Review

Total Vitamin B12 and Holotranscobalamin: Current Evidence, Limitations, and Clinical Utility

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Review

Total Vitamin B12 and Holotranscobalamin: Current Evidence, Limitations, and Clinical Utility

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Abstract

Vitamin B12 is an essential water-soluble vitamin required for critical biological processes such as DNA synthesis, erythrocyte maturation, and maintenance of nervous system integrity. Deficiency of vitamin B12 can lead to serious clinical outcomes, including megaloblastic anemia, and potentially irreversible neurological damage. Conversely, hypercobalaminemia may be associated with severe disorders, including solid neoplasms, hematological malignancies and, in some cases, may result from inappropriate supplementation or immunoglobulin–B12 macro-complexes. Although current guidelines recommend total serum vitamin B12 and holotranscobalamin (holoTC) as first-line biomarkers, total serum vitamin B12 remains the most widely used test in routine clinical practice. However, since holoTC represents the biologically active fraction of vitamin B12 available for receptor-mediated cellular uptake, it appears to provide a more reliable assessment of cobalamin status, particularly in specific clinical contexts. Compared with total vitamin B12 measurement, holoTC is assessed using a more limited number of analytical methods and the majority of available kit are aligned with the WHO reference standard, thereby improving inter-assay harmonization. This review explores literature data about the role of vitamin B12 and holoTC, discussing analytical challenges and clinical interpretation, highlights the potential advantages of holoTC over total serum B12.

Keywords: holotranscobalamin; vitamin B12; analytical immunoassays; clinical issues

1. Introduction

Vitamin B12, also known as cobalamin, is an essential water-soluble vitamin, and in humans its availability depends entirely on dietary intake [1].

Cobalamin plays multiple roles in key biological processes, including DNA synthesis [2], hematopoiesis [3], myelin synthesis and maintenance [4], and fetal nervous system development [5]. Furthermore, it is indispensable for amino acid and homocysteine metabolism [6], nucleic acid methylation reactions [7], fatty acid metabolism [8], cognitive function [9], and folate activation [10].

Given its broad physiological roles, a deficiency in vitamin B12 can result in important clinical consequences, including megaloblastic anemia, cognitive impairment, and potentially irreversible neurological damage [11].

Vitamin B12 deficiency affects approximately 1–2% of the general population, rising to more than 10% in individuals over 65 years of age. This condition is especially common among high-risk groups, including older adults, individuals with insufficient intake of animal-based proteins, pregnant women, children, and patients with a history of gastrointestinal surgery [2].

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However, the prevalence of cobalamin deficiency is highly dependent on the diagnostic criteria applied, including the distinction between clinical and subclinical deficiency, the biomarker threshold values used, and the specific characteristics of the studied population [12].

One of the principal challenges in diagnosing vitamin B12 deficiency is the nonspecific nature of early symptoms, which often leads to delays in detection and underscores the importance of early recognition to prevent serious complications [13].

Although clinical attention is traditionally focused on vitamin B12 deficiency, elevated serum cobalamin concentrations also warrant consideration. Persistently high levels have been associated with serious underlying disorders, including solid neoplasms, hematological malignancies, and liver disease [14]. They may also result from excessive or inappropriate intake of vitamin supplements [15], or, in some cases, reflect analytical artifacts such as immunoglobulin-B12 macro-complexes [16].

The 2024 National Institute for Health and Care Excellence (NICE) guideline (NG239) [17] recommends performing an initial diagnostic test for vitamin B12 deficiency in individuals presenting with at least one suggestive symptom associated with a risk factor. Key signs and symptoms include anemia or macrocytosis, cognitive disturbances, ophthalmic manifestations, glossitis, peripheral or central neurological signs, unexplained fatigue, and lack of response to iron therapy during pregnancy or lactation. Major risk factors include a diet low in B12, autoimmune (pernicious anemia) or familial diseases, malabsorption, chronic use of medications interfering with vitamin absorption, previous radiotherapy or gastrointestinal surgery, and recreational nitrous oxide use, which inactivates vitamin B12 by oxidizing its central cobalt atom [18].

In fact, the guideline specifies that holoTC should be preferred especially in certain clinical contexts, such as pregnancy, due to physiological fluctuations that can affect total serum B12 levels.

Over the past years, other molecules involved in the vitamin B12 metabolic pathway, such as methylmalonic acid (MMA) (Figure 1) and homocysteine (Hcy) (Figure 2), have been shown to play an essential role in identifying patients with impaired vitamin B12 status [19].

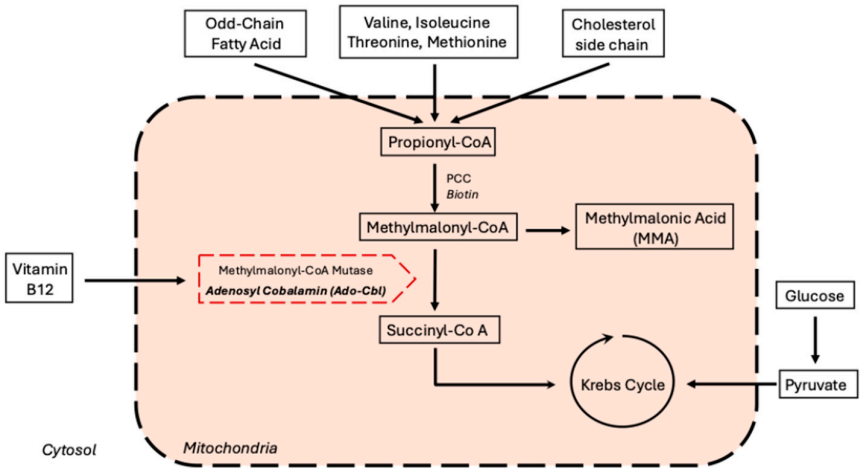

Figure 1. Vitamin B12 and methylmalonic acid. Methylmalonic acid accumulates as a result of impaired conversion of methylmalonyl-CoA to succinyl-CoA, a reaction catalyzed by methylmalonyl-CoA mutase and dependent on vitamin B12. Image created by the authors. CoA: Coenzyme-A; PCC: Propionyl-CoA carboxylase; Ado-Cbl: Adenosyl cobalamin; MMA: Methylmalonic acid.

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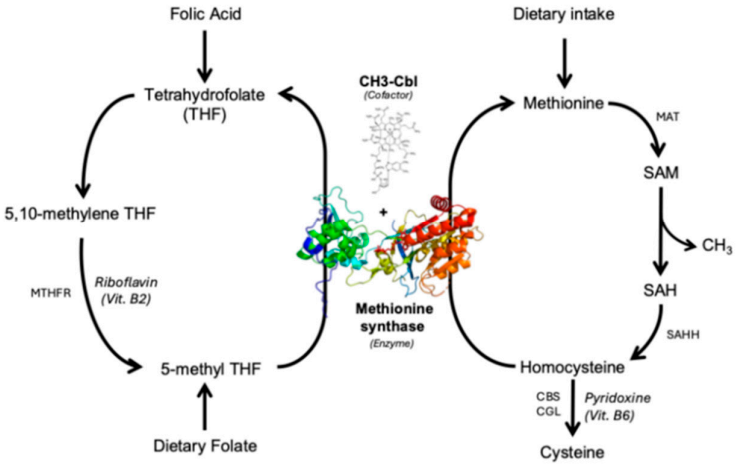

Figure 2. Vitamin B12 and homocysteine. In the absence of vitamin B12, methionine synthase activity is impaired, leading to homocysteine accumulation and folate trapping as 5-methyltetrahydrofolate, thereby reducing the availability of tetrahydrofolate for DNA synthesis. Image created by the authors. THF: Tetrahydrofolate; Cbl: Cobalamin; SAM: S-adenosyl-methionine; SAH: S-adenosyl-homocysteine.

MMA reflects altered adenosylcobalamin-dependent metabolism and often rises before serum cobalamin declines [20,21], while, homocysteine, although useful as a metabolic marker, is influenced by folate and vitamin B6 status as well as renal function.

However, MMA and Hcy routine use is limited by high analytical costs and restricted availability, resulting in variable implementation across laboratories and geographic settings.

Current guidelines NICE 2024 define specific reference ranges for total vitamin B12 and holoTC. For total vitamin B12, concentrations <200 ng/L (<148 pmol/L) indicate deficiency, 200–300 ng/L (148–221 pmol/L) are considered indeterminate, and >300 ng/L (>221 pmol/L) make deficiency unlikely. For holoTC, levels <25 pmol/L suggest deficiency, 25–50 pmol/L are indeterminate, and >50 pmol/L are unlikely to reflect deficiency [17]. However, interpretative challenges remain due to the lack of harmonization among different commercial assay kits; consequently, a bias persists between methods, most likely related to differences in analytical detection principles and calibrator traceability.

This review aims to examine the roles of vitamin B12 and holotranscobalamin starting from their biochemical properties and metabolic pathways, highlighting analytical challenges, limitations, and issues in clinical interpretation. In addition, it focuses on the role of holoTC in assessing true vitamin B12 status and discusses its advantages over total serum vitamin B12.

2. Methods

This review considered articles indexed in PubMed, published between 2005 and 2025. Publications released before 2005 were included only when they provided significant historical background or methodological insight. The literature search was performed using the following terms, either alone or in various combinations: vitamin B12, holotranscobalamin (holoTC), B12 deficiency, biomarker, total serum vitamin B12, analytical limitations, and clinical application. Boolean operators (AND, OR) were applied to optimize the search strategy. Information obtained from the selected studies addressed the physiological role of vitamin B12 and holoTC, analytical techniques for their assessment, and their relevance in clinical practice.

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3. Vitamin B12 and Holotranscobalamin (holoTC)

3.1. Biochemical Properties

The molecular structure of vitamin B12 (Figure 3) was elucidated by X-ray crystallographic studies, which identified the compound as a cyanide-ligated, cobalt-containing organometallic corrinoid [22]. The cobalt ion in vitamin B12 is centrally positioned within a contracted tetrapyrrole macrocycle, known as the corrin ring, composed of four reduced pyrrole units that coordinate the metal through their nitrogen atoms [23].

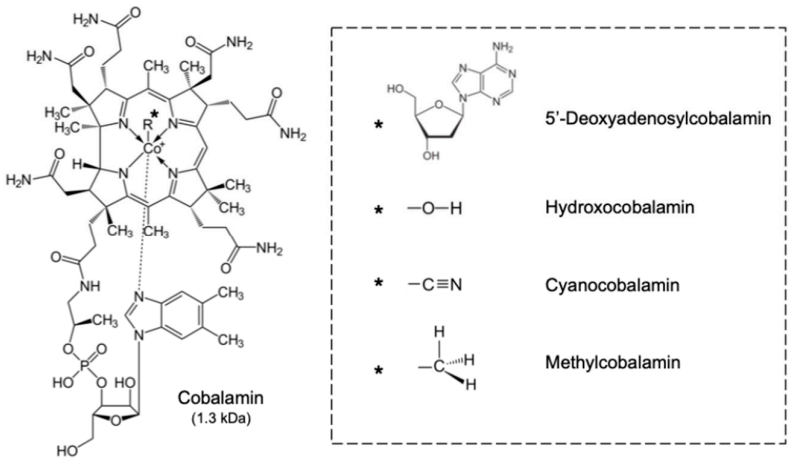

Figure 3. Chemical structure of cobalamin (vitamin B12). The corrin macrocycle confers unique chemical and biological properties to vitamin B12 [23,24]. The central cobalt atom is coordinated by the corrin ring and axially by a nucleotide base, typically 5,6-dimethylbenzimidazole, and a variable upper ligand, which defines the different cobalamin species. Methylcobalamin and 5′-deoxyadenosylcobalamin are the biologically active coenzyme forms, while Cyanocobalamin and Hydroxocobalamin serve mainly for transport or storage [25]. Image created by the authors.

Variability in axial ligation contributes to the functional versatility of vitamin B12 and modulates its interaction with cobalamin-binding proteins, underscoring its essential role in selective recognition and transport by transcobalamin [26].

Transcobalamin (TC), also known as transcobalamin II [27], is a soluble 43 kDa glycoprotein encoded by the TCN2 gene and is the principal carrier responsible for delivering vitamin B12 to cells [26]. At the molecular level, TC consists of two structurally distinct domains, termed the α- and β-domains, which are connected by a flexible linker [28]. These domains share structural homology with the other cobalamin-binding proteins, such as intrinsic factor (IF) and haptocorrin (HC), also known as transcobalamin I [29], reflecting their origin from a common ancestral gene. The binding of cobalamins requires the cooperative contribution of both TC domains and results in an extremely high binding affinity, in the sub-picomolar range [24]. The TC-Cobalamin complex, referred to as holotranscobalamin (holoTC), represents the biologically active fraction of plasma vitamin B12 due to its rapid cellular uptake via receptor-mediated endocytosis [17,26].

3.2. Metabolic Aspects and Plasma Levels

Despite its involvement in a broad spectrum of essential biological processes in humans, the ability to synthesize vitamin B12 is restricted to certain bacteria and archaea [30]. Animals are unable to produce it endogenously; however, some species host symbiotic microorganisms capable of synthesizing it [31]. In humans, these bacteria reside predominantly in the colon, whereas B12 absorption occurs in the ileum. Because microbial synthesis occurs distal to the site of absorption, B12 produced by the intestinal microbiota is not bioavailable to the human host [32]. Therefore, to meet

 doi:10.20944/preprints202603.0826.v1

the daily requirement of approximately 2.4 µg and to prevent clinical manifestations of deficiency, vitamin B12 must be obtained through the diet [33]. After dietary intake, vitamin B12 is absorbed through an intrinsic factor-dependent mechanism and subsequently transported and converted intracellularly into its biologically active form (Figure 4). In plasma, vitamin B12 binds mainly to HC (~80%) or TC (~20-25%), the latter mediating cellular uptake [34].

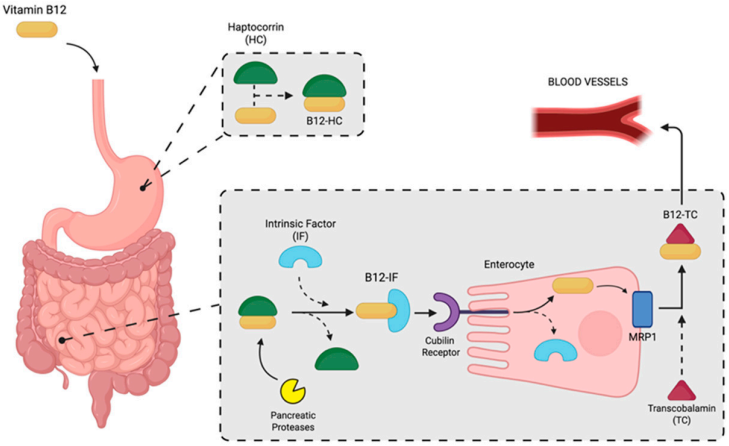

Figure 4. Vitamin B12 metabolism. Following ingestion, vitamin B12 binds to haptocorrin (HC) in the stomach and subsequently to intrinsic factor (IF) in the duodenum, enabling receptor-mediated uptake in the terminal ileum via cubilin [35]. In enterocytes, vitamin B12 is dissociated from intrinsic factor (IF) and subsequently transported into the bloodstream; this process appears to be mediated by several membrane exporters, including multidrug resistance protein 1 (MRP1) [30]. Image created in BioRender.com. HC: Haptocorrin; IF: Intrinsic Factor; MRP1: Multidrug resistance protein 1; TC: Transcobalamin.

Within enterocytes, vitamin B12 is converted into its biologically active coenzyme forms, adenosylcobalamin in the mitochondria and methylcobalamin in the cytosol [26,36] (Figure 5).

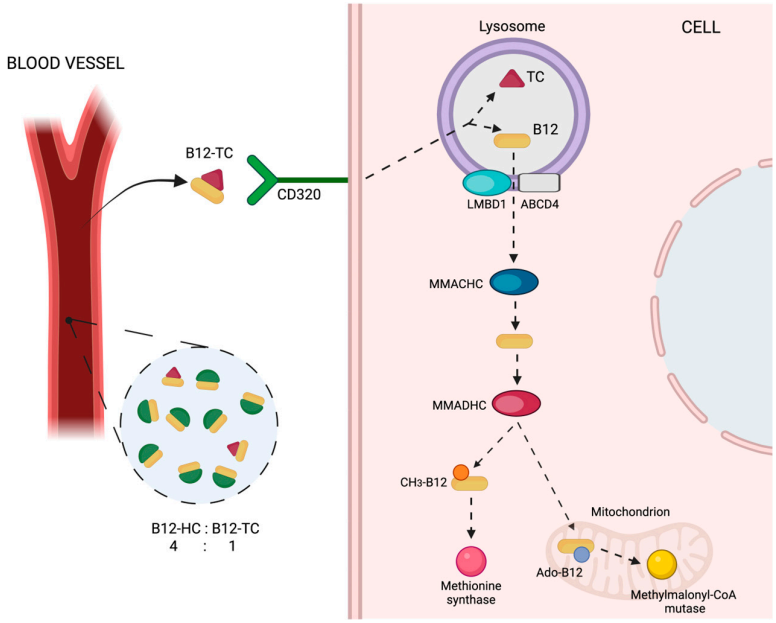

Figure 5. HoloTC cellular uptake. The TC–vit B12 complex binds to its receptor CD320. After endocytosis, the complex enters the lysosome, where the complex is degraded and free B12 is exported to the cytosol by ABCD4

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and LMBD1 [30,37]. Once released, cobalamin is processed by the chaperone proteins methylmalonic aciduria and homocystinuria type C (MMACHC) and type D (MMADHC). In the cytoplasm, vitamin B12 is converted into methylcobalamin (CH3-B12), whereas adenosylcobalamin (Ado-B12) is synthesized in the mitochondria [26,36]. Image created in BioRender.com. TC: Transcobalamin; ABCD4: ATP Binding Cassette Subfamily D Member 4; LMBD1: LMBR1 Domain-Containing Protein 1; MMACHC: methylmalonic aciduria and homocystinuria type C; MMADHC: methylmalonic aciduria and homocystinuria type D; CH3-B12: Methylcobalamin; Ado-B12: adenosylcobalamin.

The literature indicates that interindividual variability in serum holoTC and vitamin B12 concentrations is only partly explained by diet and lifestyle factors and is also associated with differences in metabolic pathway and liver function [14,38]. Major dietary sources of cobalamin are of animal origin [39], however, the strength of the associations between different animal-derived food products and cobalamin, varies across food groups and remains inconsistent in the published literature [40–43].

Variations related to age and sex have been observed in various studies, but the results are heterogeneous [38,44–46]. These discrepancies may partly reflect differences in the demographic, clinical, and environmental characteristics of the populations analyzed.

Moreover, holoTC concentrations remain stable during pregnancy, in contrast to total vitamin B12, which declines nearly 50% over the course of gestation. This difference suggests that holotranscobalamin may be a more reliable indicator of vitamin B12 deficiency during pregnancy than total serum cobalamin [47].

Additional determinants, including genetic background (such as polymorphisms in the transcobalamin gene TCN2), which influence TC expression and function [48,49] and possibly gut microbiota composition [50], may also play a role in cobalamin availability and metabolism.

3.3. Analytical Methods

Early approaches for total vitamin B12 determination relied primarily on microbiological assays based on the growth response of vitamin B12–dependent Lactobacillus species [51–53], in which extracted serum cobalamin served as the sole growth factor for bacterial proliferation. Although highly sensitive and traditionally considered reference methods, these assays were poorly reproducible [54], and susceptible to interference [55].

In the 1970s, radioimmunoassays (RIA) were developed [56,57], offering higher specificity and throughput than microbiological assays. These methods were later adapted for holoTC determination, involving separation of transcobalamin followed by quantification of bound vitamin B12 [58,59].

In the early 2000s, enzyme-linked immunosorbent assays (ELISA) were introduced for the quantification of both total B12 [60] and holoTC [61], representing a safer and more versatile alternative to RIA.

Subsequent methodological advances resulted in the development of automated immunoassays, which currently constitute the most widely used approach in routine clinical practice [62–64]. Currently available automated immunoassays for measuring total vitamin B12 and holoTC are based on competitive and sandwich assays (Figure 6), respectively.

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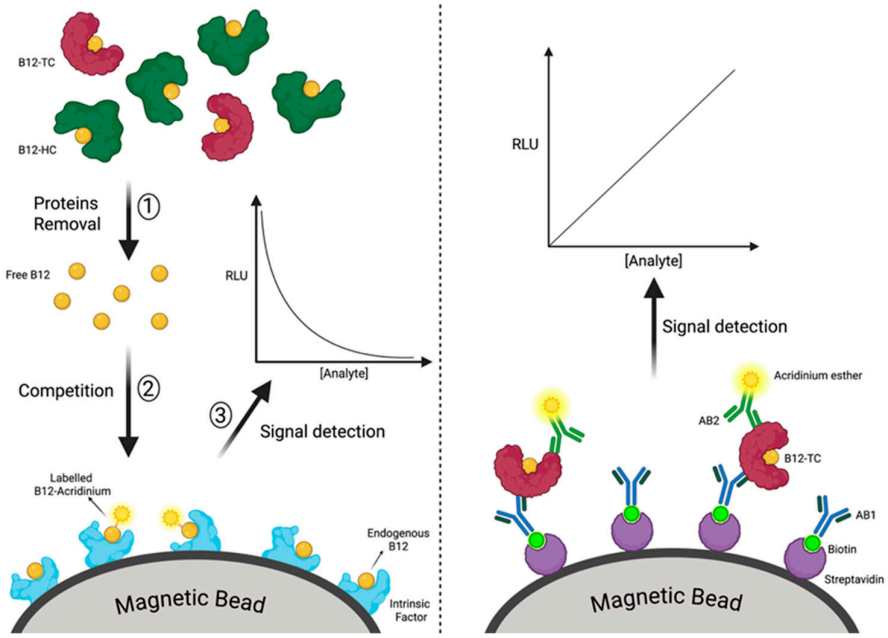

Figure 6. Principles of automated immunoassays for vitamin B12 and holoTC. The figure illustrates the most commonly used methods for measuring total vitamin B12 (left) and holo-transcobalamin (right). In the total B12 assay, the method separates the transport proteins (haptocorrin and transcobalamin) from vitamin B12 and then allows endogenous B12 to compete with acridinium-labeled B12 for binding sites. In the holo-transcobalamin assay, the technology employs two anti-transcobalamin antibodies, forming a sandwich complex for specific quantification. TC: Transcobalamin; HC: Haptocorrin; AB1: Antibody 1 (Capture Antibody); AB2: Antibody 2 (Detection Antibody). Image created in BioRender.com.

These assays provide practical, sensitive, and robust tools for the timely assessment of vitamin B12 status; however, as shown in Table 1, discrepancies exist between methods due to differences in both the standards employed and the expected values.

More recently, liquid chromatography–tandem mass spectrometry (LC–MS/MS) has emerged as a highly specific method for the accurate, high-sensitivity quantification of total vitamin B12 [65], and other markers of cobalamin status, such as MMA [66] and Hcy [67].

Although mass spectrometry has the potential to provide a more comprehensive assessment of cobalamin status, no clinically validated method currently seems to exist for holotranscobalamin, and LC–MS/MS remains a specialized, second-line technique that is not widely used for routine vitamin B12 measurement.

Emerging technologies have led to the development of point-of-care testing (POCT) devices for total vitamin B12, which are now commercially available and offer rapid measurements outside the central laboratory.

However, these platforms generally provide lower analytical sensitivity and specificity, compared with automated laboratory immunoassays [68]. Furthermore, POCT systems are generally unable to achieve the same high analytical precision as laboratory-based methods, and consequently tend to exhibit higher coefficients of variation, limiting their use to preliminary screening rather than definitive diagnosis [69].

Novel technologies, such as optical fiber–based and nanostructured electrochemical biosensors, have been developed for holoTC detection, employing antibodies or cobalamin-binding proteins combined with optical or electrochemical transduction [70,71]. Despite their high sensitivity, compact size, label-free operation, and potential for real-time or point-of-care use, these approaches remain largely limited to experimental or preclinical settings [72].

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Table 1. Commercially available automated immunoassays for total vitamin B12 and holoTC.

Analyte	Assay	Manufacturer	Platforms	Method	Assay range	Expected values	Standardization
Total B12	Mindray Vitamin B12 Assay [73]	Mindray Medical International Limited	Mindray CL series	CLIA	50.0-2000.0 pg/mL	180.0-916.0 pg/mL	Against a commercial VB12 test (CLIA)
	DiaSorin LIAISON Vitamin B12 [74]	DiaSorin S.p.a.	Liaison/XL	CLIA	55.0-1500.0 pg/mL	107.2-653.3 pg/mL	In-house Standard preparation
	MAGLUMI Vitamin B12 [75]	Snibe	MAGLUMI	CLIA	12.5-2000.0 pg/mL	200.0-1100.0 pg/mL	WHO IS 03/178
	TOSOH AIA-PACK B12 [76]	Tosoh Bioscience	Tosoh AIA	EIA	50.0-2000.0 pg/mL	230.0-1050.0 pg/mL	In-house Standard preparation
	VIDAS Vitamin B12 total [77]	Biomeriéux	VIDAS	ELFA	100.0-1200.0 pg/mL	166.9-582.8 pg/mL	WHO IS 03/178
	Alinity i B12 [78]	Abbott Diagnostics	Alinity i	CMIA	146.0-2000.0 pg/mL	187.0-883.0 pg/mL	WHO IS 03/178
	Atellica IM Vitamin B12 (VB12) [79]	Siemens Healthineers	Atellica IM	CLIA	45.0-2000.0 pg/mL	211.0-911.0 pg/mL	Cyanocobalamin (B12) USP Reference Standard
	Elecsys Vitamin B12 II [80]	Roche Diagnostics	Cobas E series	ECLIA	150.0-2000.0 pg/mL	232.0-1245.0 pg/mL	WHO IS 03/178
	Access Vitamin B12 [81]	Beckman Coulter	Access/ UniCel DxI	CLIA	105.0-2100.0 pg/mL	180.0-914.0 pg/mL	EN ISO 17511

 doi:10.20944/preprints202603.0826.v1

HoloTC	Alinity i Active-B12 (holoTC) [82]	Abbott Diagnostics	Alinity i	CMIA	5.0-128.0 pmol/L	25.1-165.0 pmol/L	WHO IS 03/178
	Atellica Active-B12 [83]	Siemens Healthineers	Atellica IM	CLIA	4.25–146.0 pmol/L	27.2-169.6 pmol/L	WHO IS 03/178
	Elecsys Active B12 [84]	Roche Diagnostics	Cobas E series	ECLIA	3.0-150.0 pmol/L	37.5-188.0 pmol/L	WHO IS 03/178
	Access Active-B12 (holoTC) [85]	Beckman Coulter	Access/ UniCel DxI	CLIA	3.0-160.0 pmol/L	32.2-152.6 pmol/L	EN ISO 17511

holoTC: holotranscobalamin; EIA: Enzyme Immunoassay; ELFA: Enzyme-Linked Fluorescent Assay; CMIA: chemiluminescent microparticle immunoassays; CLIA: chemiluminescent immunoassays; ECLIA: electrochemiluminescence immunoassays; WHO IS: World Health Organization International Standard; USP: United States Pharmacopeia; EN ISO: European Norm International Organization for Standardization.

3.3.1. Reference Materials and Assay Alignment

Although an international reference material (WHO/NIBSC 03/178) exists for total serum vitamin B12 (480 pg/mL) and holotranscobalamin (107 pmol/L) [86] not all commercially available methods are calibrated against this standard [87], thereby introducing an important source of analytical variability [68].

As reported in Table 1, total vitamin B12 assays exhibit heterogeneous traceability. Some methods are independently aligned to in-house standard preparations, others are standardized against internal reference materials produced using United States Pharmacopeia (USP)-approved materials, while additional assays are aligned to the WHO international standard or use manufacturer calibrators prepared from purified cyanocobalamin, with target values assigned spectrophotometrically according to EN ISO 17511.

In contrast to total vitamin B12, holoTC assays are currently available on only a limited number of analytical platforms (Table 1). Among these, most assays explicitly state traceability to the WHO International Standard 03/178, whereas only the Access Vitamin B12 assay (Beckman Coulter) reports traceability in accordance with EN ISO 17511, potentially supporting greater inter-assay harmonization for holoTC compared with total vitamin B12.

4. Discussion

Assessment of vitamin B12 status is a key aspect of clinical practice, particularly when deficiency is suspected. Evaluation should include potential metabolic abnormalities and genetic variants that may impair cobalamin metabolism and utilization. Total vitamin B12 deficiency can result in significant clinical consequences, including megaloblastic anemia, cognitive decline, potentially irreversible neurological damage, and metabolic disturbances. Conversely, hypercobalaminemia also requires careful interpretation, as high levels may be due to inappropriate supplementation or can also arise from analytical interference, circulating macrocomplexes formation, or underlying disorders, including paraneoplastic syndromes.

Despite numerous studies consistently demonstrating that no single biomarker possesses the necessary performance characteristics to establish deficiency in all patients, measurement of serum vitamin B12 remains the most widely used test in routine practice [68,88].

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As recommended by current guidelines, vitamin B12 status is typically assessed using first-line biomarkers such as total serum vitamin B12 and holotranscobalamin, with well-defined deficiency cut-off, [68,88]. Second-line tests include MMA and Hcy which should be considered when first-line test results are inconclusive or borderline [17].

Nevertheless, this approach may be inappropriate, as several issues still exist. Vitamin B12 is a biochemically complex molecule with circulating levels that are highly variable and influenced by multiple factors, including nutritional status, particularly vitamin and protein intake, hepatic and intestinal function, and inter-individual variability, which the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) estimates at approximately 37% [89].

Moreover, several analytical variables may significantly affect the results, including inter-method variability and the use of different reference standards. Additional discrepancies arise from manufacturer-specific reference intervals, which are not interchangeable. Furthermore, reference ranges reported in the literature vary widely due to population-specific characteristics, such as age, sex, pregnancy [90], ethnicity and cultural dietary habits [68,87] and macro-B12 formation [91].

Therefore, it's important to consider these factors, as guidelines define specific cut-off values that might not be always appropriate across different populations or analytical methods.

Evidence from studies conducted in different cohorts has consistently demonstrated this inter-method variability in total vitamin B12 cut-off values (Table 2), further limiting the accurate interpretation of clinical results.

Table 2. Inter-method and population variability of Total B12.

Authors/year	Individuals	Population	Platform	Literature RI	Manufacturers RI
Andersen SL *et al.*, 2023 [92]	41,091 Healthy donors 129 (60 m; 69 f)	Danish Healthy adults	Abbott Alinity Roche Cobas 6000 Siemens Atellica IM	**Healthy donors** 168-553 pmol/L 202-641 pmol/L 211-551 pmol/L **General population** 133-541 pmol/L 172-619 pmol/L 182-553 pmol/L	138-652 pmol/L 145-569 pmol/L 156-672 pmol/L
Jiang W *et al.*, 2020 [93]	389 (198 m; 191 f)	Chinese Healthy adults (21-80 y.o.)	Roche Cobas E602	250.8-957.1 pg/mL	197-771 pg/mL
Solé-Enrech G *et al.*, 2019 [94]	123 (59 m; 67 f)	Spanish Healthy adults	Roche Cobas 8000 e801	150-695 pg/mL (111-513 pmol/L)	197-771 pg/mL (145-569 pmol/L)
Sobczyńska-Malefora A *et al.*, 2023 [95]	35,988 (11,642 m; 24,346 f)	White 18,508 Black/British Black: 10,182 Asian/British Asian:	ARCHITECT Abbott	**0-1 years**: 159-1025 pmol/L **2-5 years** 276-1102 pmol/L **6-9 years** 245-798 pmol/L **10-13 years** 187-643 pmol/L **>13 years,**	138-652 pmol/L

 doi:10.20944/preprints202603.0826.v1

		2595		**Black** 166-805 pmol/L **> 13 years,** **white/asian** 134-511 pmol/L	
Alpdemir M. *et al.*, 2020 [96]	36,284 (10,795 m; 25,489 f)	Turkish	Abbott ARCHITECT i2000sr	97-397 pmol/L	138-654 pmol/L
Gavars. *et a*l., 2022 [97]	132,379	Latvian	Elecsys 2010 (2004-2012)	**196–942 pg/mL**	**180–900 pg/mL** (133–664 pmol/L)
			Roche Cobas e 2010b (2012-2017)		**191–663 pg/mL** (141–489 pmol/L)
			Roche Cobas e 801 (2017-2022)		197-771 pg/mL (146-569 pmol/L)
Abildgaard *A. et al.*, 2022 [98]	310 (158 m;152 f) (147 adults 18<65) 163 elderly >=65)	Danish	Siemens ADVIA Centaur XPT	**0–<1 y.o.:** 180–1400 pmol/L **1–<12 y.o.:** 260–1200 pmol/L **12–<18 y.o.:** 200–800 pmol/L **18–<65 y.o.:** 200–600 pmol/L **≥ 65 y.o.:** 200–600 pmol/L	/

m: males; f: females; RI: reference intervals; y.o: years old.

Although WHO International Standards for total vitamin B12 and holotranscobalamin were introduced to reduce inter-method variability, considerable analytical bias remains [87], highlighting ongoing challenges in assay comparability. This is particularly evident for the many total vitamin B12 assays currently available, which frequently use internal reference materials that are not directly traceable to the WHO standard. Furthermore, although many manufacturers have adapted their calibration strategies to improve agreement between assays, the use of the WHO IS in routine practice can be limited by cost and restricted availability.

Achieving both standardization and harmonization would make results obtained with different methods comparable, ensuring consistency across laboratories, even when it is difficult to use the same reference standard. However, incomplete harmonization across different immunoassays persists and heterogeneous expected values are still observed [64,99,100]. Furthermore, it should always be noted that variations between immunoassays also exist due to differences in antibody specificity and epitope recognition, as well as the type of assay (competitive or non-competitive). These variations are also clearly evident in external quality assessment (EQA) schemes, which have consistently demonstrated persistent inter-assay bias among manufacturer-specific kits, resulting in significantly different vitamin B12 values.

Cesana et al. [101] recently demonstrated a significant bias among the most used analytical platforms for total vitamin B12 measurement. Specifically, Abbott, Siemens, and Beckman assays

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showed mean differences of 2.5%, 8.4%, and up to 24.4%, respectively, when compared with Roche. These findings highlight substantial inter-method variability. Consequently, applying a universal diagnostic cut-off across different analytical platforms may increase diagnostic uncertainty in clinical practice [68,101].

Conversely, holoTC represents the biologically active fraction of vitamin B12 available for receptor-mediated cellular uptake [38]. For instance, holoTC is recommended by current guidelines because it provides a more reliable measure of vitamin B12 status than total B12, particularly in specific clinical contexts such as pregnancy, and it is less influenced by biological variability or supplementation. Moreover, in patients with megaloblastic anemia, holoTC has demonstrated significantly higher diagnostic sensitivity compared to total B12 [102]. Similarly, in populations at risk of nutritional deficiency, such as vegans or vegetarians, holoTC has been shown to more accurately reflect B12 status and its associated metabolite alterations [103]. Finally, holoTC may be particularly useful for assessing the fraction of biologically active B12 in patients with paraneoplastic syndromes, solid tumors, or myeloproliferative hematologic disorders, conditions often associated with falsely elevated total B12 levels.

For holoTC, the analytical methods currently available and applicable in routine clinical practice are fewer than those used for total vitamin B12 measurement. Most assays are standardized against the WHO reference material, allowing improved harmonization of results obtained with different assay kits. Importantly, cut-off values are similar across the available methods and are consistent with the 2024 guideline recommendations, except for Roche assay (Table 1).

A relevant issue when comparing total vitamin B12 and holoTC concerns the different units in which these analytes are commonly reported (respectively mass and molar units), both across available assays (Table 2) and in literature data [104]. This discrepancy mainly affects total vitamin B12, for which most commercial kits report pg/mL (Table 1) as the primary unit of measurement, whereas clinical guidelines indicate both ng/L and pmol/L. In contrast, greater uniformity is observed for holoTC, which is always expressed in pmol/L across different assays and current guidelines. Such heterogeneity may complicate results interpretation, as unit conversion may be challenging and potentially imprecise, particularly when assessing the expected ratio between total and biologically active vitamin B12. In fact, it is important to consider that holoTC assays quantify also the protein fraction bound to transcobalamin, unlike total vitamin B12.

Although the holoTC assay is more expensive, its measurement could help prevent misdiagnosis and enable the timely beginning of appropriate therapy. Late recognition and treatment of B12 deficiency can negatively affect quality of life and increase the risk of neurological damage [17], highlighting the clinical value of using holoTC as a first-choice marker despite its higher cost, particularly in specific settings.

5. Conclusions

Overall, harmonization of total vitamin B12 assays remains incomplete, and the different methods are not interchangeable. Consequently, diagnostic cut-offs, including those recommended by NICE, cannot be universally applied. In the absence of full standardization, method-specific decision thresholds should be adopted, and results interpreted with caution, particularly near clinical decision limits.

Therefore, the clinicians should request holo-transcobalamin together with total B12 to provide a more reliable and accurate assessment of B12 status, potentially reducing diagnostic errors and inappropriate supplementation. This would prevent higher long-term healthcare costs and suboptimal patient management.

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Abbreviations

The following abbreviations are used in this manuscript:

AB1	Antibody 1
AB2	Antibody 2
Ado-Cbl	Adenosyl Cobalamin
B6	Pyridoxine vitamine
B12	Riboflavin vitamine
Cbl	Cobalamin
CBS	Cystathionine β-synthase
CGL	Cystathionine γ-lyase
CH3	Methyl group
CH3-Cbl	Methylcobalamin
CLIA	Chemiluminescence immunoassay
CMIA	Chemiluminescent microparticle immunoassay
CoA	Coenzyme A
DNA	Deoxyribonucleic Acid
ECLIA	Electrochemiluminescence immunoassay
EFLM	European Federation of Clinical Chemistry and Laboratory Medicine
EIA	Enzyme Immunoassay
ELFA	Enzyme-linked Fluorescent Assay
ELISA	Enzyme-linked immunosorbent assays
EN ISO	European Norm International Organization for Standardization.
EQA	external quality assessment
f	females
HC	Haptocorrin
Hcy	Homocysteine
holoTC	holotranscobalamin
IF	Intrinsic Factor
IS	International Standard
kDa	kiloDalton
LC-MS/MS	liquid chromatography–tandem mass spectrometry
m	males
MAT	Methionine Adenosyle Transferase
MMA	Methylmalonic acid
MRP1	Multidrug Resistance Protein 1
MTHFR	Methylenetetrahydrofolate reductase
NG239	NICE Guideline 239
ng/L	nanograms/liter
NH2	Amino group
NIBSC	National Institute for Biological Standards and Control
NICE	National Institute for Health and Care Excellence
OH	Hydroxyl group
PCC	Propionyl-CoA Carboxylase
pg/mL	picograms/milliliter
POCT	Point-Of-Care Testing
pmol/L	picomoles/liter
RI	Reference Intervals
RIA	radioimmunoassays
RLU	Relative Lights Units
SAH	S-adenosyl-homocysteine

 doi:10.20944/preprints202603.0826.v1

SAHH	S-adenosyl-homocysteine hydrolase
SAM	S-adenosyl-methionine
TC	Transcobalamin
TCN2	Transcobalamin 2
THF	Tetrahydrofolate
USP	Unites States Pharmacopeia
WHO	World Health Organization
y.o	years old
ug	micrograms

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