminished absorption of vitamin B₁₂ and, consequently, B₁₂ deficiency in some patients. About 30% of patients following a partial gastrectomy will develop vitamin B₁₂ insufficiency [32]. Vitamin B₁₂ deficiency was found in 48% and 65% of patients with ileal resections of ≤ 20 cm and > 20 cm, respectively [188].

B₁₂ deficiency is also common in patients undergoing bariatric surgery, causing poor absorption of

food-bound B₁₂ postoperatively. The prevalence of deficiency following bariatric surgery varies between studies but may depend on the type of surgery. The highest prevalence of B₁₂ deficiency, ranging from ~35% to 75%, was reported following Roux-en-Y gastric bypass (RYGB), as no gastric acid remains in the gastric pouch [191], compared to post-sleeve gastrectomy (SG) [47]. It was also recently found that MMA increased as early as two months following surgery, and holoTC decreased in patients following RYGB and SG, indicating a negative B₁₂ homeostasis immediately following surgery [47]. However, changes in serum B₁₂ were observed after 6 months in this study indicating that holoTC and MMA may be more appropriate for monitoring B₁₂ status post-bariatric surgery.

Surgical anastomoses, which create gastrointestinal cul de sacs, can lead to severe bacterial overgrowth [192] and, consequently, B₁₂ inadequacy.

Pharmaceutical interactions.

Metformin. Metformin is a medication for the treatment of type 2 diabetes (T2D), prediabetes, and polycystic ovary syndrome. It is a drug known to affect mitochondrial function and glucose metabolism [193]. A range of evidence since 2003, including observational, experimental, and meta-analyses, suggests that the use of this oral hypoglycemic medication interacts negatively with and leads to a marked reduction in B₁₂ status [194,195]. However, the associations between B₁₂ levels and metformin appear not to be linked to the duration of therapy and dosage [196]. Not many metformin-related studies have used functional markers of B₁₂ status. In those that have, elevations in tHcy and MMA were found in addition to low serum B₁₂ concentrations [197]. In other studies, no difference or lower MMA was found in T2D patients using metformin [198,199].

Although it is widely accepted that metformin induces malabsorption of B₁₂ *via* inhibition of the Ca²⁺-dependent binding of the IF-Cbl complex to cubam receptors in the ileum (an effect that can be reversed by increasing calcium intake [200]), other mechanisms affecting B₁₂ status are also possible. Notably, Liu et al. [201] showed that adaptations in mitochondrial substrate utilization underlie metformin sensitivity/resistance and can result in perturbations in the tricarboxylic acid cycle and ATP production, generation of reactive oxygen species, and alterations to nucleotide/lipid synthesis. It was also shown that genetic variation in *HIBCH*, *MUT* genes, as well as genes encoding for proteins/enzymes in the branched chain amino acids pathway, affected the mitochondria as a direct consequence of metformin exposure [201]. We suggest that current

data support the view that patients on long-term metformin treatment should have their functional B₁₂ status checked at regular intervals.

Protein pump inhibitors and H₂-receptor antagonists. Proton pump inhibitors (PPIs), such as omeprazole, lansoprazole, and esomeprazole, and H₂-receptor antagonists, such as ranitidine, cimetidine, famotidine, and nizatidine, are used to reduce gastric acid secretion for the treatment of gastroesophageal reflux disease and peptic ulcers [194]. Similar to metformin, associations of these drugs with lower serum B₁₂ have been found. However, none of these studies were a randomized controlled trial, nor did they assess changes in B₁₂ markers or the onset of clinical indications of deficiency [194]. A higher incidence of B₁₂ deficiency has been found in older adults using PPIs for more than 12 months in case-control studies of [202,203]. The same was not concluded in a study of elderly patients taking PPIs for more than three years [204]; however, the cross-sectional design of this work, as well as a slightly higher proportion of controls positive for *Helicobacter pylori* infection, albeit not significant ($p = .09$), may have accounted for the lack of difference in B₁₂ status between PPI users and controls. The use of omeprazole in patients with duodenal ulcers with concurrent *Helicobacter pylori* infections augments the pH-increasing effect of PPIs [205].

Genetic polymorphisms that diminish the microsomal cytochrome P450 driven catabolism of omeprazole have been shown to have an impact on serum B₁₂ concentration [206,207]. Those with a heterozygous mutation in the CYP2C19 polymorphism with a prevalence of 47% in Orientals and 30% in Caucasians had much lower B₁₂ concentrations compared to homozygotes (wt/wt) after >1 year therapy with 20 mg omeprazole daily [206]. Drinking acidic fruit juice concurrently with B₁₂ may improve B₁₂ absorption in PPIs users [208].

Oral contraceptives. Oral contraceptives (OCs) are among the most commonly used drugs in developing countries. They frequently contain both estrogen and progestin or progestin-only pills. The serum concentration of B₁₂ has consistently been reported as lower in OC users when compared to nonusers [209]; however, the values were still within normal limits in most women [210]. The findings were the same for users of the combined pills [209,211] as well as progestin-only pills [210]. Lower vitamin binding capacity and lower haptocorrin levels have been suggested as potential causes for lower B₁₂ in OC users [209]. It has been hypothesized that hormones used in OCs may affect

the synthesis of haptocorrin, since the same is observed in pregnancy as well [209]. Some studies also measured holoTC in addition to total B₁₂; in one cross-sectional study of 264 females, holoTC was found to be 25% lower in OC users than in controls [211]. The women in this study were most frequently on combination tablets containing the synthetic estrogen ethinylestradiol and the progestogens, levonorgestrel or drospirenon [211]. The proportional decreases in both cobalamin markers in this study indicates that cobalamin bound to HC and to TC was equally affected in OC users. However, the same group has previously shown that total TC is not significantly lower in OC users [212]. Hence, the mechanism for the observed decrease in plasma holoTC is not clear and warrants further investigation.

Consequently, it seems reasonable to take OC usage into consideration when interpreting B₁₂ markers, especially total B₁₂ and holoTC. Nonetheless, this fall in abundance does not appear to reflect a functional deficient state, as no differences in tHcy or MMA have been observed [211]. Therefore, redistribution of B₁₂, rather than depletion of intracellular cobalamin, has been proposed in OC users [211].

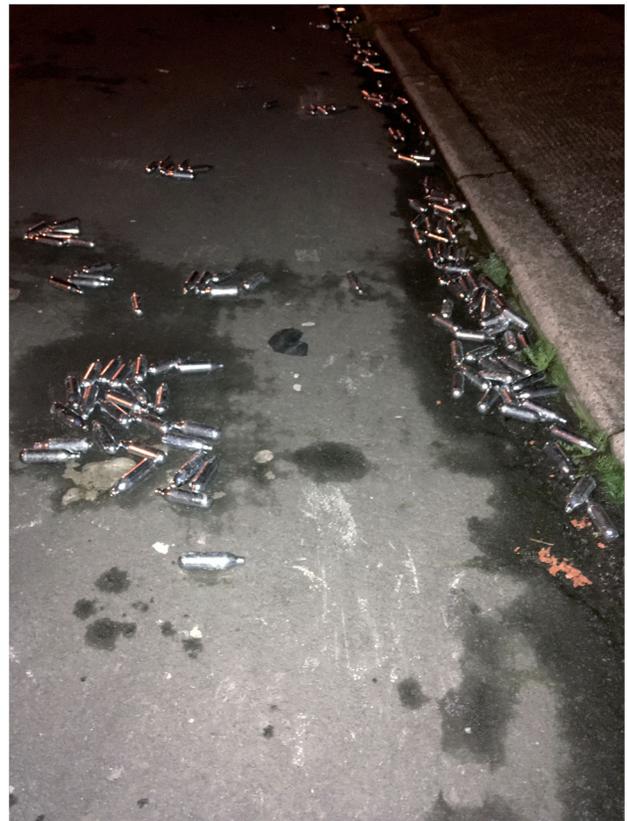


Figure 6. Empty nitrous oxide canisters in a London residential street, February 2020.

Nitrous oxide. Nitrous oxide (N₂O) is commonly used for sedation and pain relief, but it is also abused. The gas is also a food additive when used as a propellant for whipped cream, hence its low price and easy access [213]. The gas is inhaled, typically by discharging N₂O cartridges into another object, such as a balloon, or directly into the mouth. Empty silver canisters of N₂O have become a common sight not only outside night clubs but also on the streets in residential areas in the UK (Figure 6).

Nitrous oxide oxidizes the active cobalt atom of cob(II)alamin from the 1+ to the 3+ valence state at a high rate, outperforming the reductive recovery system *via* cblC and MS-reductase [214,215]. This leads to a stable shift toward cob(III)alamin, followed by dissociation of Cbl from MS and inactivation of apo-MS (Figure 2) [216]. Inactivation of the MS enzyme will lead to the impairment of homocysteine remethylation to methionine and, subsequently, all methylation reactions become affected due to a low level of SAM. In contrast, the second Cbl-dependent enzyme, MCM, is unaffected by N₂O from the start, because MCM does not generate cob(II)alamin but instead generates cob(III)alamin, which is insensitive to N₂O. However, long-term abuse of N₂O leads to repeated reductions and oxidations of Cbl, giving off reactive oxygen species upon oxidation (e.g. H₂O₂ [214]). These reactive molecules can convert Cbl to inactive “stable yellow corrinoids” with a broken pattern of the conjugated bonds [217]. Such degradation of Cbl causes gradual inactivation of MCM as well. Therefore, biochemically, highly elevated tHcy is seen first in patients who abuse N₂O, followed by a slow decrease in serum B₁₂ and holoTC, as well as slight elevations in MMA. Serum B₁₂ is often normal at presentation [218,219], which may lead to an incorrect differential diagnosis. It has been also suggested that inactive Me-Cbl is converted to Ado-Cbl [216].

Some of the short-term effects of N₂O include euphoria, numbness, sedation, giddiness, uncontrolled laughter, uncoordinated movements, blurred vision, confusion, dizziness and/or lightheadedness, sweating, and tiredness. If taken in large doses, N₂O can cause loss of blood pressure, heart attack, or death due to hypoxia. Long-term effects lead not only to severe B₁₂ deficiency but also memory loss, myelopathy, incontinence, demyelinating polyneuropathy, subacute combined degeneration, limb spasms, weakened immune system, disruption to reproductive systems, depression, and psychosis [213,218]. In the authors' hospital, which is located in central London, N₂O poisoning cases are seen regularly. Most patients are in their early twenties and often present to the A & E department with lower

leg weakness and sensory loss, urinary incontinence, tingling in the fingertips, and difficulty walking. Their plasma tHcy is frequently >100 μmol/L. Some patients reported ordering canisters off the internet in bulk and taking ~100 canisters when at a party or an event. A marked improvement in neurological symptoms is often seen following IM hydroxycobalamin injections and homocysteine normalization.

A single use of N₂O for anesthetic purposes should not pose a health risk; however, more work may be required to confirm this since some studies suggested possible adverse health effects. In a study by Zanardo et al. [220] both plasma homocysteine of mothers and cord blood of the respective offspring were higher in 25 women undergoing an elective cesarean section under nitrous oxide general anesthesia than in 25 undergoing vaginal delivery. Moreover, maternal homocysteine levels significantly correlated with cord levels of cesarean and vaginally delivered neonates ($r=0.57$; $p<.01$ and $r=0.66$; $p<.001$, respectively) [220].

In a different study involving 394 patients, postoperative hyperhomocysteinemia was associated with an increased risk of major complications with a risk ratio (RR) of 2.8 (95% CI: 1.4–5.4, $p=.002$) and cardiovascular events, with an RR of 5.1 (95% CI: 3.1–8.5, $p<.0005$) in the N₂O group [221].

Data on occupational exposure to N₂O is scarce. One study reported increased oxidative DNA damage in medical personnel of operating theaters. The extent of genetic injury was especially evident among nurses and anesthesiologists exposed to N₂O in concentrations exceeding occupational exposure limits (180 mg/m³) [222].

Treatment and prevention regimens

The treatment choice for clinical deficiency depends on whether there is neurological involvement; a specialist should manage such patients. If a specialist is not immediately available, 1 mg of hydroxocobalamin should be given intramuscularly on alternate days until there is no further improvement, then intramuscularly every 2 months [223]. For those without neurological involvement, 1 mg hydroxocobalamin should be administered intramuscularly three times a week for 2 weeks to replenish stores. This should result in retention of up to 3–4 mg of B₁₂, sufficient to bring the body's stores to within the normal range [33]. On-going maintenance dosage depends on whether the deficiency is diet-related. For patients with deficiency that is not of dietary or drug-related origins, but caused by life-long malabsorption, one should continue with 1 mg intramuscularly every 2 months for life [223].

Patients who have undergone bariatric surgery are recommended oral, sublingual, or liquid B₁₂ with 350–1000 µg daily, as well as nasal sprays as directed by manufacturer or parenteral (IM or subcutaneous) with 1000 µg monthly prevent deficiency [224]. A 1000 µg/d of B₁₂ to achieve normal levels is recommended for bariatric patients with deficiency [224]. An oral vitamin B₁₂ with 350 µg deemed appropriate to correct low B₁₂ concentrations in many patients following bariatric surgery [225].

Those whose deficiency is likely diet-related can consider oral supplementation with 50–150 µg B₁₂ daily or have a twice-yearly 1 mg hydroxocobalamin injection [223]. In severe cases, one could also consider commencing treatment with several injections to replenish B₁₂ stores. In vegans, treatment may need to be life-long. In others, replacement can be stopped once B₁₂ levels are corrected and the diet has improved; it is important to give dietary advice about foods rich in B₁₂ such as meat, fish, eggs and dairy products.

If there are doubts regarding the formal diagnosis of deficiency, there is little to be lost, and much to be gained, by instigating a trial of replacement. Alternative administration routes (see further) can be considered instead of injections to keep costs low [226].

Dosage, formulation, and schedule of administration vary between countries, probably reflecting a paucity of randomized research fulfilling the strict criteria of evidence-based medicine [59]. Cyanocobalamin and hydroxocobalamin are the most commonly used formulations, but methylcobalamin and adenosylcobalamin are also available, especially *via* the internet. In some countries, the market has been flooded with the methylcobalamin form of vitamin B₁₂, promulgating it as the preferred formulation for treatment [227]. However, using unstable photo-sensitive methylcobalamin and adenosylcobalamin may not be advantageous over cyanocobalamin and hydroxocobalamin, as they are likely to be converted to hydroxocobalamin *in vivo* during intracellular processing. The newly internalized alkylcobalamins (i.e. methylcobalamin and adenosylcobalamin) probably undergo dealkylation by the enzyme MMACHC (cblC) [50]. It is therefore likely that these forms of B₁₂ are no superior to cyanocobalamin in daily oral therapy regimes [228].

Moreover, the solvent vehicle the vitamin is dissolved in can have an impact on treatment frequency. It was shown that cyanocobalamin in an oily suspension (Betolvex) had a longer treatment response than cyanocobalamin in aqueous solution (Cytobion) [229].

Cyanocobalamin and hydroxocobalamin have differing pharmacokinetic properties. Hydroxocobalamin is

retained far more efficiently than cyanocobalamin. It disperses more slowly from the injection site, binds more strongly to plasma, and has a slower diffusion rate than cyanocobalamin [230–232]. Hence, cyanocobalamin is administered at 1000 µg once a month, while the same dose of hydroxocobalamin can be administered every two to three months. Treatment for PA in France involves daily administration of 1 mg of cyanocobalamin for one week, followed by 1 mg per week for 1 month, and then by monthly 1 mg injections for life [59]. In some other countries, such as Sweden and Canada, a high oral dose is the treatment of choice [233]. Oral doses ≥ 500 µg per day of cyanocobalamin have been found effective at treating B₁₂ deficiency, as $\sim 1\%$ of this quantity is absorbed *via* passive diffusion [30]. A recent pragmatic randomized multi-center trial of the effectiveness of oral vs intramuscular B₁₂ in elderly deficient patients showed that 1 mg oral administration was no less effective than IM administration at 8 weeks, although small differences were found between administration routes after 1 year (73.6% achieved normal B₁₂ in the oral arm vs 80.4% in the IM arm [234]. This study confirms the findings of other earlier studies, which found that oral cobalamin treatment with large vitamin B₁₂ doses (1–2 mg daily) was as effective as IM cobalamin treatment [235,236].

Vitamin B₁₂ administered sublingually and intranasally has also been shown to be effective at treating B₁₂ deficiency [237,238]. A sublingual dose of 50 µg/d (350 µg/week) of cobalamin, instead of the commonly used 2000 µg/week provided in a single dose was suggested by Del Bo et al. [239] to reach a state of nutritional adequacy of B₁₂ in vegetarians and vegans.

A recent survey found that one in ten of Pernicious Anemia Society members resort to unlicensed formulations to “supplement” their prescribed treatment regime [92]. These include nasal-sprays, sublingual drops and sprays, transdermal patches, suppositories, self-administered subcutaneous injections, and even intravenous infusions. None can be deemed to be any superior, as there is very little evidence base for these preparations.

It is noteworthy that early publications concerning parenteral B₁₂ refer to IM and subcutaneous routes as modes of administration. Self-administered B₁₂ *via* subcutaneous injection should perhaps be explored as an alternative to current treatment regimes. This would significantly reduce costs and undoubtedly benefit developing countries where deficiency is highly prevalent but nursing care is scarce. However, there is an inadequate research base differentiating between “IM” and “subcutaneous” routes, and further work is required to fully evaluate their relative efficacies.

Many people choose to supplement with vitamin B₁₂ as a conscious health decision, and supplementation of vitamin B₁₂ has been shown to be well-tolerated without significant adverse effects when consumed by healthy adults or B₁₂-deficient patients [240]. The elderly, vegans, and vegetarians may likely benefit from voluntary supplementation. Vitamin B₁₂ supplementation is also highly recommended during pregnancy; however, the recommendations for the dose and the duration have not been fully validated. Doses of 50 µg and 250 µg daily have been effectively used in clinical trials in countries with a high prevalence of vitamin B₁₂ deficiency [149,150], but whether a physiological dose of B₁₂ will be protective against deficiency during pregnancy remains to be established [241]. In their study in Indian lactovegetarians with low vitamin B₁₂, Naik et al. [242] have shown that the physiological dose of B₁₂ (2 × 2.8 µg/d) for eight weeks cannot provide a fast replenishment of cobalamin stores and restoration of a normal metabolic status.

Considering associated risks

It is beyond the scope and size of this review to address all the potential risks associated with low B₁₂ status thus far. Here we can count (i) hyperhomocysteinemia, which results from vitamin B₁₂ deficiency among other causes; (ii) its impact on disease risk [243] and; (iii) cobalamin interactions with folate [244].

Importantly, in a hospital setting, there are twice as many patients with high serum B₁₂ than with its low concentrations [116]. Whilst many of these patients are on B₁₂ supplements, some cases of high B₁₂ can be indicative of disease state/risk [245,246].

Only the selected health risk/diseases will be discussed in the following section.

Vitamin B12, PA, and cancer risk

Pernicious anemia is considered to be a premalignant condition. Some tumors in PA patients arise as a consequence of gastric atrophy, and subsequently, the prolonged elevated gastrin levels have a trophic effect on cells that gastric tumors originate from [247]. Patients with PA have a significantly increased risk of gastric carcinoid tumors, adenocarcinomas, tonsillar cancer, hypopharyngeal cancer, esophageal squamous cell carcinoma, small intestinal cancer, liver, myeloma, acute myeloid leukemia, and myelodysplastic syndrome, but a lower risk of rectal cancer [247]. Based on their study in PA patients, Kokkola et al. [248] confirmed a high risk for gastric carcinoids in this patient group, though discrepancies exist regarding advice about regular

gastroscopic follow-up of PA patients [247]. However, it would be prudent to consider thrice-yearly endoscopic surveillance of those PA patients with a family history of gastric cancer, or advanced age (≥70 years), pending further prospective cohort studies and cost effectiveness analyses [249].

It is unknown if vitamin B₁₂ deficiency contributes to cancer risk by affecting DNA synthesis and methylation potential (*via* the remethylation pathway), or if an excess of cobalamin is associated with cancer risk. Similar mechanisms were purported in folate deficiency and excess in relation to cancer risk [244]. The associations of high B₁₂ with cancer or liver diseases have been explained by a release of HC from proliferating leukocytes/malignant tissues/damaged hepatocytes, as well as by an increased synthesis or decreased clearance of TC and HC proteins [245,246], hence high B₁₂ concentrations are accumulated in blood. A screening strategy for addressing high serum B₁₂ values was proposed [246]; however, this strategy did not suggest using cobalamin as a diagnostic marker for cancer, but only suggested following it, if unexplainable high concentrations were encountered. A value of 1000 pmol/L has been suggested as a cutoff for high serum/plasma B₁₂ concentrations if the elevated level did not originate from regular intakes of high vitamin doses [246]. Importantly, Arendt et al. [250] showed that high plasma B₁₂ increased the risk of subsequently diagnosed cancer, mostly within the first year of follow-up. Moreover, a recent large study, which used pre-diagnostic biomarker data from 5183 case-control pairs nested within 20 prospective cohorts and genetic data from 29,266 cases and 56,450 controls, found that higher vitamin B₁₂ concentrations were positively associated with overall lung cancer risk, with the authors concluding that high vitamin B₁₂ status increases the risk of lung cancer [251]. Of note is the fact that patients with values of B₁₂ >850 pmol/L were excluded from analysis in this study.

Vitamin B12, cognition, and dementia

B₁₂ deficiency can present as “cognitive impairment” in some individuals and is a recognized cause of reversible dementia [252]. Its relative contribution to the etiology of specific dementias, including Alzheimer’s disease (AD), is of high interest given the dearth of current prophylaxis for dementias. Associations between B₁₂ deficiency and AD emerged in the 1980s [253,254], and it was unclear whether the two were definitely related. The discovery of low B₁₂ concentrations in a family with genetically determined AD confirmed a genuine association [255]. The advent of homocysteine as a marker for

B₁₂ deficiency resulted in an avalanche of literature describing its association with AD, vascular dementia, and other neurological disorders [243,256]. Lowering homocysteine with high dose B vitamins, including B₁₂, has proven effective in slowing cognitive decline and brain atrophy [257,258]. Credible mechanisms underlie the association, which fulfills Bradford-Hill's criteria suggesting causality [259]. Further trials are needed, but the physician should be especially alert to middle-aged and elderly patients with insufficiency of B₁₂, in order to potentially slow cognitive decline and perhaps avert or delay a devastating dementia.

Vitamin B12 and multiple sclerosis

Multiple sclerosis (MS) is an autoimmune neurodegenerative disease of the central nervous system leading to axonal loss and demyelination. Cobalamin deficiency is prevalent in MS patients, although not all studies, which used serum B₁₂ as a deficiency marker, have demonstrated this, thereby questioning the role of B₁₂ in MS [260,261]. Significantly higher unsaturated R-binder capacities were also found in MS, compared to patients with other neurological impairments or normal controls [262]. It is possible that B₁₂ deficiency, whatever its cause might be, could render the patient more vulnerable to the putative viral and/or immunologic mechanisms, which have been suggested as the pathogenesis of this disease [260]. Conversely, chronic immune reactions or recurrent myelin repair processes in MS may increase the demand for vitamin B₁₂ [262]. More importantly, Nijst et al. [263] showed significantly lower B₁₂ in cerebrospinal fluid (CSF) in MS patients than in reference patients, but the same was not seen for serum B₁₂. Serum and CSF B₁₂ correlated positively in 293 neurological patients, including 58 with MS in this study, but in individual patients, CSF B₁₂ concentrations varied considerably for a given serum concentration in some patients. Of note is the fact that CSF B₁₂ was very low, while serum B₁₂ was normal, suggesting a blood-brain barrier transport defect [263]. A resemblance of MS to PA was also suggested (despite age of presentation differences), because the sex, racial, and geographical distribution of MS and PA are similar [264].

The studies using B₁₂ supplementation in MS are scarce and the benefits of regular B₁₂ supplementation to MS patients are yet to be demonstrated. For example, high methylcobalamin supplementation improved visual and brainstem auditory evoked potentials in one study [265], while adenosylcobalamin injections restored memory and speech as well as improved

balance and mobility in another study, though only one patient was involved [266].

Conclusions and future directions

Despite significant advances in elucidating mechanisms of vitamin B₁₂ absorption and intracellular processing, challenges remain in the diagnosis and prognosis of vitamin B₁₂ deficiency/insufficiency, as well as the frequency and the type of treatment being administered. We recommend that robust assessment of B₁₂ status should include at least two markers, especially in instances where discrepancies between values and clinical presentation exist, and the combined index cB₁₂ may also be calculated. Finding the cause of deficiency before commencing treatment is detrimental to the future management of patients. In the event where treatment should be initiated promptly due to severe presentation, blood samples should be taken and saved for future analysis as appropriate.

The elucidation of the mechanism of B₁₂ crossing the blood-brain barrier, a better understanding of the role of the microbiome and corrinoids on vitamin B₁₂ status and their impact on markers of B₁₂, validation of the CobaSorb or development of new absorption tests for use in diagnostic settings, as well as a better understanding of the impact of long term vitamin B₁₂ injections on B₁₂ status/markers as well as cobalamin's role in cancer, MS, and dementia are needed to improve B₁₂ diagnosis and health interventions.

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ORCID

Agata Sobczyńska-Malefora  <http://orcid.org/0000-0001-7349-9517>

Dominic J. Harrington  <http://orcid.org/0000-0003-4786-9240>

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