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CAT
Critically Appraised Topic

Methylmalonic acid as indicator of cobalamin deficiency

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CLINICAL BOTTOM LINE

The timely diagnosis and treatment of vitamin B12 deficiency is paramount to prevent neurological or haematological deterioration and irreversible damage. However, the reliable use of stand-alone biochemical markers for vitamin B12 deficiency (total cobalamin, holotranscobalamin, methylmalonic acid (MMA) and homocysteine) is hampered by their inadequate sensitivity and/or specificity. Currently, only two biochemical markers (total cobalamin and homocysteine) are available in the clinical laboratory of AZ Groeninge. Based on a literature review, MMA seems to be the most sensitive and specific marker for diagnosing B12 deficiency, provided that non-B12-related modifiers (e.g., renal function and age) of MMA are taken into account. Therefore, an LC-MS/MS method to measure MMA in serum was implemented in the clinical lab of AZ Groeninge. MMA reference values reported in literature (60-360 nmol/L) were verified and a grey zone for MMA (360 – 800 nmol/L) was applied to consider mildly increased MMA levels caused by renal impairment, age or other unknown factors. Next, MMA was measured in a delineated hospital-specific patient population with low cobalamin levels (< 400 ng/L) (*n*=80). Our data show that only 20% (if eGFR > 90 mL/min/1.73m²) to 27% (if eGFR < 90 mL/min/1.73m²) of the patients with a B12 concentration lower than the presumed cut-off level of 200 ng/L have a MMA concentration indicative of B12 deficiency. Moreover, no significant correlation was observed between MMA and total cobalamin in patients without renal impairment. Therefore, MMA may elucidate the actual B12 status of patients with low B12 concentrations without a functional B12 deficiency. Furthermore, 5 to 17% of the patients with normal cobalamin concentrations (200-400 ng/L) have an MMA level indicative of B12 deficiency. Therefore, the measurement of MMA in patients with clinical symptoms and cobalamin levels above the presumed cut-off of 200 ng/L is useful to reliably confirm the diagnosis of B12 deficiency. In conclusion, the implementation of MMA analysis in the clinical laboratory of AZ Groeninge will considerably improve the diagnostics of B12 deficiency.

CLINICAL/DIAGNOSTIC SCENARIO

Vitamin B12 (B12) or cobalamin is an essential water-soluble micronutrient [1]. Since human cells are unable to synthesize B12, a sufficient dietary intake containing B12 such as dairy products, eggs, fish, and meat is required [1, 2]. The daily B12 requirement is 2-3 µg, which is often not reached in persons consuming low amounts of animal-derived products and not taking any supplements. Nevertheless, a large body storage of B12 (~ 2-5 mg), of which approximately 1 mg is stored in the liver (i.e. a quantity equivalent to the daily metabolic requirement for 2000 days), prevents a rapid progression towards B12 deficiency during short periods of insufficient intake [3, 4]. If there is, however, a persistent long-term (i.e. several years) underlying cause, B12 body storage might get depleted and the body will turn into a state of cobalamin deficiency [3]. The underlying cause of cobalamin deficiency is usually multifactorial comprising insufficient cobalamin intake (e.g., malnutrition) (= nutritional deficiency) in combination with an acquired or inherited disruption of B12 absorption, transport or intracellular processing pathways (= functional deficiency) [1]. The most frequent causes and risk factors are enlisted in Table 1.

B12 deficiency can present as a clinical or subclinical deficiency (SCCD) [3]. A clinical deficiency is typically marked by macrocytosis and hypersegmentation of the neutrophils caused by defective DNA synthesis, and/or neurological symptoms (including sensory and motor disturbances, spasticity and paralysis, and cognitive decline) caused by demyelination of peripheral and central neurons (the so-called *subacute combined degeneration of the spinal cord*) [2–4]. Intriguingly, an inverse relationship between the haematological and neurological symptoms has been observed [4]. However, these clinical signs are shared with other diseases (e.g. myelodysplastic syndrome, dementia, …) and the haematological symptoms are indistinguishable from folate deficiency, hampering a swift diagnosis of B12 deficiency, especially in elderly individuals [4]. Nonetheless, a timely diagnosis and treatment is paramount, as neurological damage may not entirely reverse following treatment [2]. In addition to the clinical picture, biochemical serum biomarkers (total cobalamin, holotranscobalamin (holoTC), methylmalonic acid (MMA) and homocysteine) can be useful to elucidate a patient's B12 status. The prevalence of clinical deficiency is estimated to be 2-3% in the older population (> 65 years) [5].

Patients with SCCD represent a larger subgroup in comparison to clinical deficiency (~3-26% in the general population depending on the B12 cut-off used) [6]. SCCD patients sometimes experience non-characteristic symptoms, but the actual clinical impact of SCCD and the progression rate towards a clinical deficiency remains to be fully elucidated [3]. In contrast to a clinical deficiency, the diagnosis of SCCD solely relies on biochemical testing, which might be challenging in some patients because of the low specificity and sensitivity of individual biochemical markers [3]. Therefore, stand-alone markers (e.g. cobalamin in serum) are considered insufficient for an adequate diagnosis of B12 deficiency. For example, cobalamin levels below the lower reference limit may not mirror a true B12 deficiency, whereas patients with true B12 deficiency may show normal cobalamin levels [2]. Therefore, additional testing for MMA and/or homocysteine levels is recommended as second-line tests, since these metabolites might better represent the functional activity of B12 [5, 7]. In an attempt to improve the diagnostics of B12 deficiency, numerous definitions, various cut-offs of the biochemical markers for B12 deficiency and several algorithms integrating at least two biomarkers at the time, have been published worldwide. Altogether, an optimized diagnostic process to identify true cobalamin deficiency in an early phase is paramount to ensure a timely and effective therapeutic intervention and prevent (further) neurological or haematological deterioration and irreversible damage. In addition, an accurate diagnosis of SCCD will identify the subpopulation at risk for the development of clinical symptoms, thereby preventing overtreatment of the overall population.

Currently, only two biochemical markers for B12 deficiency (total cobalamin and homocysteine) are available in the clinical laboratory of AZ Groeninge. In order to improve the diagnostic process of cobalamin deficiency in AZ Groeninge, we reviewed the literature to document the current knowledge on the different B12 biochemical markers (MMA, homocysteine, cobalamin and holoTC). Next, the measurement of MMA was implemented in the clinical laboratory of AZ Groeninge and its relationship with other B12 biochemical markers was evaluated in a hospital-specific patient population.

Table 1: An overview of the most frequent causes and risk factors of cobalamin deficiency. Adapted from Green et al. [2].

Inadequate intake	Chemical inactivation of B12	Inherited disorders
▪ Chronic alcohol consumption ▪ Vegetarian or vegan diet ▪ General malnutrition	▪ Recreational use of the gas nitrous oxide: irreversible oxidation of the active cofactor methylcobalamin	▪ Decreased expression, binding activity or affinity of receptors and proteins involved in B12 trafficking and metabolism

Malabsorption
▪ Pernicious anaemia: deficiency of intrinsic factor or auto-antibodies directed against gastric parietal cells and intrinsic factor causing chronic atrophic gastritis ▪ Chronic atrophic gastritis causing loss of the gastric-acid producing cells: B12 is not released from the food matrix ▪ Drugs affecting gastric acid secretion: proton pump inhibitors, histamine receptor 2 antagonists, antacids: B12 is not released from the food matrix ▪ Pancreatic disease or pancreatectomy: B12 is not released from haptocorrin ▪ Inflammatory bowel disease, coeliac disease, ileal resection, parasitic infestations and bacterial overgrowth, all affecting adequate absorption of the B12-IF complex ▪ Medications affecting B12 absorption or metabolism trough known or unknown mechanisms (e.g., colestyramine and metformin)

QUESTION(S)

1) *Question I: Which biomarkers can be used in the diagnosis of cobalamin deficiency?*
2) *Question II: Is it possible to improve the diagnostic process of cobalamin deficiency by implementing the analysis of methylmalonic acid in the clinical laboratory of AZ Groeninge?*

SEARCH TERMS

1) MeSH Database (PubMed): MeSH terms
2) PubMed Clinical Queries (from 1966; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi): Systematic Reviews;
Clinical Queries using Research Methodology Filters (diagnosis + specific, diagnosis + sensitive, prognosis + specific)
3) Pubmed (Medline; from 1966), SUMSearch (http://sumsearch.uthscsa.edu/), The National Institute for Clinical Excellence (http://www.nice.org.uk/), Cochrane (http://www.update-software.com/cochrane)
4) International organizations: e.g. National Committee for Clinical Laboratory Standards (NCCLS; http://www.nccls.org/), International Federation of Clinical Chemistry (IFCC; http://www.ifcc.org/ifcc.asp), American Diabetes Association (ADA; http://www.diabetes.org/home.jsp)
5) UpToDate Online

APPRAISAL

Question 1: Which biomarkers can be used in the diagnosis of cobalamin deficiency?

1. Total cobalamin (total vitamin B12)

- *Metabolic pathway*

In the acidic environment of the stomach and in the presence of pepsin, cobalamin is released from food proteins and binds to haptocorrin, which protects B12 against the acidic environment. In the duodenum, the cobalamin-haptocorrin complex is degraded by pancreatic enzymes and free cobalamin binds to intrinsic factor released from gastric parietal cells. The latter complex is taken up by epithelial cells, whereafter free cobalamin enters the circulation [4, 8]. The majority of cobalamin (~80-94%) binds to haptocorrin (previously known as transcobalamin I), whereas the remainder fraction binds to transcobalamin (TC) (previously known as transcobalamin II) [4, 9]. Despite the overbalance of cobalamin-haptocorrin, this complex may only attach to cell surface receptors on the liver and seems not to be involved in the cellular cobalamin uptake and supply [9]. The cobalamin-TC complex (= holotranscobalamin), on the other hand, is the bioactive fraction since it is transported from the liver to tissues and internalized by the cells after binding to the TC receptor. In the cells, cobalamin is converted into its active cofactors (methylcobalamin and adenosylcobalamin) through a series of biochemical modifications classified as cobalamin complementation groups A-J [4, 8].

Methylcobalamin is an essential cofactor in the reaction that converts homocysteine to methionine catalyzed by the enzyme methionine synthase. During this reaction, tetrahydrofolate is regenerated from N^5-methyl-tetrahydrofolate, which is on its turn an essential cofactor for the *de novo* biosynthesis of nucleic acids. Adenosylcobalamin plays a role in the biosynthesis of succinyl-CoA from methylmalonyl-CoA (a product of amino acid and fatty acid catabolism) catalysed by the enzyme methylmalonyl-CoA synthase. Considering this, concentrations of MMA and/or homocysteine will increase upon insufficient cobalamin supply or genetic and inherited defects in the absorption, transport or intracellular processing of cobalamin [1]. The function of B12 and folate is schematically represented in Figure 1 and the chemical formula of cobalamin, MMA and homocysteine in Figure 2.

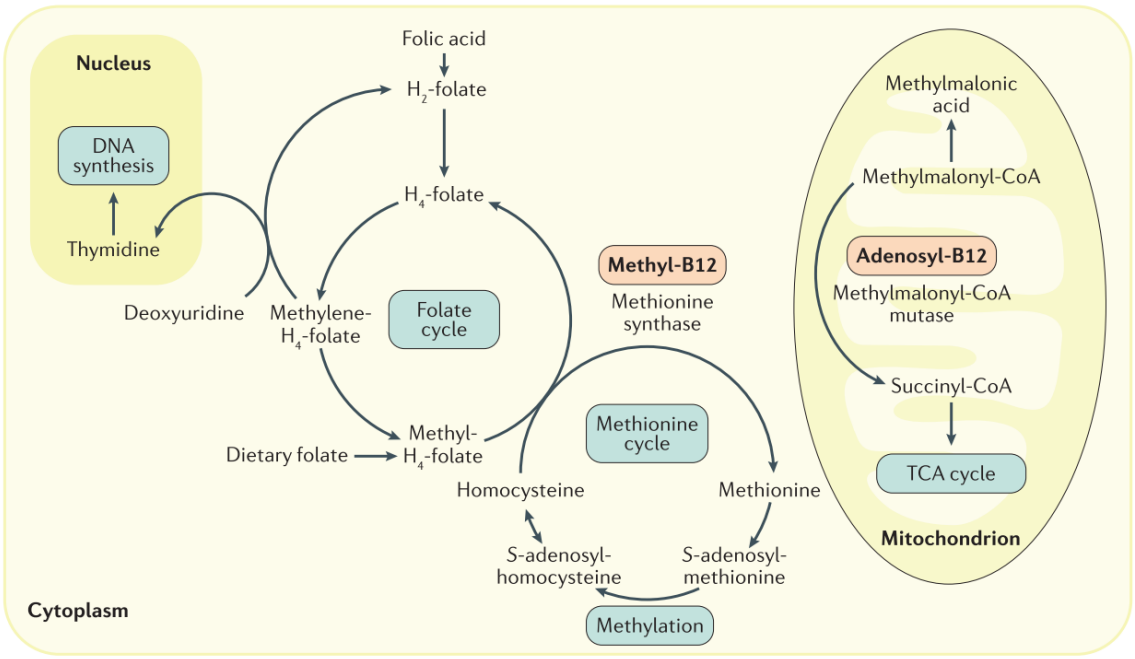

Figure 1: Vitamin B12 and folate metabolism and function [2]**.**

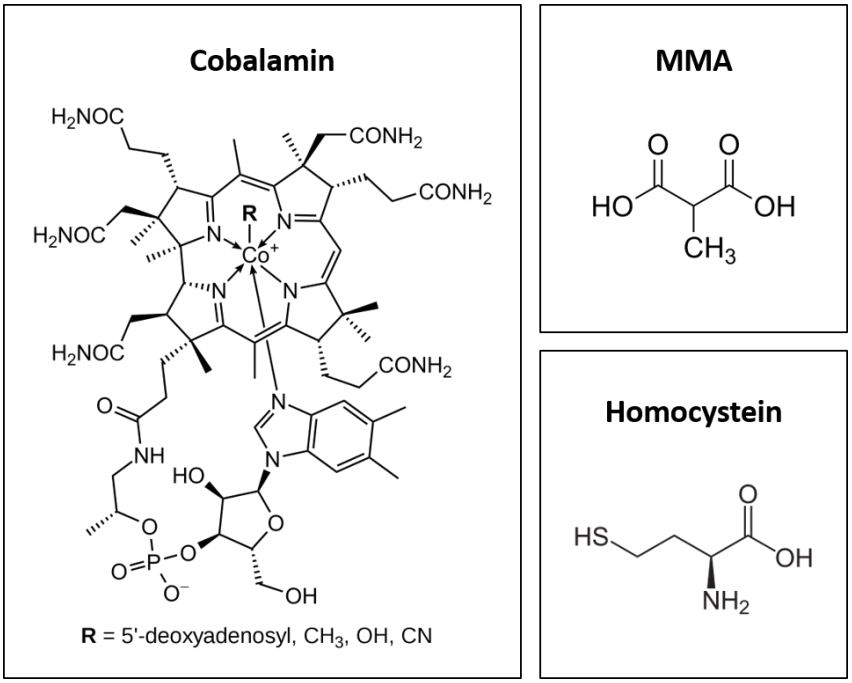

Figure 2: The chemical formula of cobalamin, methylmalonic acid (MMA) and homocysteine.

- *Measurement of total cobalamin*

A number of methods have been introduced throughout the years to quantify total cobalamin in serum [10]. Nowadays, immunoassays using solid-phase separation based on immobilized nonhuman intrinsic factor on beads or magnetic particles are highly accessible and most commonly used [4]. In AZ Groeninge, the Elecsys Vitamin B12 II assay (Roche®) is used, which is based on the competition principle. In a first step, bound B12 is released from the transporter proteins haptocorrin and transcobalamin. As such, total circulating B12 is quantified, including the inactive fraction of vitamin B12 bound to haptocorrin which is not available for cellular uptake [1]. Afterwards, ruthenium labelled intrinsic factor is added to the sample containing released B12. Next,

streptavidin-coated microparticles and vitamin B12 labelled with biotin are added to occupy the remaining vacant sites of the ruthenium labelled intrinsic factor. The latter complex will bind to the solid phase via interaction of biotin and streptavidin. Eventually, the reaction is monitored by measuring chemiluminescent emission. Of note, the assay is designed to avoid interference of patient-derived anti-intrinsic factor antibodies. Moreover, in an attempt to harmonize the interpretation of B12 results between laboratories, the World Health Organization (WHO) International Standard 03/178 was established in 2005 with an assigned value of 480 pg/mL [11]. Using the Roche Vitamin B12 II assay, the mean recovery of the target value of this standard was reported to be 102%, as described in the kit insert.

- *(Dis)advantages of total cobalamin*

Cobalamin measurement in serum is considered the first-line assay to determine cobalamin status, although the reference intervals are still a matter of debate. As such, total cobalamin does not consistently reflect the cellular B12 status, since the inactive fraction of B12 bound to haptocorrin is measured as well [1, 2]. Moreover, concentrations of the transporter proteins haptocorrin and transcobolamin vary between individuals [1, 2]. Regarding the latter, low haptocorrin concentrations can be found in approximately 15% of the persons with low serum cobalamin and could therefore be one of the most common causes of low total cobalamin concentrations without a functional deficiency [4]. Nevertheless, because of the accessible and easy-to-use B12 assays, total B12 is often used as a screening tool, followed by a second line test (e.g., MMA analysis) if B12 is below a certain threshold in patients suspected for clinical B12 deficiency (cf. infra). A recent WHO consultation defined a serum B12 concentration less than 203 ng/L as deficient, thereby keeping in mind that B12 concentrations within the reference range may not necessarily reflect adequate B12 status and low serum B12 concentrations may not consistently be indicative of B12 deficiency [1, 4, 12, 13].

Interestingly, B12 status has been studied in the National Health and Nutrition Examination Survey (NHANES) in the United States (1999-2004) in which 12612 adult participants without serious renal impairment were included [6]. In this study, when using cut-off values of <200 ng/L, <271 ng/L and <347 ng/L, the prevalence of B12 deficiency was estimated to be 2.9%, 10.6% or 25.7%, respectively. In contrast, when MMA was used as a functional indicator of B12 status, the prevalence of B12 deficiency was only 2.3% using a cut-off value for MMA of > 0.376 µmol/L. Remarkably, only 22-44% of the participants with total B12 levels < 200 ng/L showed increased levels of MMA and only 32% had increased homocysteine concentrations [2, 6]. Furthermore, 5% of the population showed normal B12 levels (> 200 ng/L) with elevated MMA concentrations (> 271 nmol/L), probably reflecting the superior sensitivity of MMA to B12 in SCCD [6]. Considering this, patients with low total B12 concentrations might have a normal B12 activity status (indicated by a normal MMA level), whereas normal total B12 levels might mask a true cobalamin deficiency. Moreover, this study demonstrates that increasing the cut-off for B12 deficiency is not recommended as only 6–26% (depending on demographic subset) of the cohort with total B12 concentrations of 200 to 300 ng/L had abnormal MMA concentrations and only 14% had elevated homocysteine concentrations [6]. Only if one considers that all patients with SCCD require treatment and that early detection allows mislabelling many nondeficient persons, increasing the threshold may be justified [14]. Multiple publications show the difference in prevalence of B12 deficiency when a higher threshold is used in comparison with the presumed threshold of 200 ng/L (Table 2). Alternative to changing the B12 cut-off level, other studies and guidelines suggest a grey zone for total cobalamin (e.g. 122-407 ng/L [15]) in which a second marker of B12 metabolism is required to elucidate the actual B12 status [7, 15–17]. In conclusion, the low sensitivity and specificity of serum total cobalamin levels around the presumed cut-off of 200 ng/L requires a cautious interpretation of these levels.

Table 2: Comparison of the prevalence of abnormal cobalamin using different cobalamin cut-offs. Adapted from Carmel et al. [14]. For references, see Carmel et al. [14].

Source location, year	Study population	n	Compared cobalamin cut-offs (ng/L)	Frequencies of "low" cobalamin (%)
Netherlands, 1998	Elderly town dwellers	105	<203 vs <352	24.8 vs 60.1
Oklahoma City, 1998	Elderly outpatients	303	<201 vs <300	6.3 vs 16.2
United States, 1999	Disabled elderly town-dwelling women	762	<201 vs <350	6.2 vs 33.5
Los Angeles, 1999	Elderly town dwellers and outpatients	591	<190 vs <350	11.8 vs 50.4
United Kingdom, 2007	Elderly town dwellers in medical registries	2403	<203 vs <271 vs <407	8.6 vs 29.3 vs 71.7
Norway, 2009	Adults aged 47–49 y	3684	<203 vs <271 vs <542	0.4 vs 3.1 vs 64.5
Norway, 2009	Adults aged 71–74 y	3262	<203 vs <271 vs <542	3.1 vs 6.7 vs 67.6

2. Holotranscobalamin (active B12)

- *Measurement of holotranscobalamin*

Holotranscobalamin refers to the cobalamin-TC complex and represents the active cellular fraction of cobalamin (cf. supra). This fraction comprises approximately 20% of the total serum cobalamin concentration. Starting from the early 2000s, several immunoassays measuring active B12 (i.e. holotranscobalamin) were commercialized with the aim to develop a reliable test for the diagnosis of B12 deficiency [18]. In AZ Groeninge, active B12 measurement is not routinely available, but as part of this study the Elecsys Active B12 (Roche®) assay was used, which is based on the sandwich principle. In short, a biotinylated monoclonal antibody directed against holotranscobalamin and a ruthenium-labelled monoclonal antibody directed against transcobalamin are used to form a sandwich complex with holotranscobalamin. Afterwards, streptavidin-coated magnetic microparticles are added to the mixture. After applying a voltage to the electrode in the measuring cell, chemiluminescent emission is measured by a photomultiplier. Similar to cobalamin, a holoTC value was assigned to the WHO International Standard 03/178 in 2016 [19]. The Elecsys Active B12 assay has been standardized against this WHO International Standard, as described in the kit insert.

- *(Dis)advantages of holotranscobalamin*

The usefulness of holotransobalamin is highly debated within the field. Already in 1994, Herbert et al. suggested that "low concentrations of holoTC occur before low concentrations of total serum B12 or before deficiency", a statement stooled on the small percentage of total B12 consisting of holoTC, and the short half-life of holoTC (ranging from 6 minutes to 1-2h for holoTC vs. 240h for cobalamin-haptocorrin) [18]. However, holoTC is claimed to reflect (i) the daily influx from the gut (cf. malabsorption of B12) and/or (ii) inadequate availability to tissues [14]. These two diagnostic claims are rather contradicting, since transient malabsorption for only a few weeks or months might be reflected by low holoTC levels but does not necessarily result in a B12 deficient metabolic state. Considering this, holoTC might not adequately reflect B12 function in tissues [1, 14, 15, 20–22]. After a few decades of research, Golding et al. [18] performed an extensive literature review in 2016 and concluded that the holoTC immunoassay is not more reliable than total B12 to objectify the B12 status or to predict the onset of a metabolic deficiency. Indeed, the literature on holotranscobalamin is rather contradicting, with authors praising holoTC as an early and more sensitive indicator than total serum cobalamin on the one hand, and original studies showing no significant increase in sensitivity of holoTC in comparison to total B12 on the other hand [18]. In multiple studies reviewed by Golding et al., the sensitivity of holoTC ranged from 0.55–0.87 vs 0.33–0.76 for total B12, whereas the specificity ranged from 0.50–0.96 for holoTC and from 0.41–0.98 for B12 [9, 18] (Table 3). One possible explanation for the diverse results for sensitivity and specificity between studies might be the varying MMA cut-offs used to define a B12 deficient metabolic state.

Table 3: The sensitivity and specificity of holoTC versus B12 at the specified cut-offs for B12 deficiency. Adapted from Golding et al. [18]. For references, see Golding et al. [18].

Reference	holoTC vs. B12		Cut-off	AUC
	Sensitivity	Specificity		
Clarke et al. (2007) (definite deficiency)	0.771 versus 0.757	0.761 versus 0.724	MMA > 0.75 µmol/L	0.85 versus 0.76
Clarke et al. (2007) (probable deficiency)	0.647 versus 0.626	0.792 versus 0.748	MMA > 0.45 µmol/L	0.79 versus 0.87
Goringe et al. (2006)	/	/	HoloTC < 38 pmol/L	0.75 versus 0.72
Heil et al. (2012)	0.83 versus 0.64	0.60 versus 0.64	MMA > 0.45 µmol/L	0.78 versus 0.70
Herrmann et al. (2003a)	0.87 versus 0.45	0.75 versus 0.98	MMA > 0.271 µmol/L	0.879 versus 0.836
Herrmann and Obeid (2013)	0.72 versus 0.72	0.54 versus 0.41	MMA > 0.300 µmol/L	0.714 versus 0.632
Hvas and Nexo (2003)	1.0	0.89		
Hvas and Nexo (2005)	/	/	MMA > 0.75 µmol/L	0.90 versus 0.85
Lindemans et al. (2007)	/	/	MMA > 0.26 µmol/L	0.80 versus 0.68
Lloyd-Wright et al. (2003)	/	/	MMA > 0.75 µmol/L	0.87 versus 0.86
Miller et al. (2006)	/	/	holoTC < 35 pmol/L	0.828 versus 0.816
Obeid and Herrmann (2007a)	0.72	/	MMA > 0.300 µmol/L	0.71 versus 0.60
Palacios et al. (2013)	0.44 versus 0.20	0.94 versus 0.94	HoloTC < 35 pmol/L	0.75 versus 0.69
Schrempf et al. (2011)	0.563 versus 0.662	0.505 versus 0.621	MMA > 0.40 µmol/L	0.66 versus 0.72
Scott et al. (2007)	/	/	MMA > 0.75 µmol/L	0.85 versus 0.75, 0.74, 0.72
Valente et al. (2011)	0.55 versus 0.33	0.96 versus 0.95	Red cell cobalamin < 33 pmol/L	0.90 versus 0.80

Furthermore, the correlation of holoTC and total B12 is not completely understood and varies widely between studies (ranging from weak correlations to very strong ones) [18]. However, strong correlations imply a similar sensitivity in detecting B12 deficiency for holoTC and total B12, arguing against the added value of holoTC in the diagnosis of B12 deficiency. Furthermore, whether holoTC concentrations represent a long-term status or recent absorption of B12 remains an open question, with studies reporting the dependency of holoTC on recent B12 absorption as well as studies not observing this dependency [18]. Moreover, there is no consensus on the cut-off level of holoTC to define B12 deficiency, not to mention the potential grey zone quoted by several researchers [18]. Cut-offs reported in literature range from 20 to 50 pmol/L and grey zones vary between 20 and 76 pmol/L (reviewed by Golding et al. [18]) [23]. Considering this, holoTC seems to suffer from the same limitation as total cobalamin - the absence of a single cut-off value reflected by a wide indeterminate range - which diminishes the potential added value of holotranscobalamin. Of note, the holoTC assay is suggested to be useful when transcobalamin and haptocorrin deficiencies are suspected [9]. In haptocorrin deficiency considered as a benign condition, total B12 might be suspiciously low, whereas holoTC concentrations are normal to high. In transcobalamin deficiency, holoTC is unmeasurable and an early treatment is paramount. Furthermore, in contrast to the functional markers MMA and homocysteine, holotranscobalamin does not correlate with renal function as indicated by serum cystatin C [24]. In conclusion, considering the discordant literature, holotranscobalamin may complement the repertoire of B12 biomarkers, but should not be used as a stand-alone marker. Further investigation is required to reliably use active B12 as a potential first-line test.

3. Methylmalonic acid

- *Measurement of MMA*

MMA is definitely not the new kid on the block, since gas chromatographic assays for MMA in urine have been available since the late 1950s [14]. The measurement of MMA is complicated by its small molecular weight, its hydrophilic, nonvolatile nature and its low concentration in serum. Therefore, different sample extraction methods, with or without sample derivatization, have been explored to improve the detection (e.g.,

ultrafiltration, solid-phase or liquid-liquid extraction) [25]. More recently, LC-MS/MS methods allow the accurate and precise measurement of small concentrations in serum, without the need for sample extraction and derivatization. Other advantages of LC-MS/MS methods are the shorter run time and better sensitivity compared with GC-MS procedures [25]. Another hurdle to take is the potential interference from other low-mass carboxylic acids, especially the structural isomer succinic acid, which is abundantly present in serum [25]. The nearly identical mass spectra of MMA and succinic acid require a chromatographical separation, with a C18 UHPLC column showing the best resolution [25] (Fig. 3).

In the clinical laboratory of AZ Groeninge, the measurement of MMA in urine or serum is currently not implemented. In the context of this study, the commercially available MassChrom reagent kit from Chromsystems was explored using LC-MS/MS (Acquity H Class plus – Xevo TQ-XS (Waters)) to quantify MMA in serum. Sample preparation comprises addition of the internal standard and a simple filtration step using the so-called Clean-Up Tubes. The sample volume required is only 50-100 µL. A method verification was performed and MMA was measured in a selection of reference persons and patients (cf. infra).

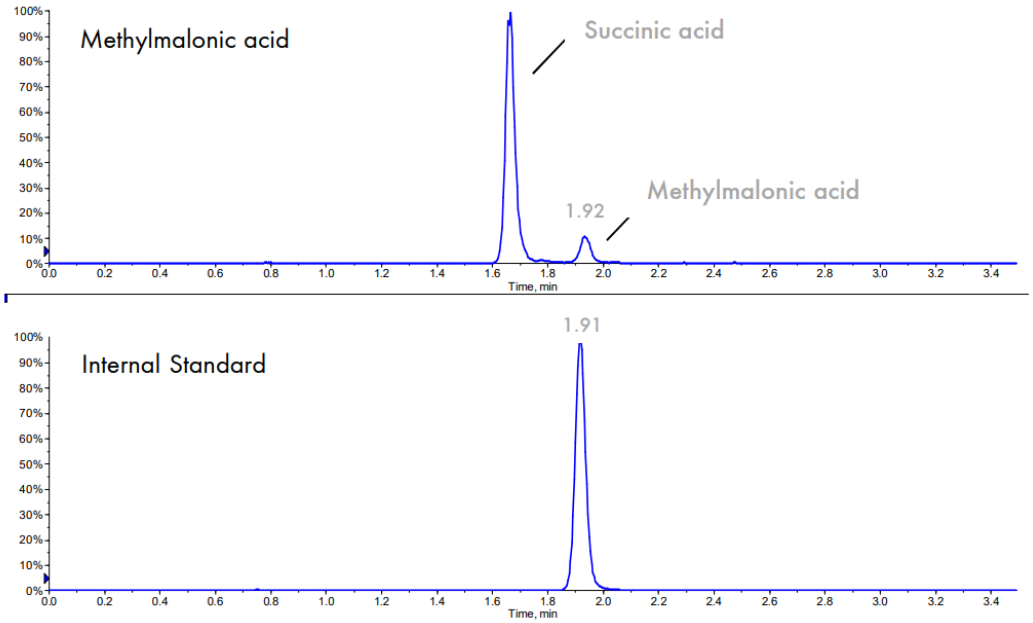

Figure 3: The chromatographical separation of MMA and succinic acid. The figure shows a representative chromatogram of a plasma calibrator with a MMA concentration of 254 nmol/L (kit insert MMA MassChrom reagent kit from Chromsystems).

- *(Dis)advantages of MMA*

Adenosylcobalamin plays a role in the biosynthesis of succinyl-CoA from methylmalonyl-CoA catalysed by the enzyme methylmalonyl-CoA synthase (Fig. 1). In the absence of sufficient cobalamin, methylmalonyl-CoA will accumulate and enter the circulation as free MMA. Since no other vitamins (e.g. folate) are involved in the reaction catalysed by methylmalonyl-CoA synthase, MMA is, in contrast to homocysteine, considered a specific marker of B12 deficiency [1]. Moreover, the significant reduction of high MMA upon B12 supplementation points unequivocally to cellular B12 deficiency in patients with high MMA levels [16, 17]. Furthermore, the prevalence of increased concentrations of MMA is higher than decreased concentrations of B12, and MMA started to increase when serum B12 concentration are lower than 542 ng/L in two studies with 6946 and 2180 participants, respectively [15, 16]. Therefore, MMA is considered the earliest marker of functional B12 deficiency and a more sensitive indicator of B12 deficiency than the serum total B12 or holoTC levels [4, 16, 17, 26–28].

On the downside, vitamin B12-unrelated factors like impaired renal function [29–33] and increasing age [29, 34–39] are associated with higher MMA levels. MMA levels increased with age, even in a population with normal

renal function [15]. Furthermore, other factors, such as intravascular volume depletion, and increased production of propionic acid from bacterial overgrowth in the intestine or increased catabolism of MMA precursors like cholesterol, branched-chain amino acids, and odd-chain fatty acids, are supposed to influence MMA concentrations as well [16]. Antibiotic treatment suppresses anaerobic gut flora and has been shown to lower MMA [16]. All this minorly declines the value of MMA as gold standard for metabolic B12 deficiency and contributes to an overestimation of B12 deficiency in the general population not selected for e.g. age or renal function. In an attempt to tackle these imperfections, several researchers have tried to establish age-specific reference ranges [29, 34–39] and, more recently, investigated the possibility of mathematically correcting MMA levels for the individual estimated glomerular filtration rate (eGFR) [15, 26].

Most published MMA reference intervals (including age-specific reference intervals) are situated between 50 and 400 nmol/L, with outliers till 510 nmol/L dependent on the demographical characteristics of the study population, such as age and renal function, and the analytical method used (Table 4). Several studies reported the (moderate) increase of MMA upon impaired renal function due to reduced clearance [24, 26, 39–43]. Van Loon et al. was able to build a three-dimensional model based on 2906 records of combined B12, MMA and eGFR measurements [15] (Fig. 4). The renal function was implemented as the eGFR according to the CKD-EPI formula (using creatinine serum concentration, gender and age as input variables). The model allowed the adjustment of MMA independent of the observed MMA concentration using the following formula: "*adjusted MMA = observed MMA – (predicted MMA for a given B12 concentration and eGFR – predicted MMA for a given B12 concentration and a GFR of 121 mL/min/1.73m² (= the threshold eGFR at which the renal function started to affect MMA according to the model)"*. The formula for the predicted MMA is $\exp(\beta_0 + \beta_1*B12 + \beta_2*eGFR + \beta_3*B12^2 + \beta_4*B12*eGFR + \beta_5*eGFR^2)$, with β_0=7.06, β_1=-2.81*10^-3, β_2=-1.96*10^-2, β_3=3.30*10^-6, β_4=-8.07*10^-6 and β_5=8.14*10^-5. The model thus calculates the difference in MMA due to decreased eGFR by subtracting the predicted MMA concentration as if renal function was normal from the predicted MMA concentration using the patient's actual eGFR [15]. Then, this difference is subtracted from the observed MMA concentration to obtain the adjusted MMA concentration. Application of this model in a population of 989 patients with a B12 concentration between 122 en 407 ng/L and a eGFR < 90 mL/min/1.73m² demonstrated that the number of B12 deficiencies defined as MMA > 430 nmol/L could be reduced by 40% (58/144) [15]. In a recent confirmatory study at another hospital (n=115245 patients), 19.7% of the patients with an eGFR < 90 mL/min/1.73m² were reclassified as B12 non-deficient (MMA < 0.43 µmol/L) [26]. The lower percentage of reclassification in the latter study might be caused amongst others by the inclusion of patients regardless of the B12 level, whereas van Loon et al. [15] only included patients with B12 concentrations within the grey zone (122-407 ng/L). Other disadvantages to bear in mind are the requirement of technical expertise to perform an LC-MS/MS method to measure MMA, making it only available in specialized, well-equipped labs. Moreover, this rather expensive analysis is entirely charged to the patient since it is currently not reimbursed in Belgium.

Altogether, it is warranted to use MMA as gold standard for B12 deficiency in a population with normal renal function. For clinical purpose, a grey zone (e.g., ranging from 400 to 800 nmol/L) may be used to indicate a "possible" B12 deficiency, thereby taking into account slight elevations in MMA commonly observed in elderly or patients with renal insufficiency [16, 17, 44, 45]. Alternatively, the recently developed mathematical model to adjust MMA concentrations for the patient's eGFR might be used, although the generalizability of this formula should still be investigated using distinct patient populations and lab equipment [15, 26].

Table 4: Selected published reference intervals for methylmalonic acid in serum or plasma. Adapted from Mineva et al. [29].

Authors (reference)	Population	Central 0.95 reference interval, nmol/L	Additional information
Allen et al., 1990 GC-MS [46]	Healthy US middle-aged adults	73–271	Men and women, n = 50; 18–65 y
Rasmussen et al., 1990 GC-MS	Healthy Danish middle-aged adults	50–370	Men and women, n = 58; 40–68 y (median: 53 y)
Rasmussen et al., 1996 GC-MS [34]	Healthy Danish middle-aged adults before and after vitamin B12 supplementation for 1 wk (in a few cases for 2 wk)	80–280	Men (n = 109) and women (n = 126); 20–84 y (men; median: 50 y) and 20–85 y (women; median: 49 y); all but 1 subject had plasma creatinine concentrations within the reference interval for healthy subjects
Joosten et al. , 1996 GC-MS [35]	Healthy Belgian, Dutch, and German middle-aged adults	62–247	Men and women, n = 99; 19–55 y (mean: 30 y)
	Healthy Dutch elderly living at home	72–476	Men and women, n = 64; 65–88 y (mean: 76 y); no participant had creatinine clearance <30 mL/min
	Healthy German elderly after B-vitamin supplementation for 3 wk	55–278	Men and women, n = 143; 65–96 y (mean: 75 y); no participant had creatinine clearance <30 mL/min
Lewerin et al., 2003 GC-MS [47]	Swedish elderly with and without B-vitamin supplementation		Men and women, n = 209; 70–88 y (women) and 70–93 y (men) (overall median: 76 y)
	Total study group at baseline	110–480	n = 208
	Healthy elderly at baseline	120–380	n = 123
	Healthy elderly after B-vitamin supplementation for 4 mo	20–340	n = 78 (vitamin B12 replete)
Milman et al., 2007	Healthy Danish pregnant women		Women (n = 434) with a normal pregnancy ≥37 wk
GC-MS [48]	18 weeks of gestation	40–290	n = 413
	32 weeks of gestation	50–340	n = 390
	39 weeks of gestation	60–360	n = 250
	8 wk postpartum	80–350	n = 160
Vogiatzoglou et al., 2009 [16]	Norwegian middle-aged adults		Men and women, n = 3684; 47–49 y
GC-MS	Unselected	100–320	n = 3684
	Vitamin B12 ≥200 pmol/L	100–300	n = 3568
	Vitamin B12 ≥400 pmol/L	100–280	n = 1306 (vitamin B12–replete)
	Norwegian elderly		Men and women, n = 3262; 71–74 y
	Unselected	110–490	n = 3262
	Vitamin B12 ≥200 pmol/L	110–410	n = 3043
	Vitamin B12 ≥400 pmol/L	100–360	n = 1058 (vitamin B12 replete)
Erdogan et al., 2010 [36]	Healthy US adults	60–360	Men (n = 16) and women (n = 24)
LC-MS/MS	US persons tested for MMA (unknown clinical history)		Males and females (n = 4944); highest 10% of results disregarded (potentially unhealthy persons)
	0–10 y	0–510	n = 28
	11–20 y	30–260	n = 39
	21–30 y	50–330	n = 165
	31–40 y	50–400	n = 287
	41–50 y	50–400	n = 545
	51–60 y	50–420	n = 813
	61–70 y	50–440	n = 918
	≥71 y	50–480	n = 2149

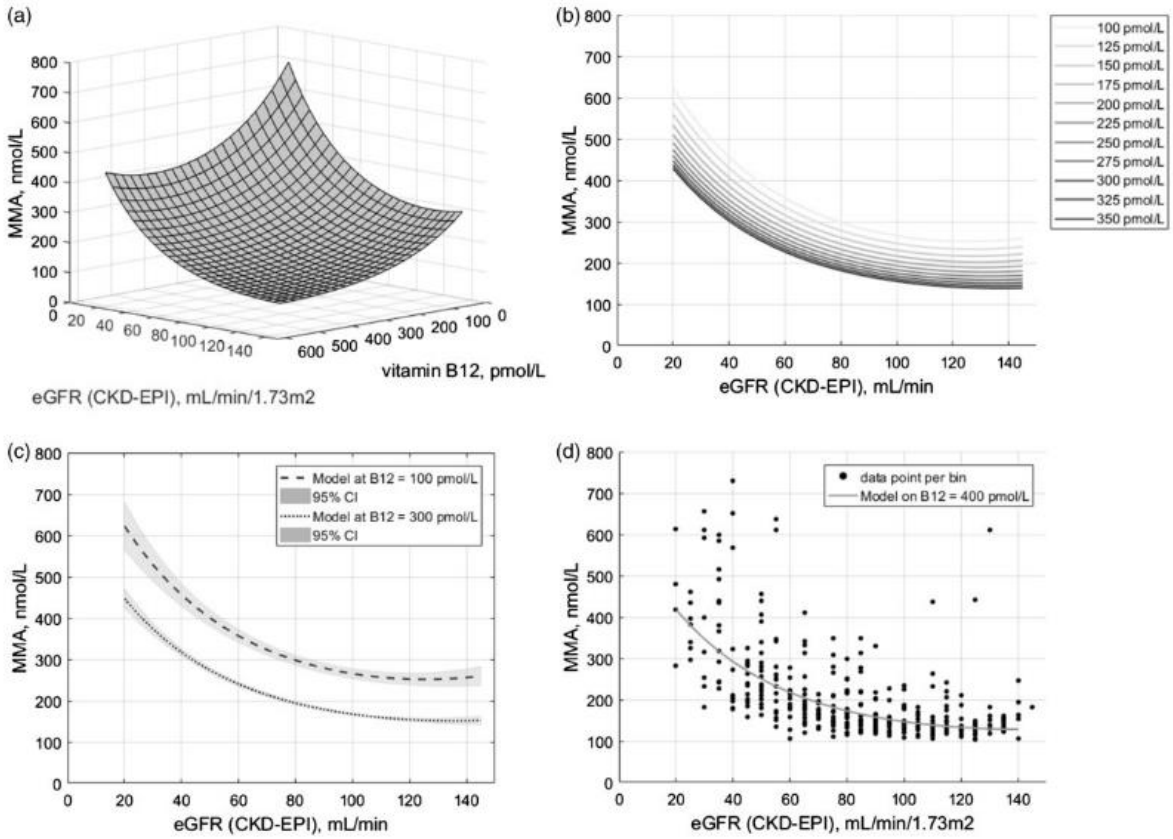

Figure 4 [15]**: Model developed by Van Loon et al.** (a) 3D representation of the model plotted against vitamin B12 and eGFR, showing a curved plane. (b) 2D representation of the model, plotted against eGFR at different concentrations of vitamin B12. (c) 95% confidence interval (CI) at different concentrations of vitamin B12 plotted against eGFR. (d) Isolated effect of renal function on MMA showed the model is in agreement with the data. MMA: methylmalonic acid; eGFR: glomerular filtration rate [15].

4. Homocysteine

- *Measurement of homocysteine*

Analytical methods to measure total homocysteine were introduced since the 1980s. To obtain reliable results, samples need to be treated with a reducing agent before analysis to convert the unstable oxidized homocysteine species (bound to plasma proteins or present as disulfide homocysteine) to the reduced free sulfhydryl form of homocysteine, which is then measured either directly or after derivatization [49]. Homocysteine can be measured by chromatographic methods or immunoassays [49]. In the clinical lab of AZ Groeninge, homocysteine is currently measured using LC-MS/MS. The sample preparation includes the addition of the internal standard, a reduction step with dithiothreitol (DTT), and a protein precipitation using acetonitrile.

- *(Dis)advantages of homocysteine*

As previously mentioned, methylcobalamin is an essential cofactor in the reaction that converts homocysteine to methionine catalyzed by the enzyme methionine synthase (Fig. 1). During this reaction, tetrahydrofolate is regenerated from N^5-methyl-tetrahydrofolate. Since both B12 and folate are required in the conversion of homocysteine to methionine, homocysteine will accumulate upon a deficiency in either one of these micronutrients [1]. Furthermore, deficiencies of vitamin B2 and B6 may contribute to increased homocysteine as well [4]. Despite the establishment of gender- and age-specific reference ranges in some studies, the normal range of total plasma homocysteine is generally considered 5 – 15 µmol/L [1, 23]. Of note, homocysteine concentrations are sensitive to preanalytical variables such as a high protein meal and venous stasis which will

increase the concentration [4]. EDTA plasma is the recommended sample type. The EDTA sample should be kept on ice until centrifugation and should preferably be centrifuged within one hour. The latter is important since homocysteine is continuously released from the red blood cells, resulting in an increase of approximately 1 $\mu mol*L^{-1}*h^{-1}$, independent of the initial concentration [49]. Therefore, levels are higher in serum compared to plasma as serum is usually left at room temperature for 30-60 minutes to allow coagulation. Last but not least, homocysteine levels increase, just like MMA, upon renal impairment and with age [24, 49]. Despite being a sensitive functional marker, the non-specific character of homocysteine, which is, on top, prone to pre-analytical errors, makes it less suitable as a good (stand-alone) biochemical marker for the diagnosis of B12 deficiency.

5. Algorithms for the diagnosis of B12 deficiency

As presented in the sections above, every biochemical marker evaluating the B12 status comes with pros and cons. Therefore, in an attempt to accurately interpret a patient's B12 status, researchers from over the world have developed algorithms that combine the use of two or more markers, thereby taking advantage of their individual strengths (reviewed by Hannibal et al. [1]). Of note, the clinician should always bear in mind that the lab reference values are a guidance rather than a firm indicator of the patients B12 status in order to prevent misdiagnosing patients [9]. Sobczyńska-Malefora et al. recently published a critical review on the diagnosis of B12 deficiency and insufficiency in which they provide a clear overview of the expected B12 biochemical marker patterns in distinct clinical scenario's (Table 5). In the following section, a selection of published algorithms are discussed.

Table 5: The commonly seen vitamin B12 biomarker patterns in selected clinical scenarios according to Sobczyńska-Malefora et al. [9]**.**

Serum holoTC	Serum total B12	Plasma MMA	Plasma tHcy	Possible diagnosis
N	N	↑	↑	Suboptimal B12 status
↓	↓	N or ↑	↑	Mild B12 deficiency, on antibiotics
N	N	↑	N	Bacterial overgrowth, B12 replete
↓↓↓	↓↓↓	↑↑	↑↑	Pernicious anemia
N or ↓	↓	N or ↑	N	Pregnancy, B12 replete
↓	↓ ↓	↑	N or ↑	Pregnancy, B12 deficiency
N	N	N	↑ - ↑↑↑	Mild to severe folate deficiency, B12 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 B12 injections
↑↑↑	↑↑↑	N	N	Chronic myeloid leukemia

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

- *British Society for Haematology*

In 2014, the British Society for Haematology guidelines on investigation and diagnosis of cobalamin and folate deficiencies were revised (Fig. 5) [7]. According to these guidelines, the clinical picture must definitely be considered when interpreting biomarker results, as no gold standard test is currently available. The first-line test remains serum cobalamin with MMA as additional second-line test. Individuals classified as subclinical deficiency according to the algorithm should receive empirical therapy and have their serum levels re-checked after three

months. Of note, treatment should promptly be started in the presence of strong clinical signs of deficiency, even if the biochemical markers do not point into the direction of a B12 deficiency. Moreover, they advise the establishment of local reference ranges given the wide range of reference values reported and methodologies used in literature.

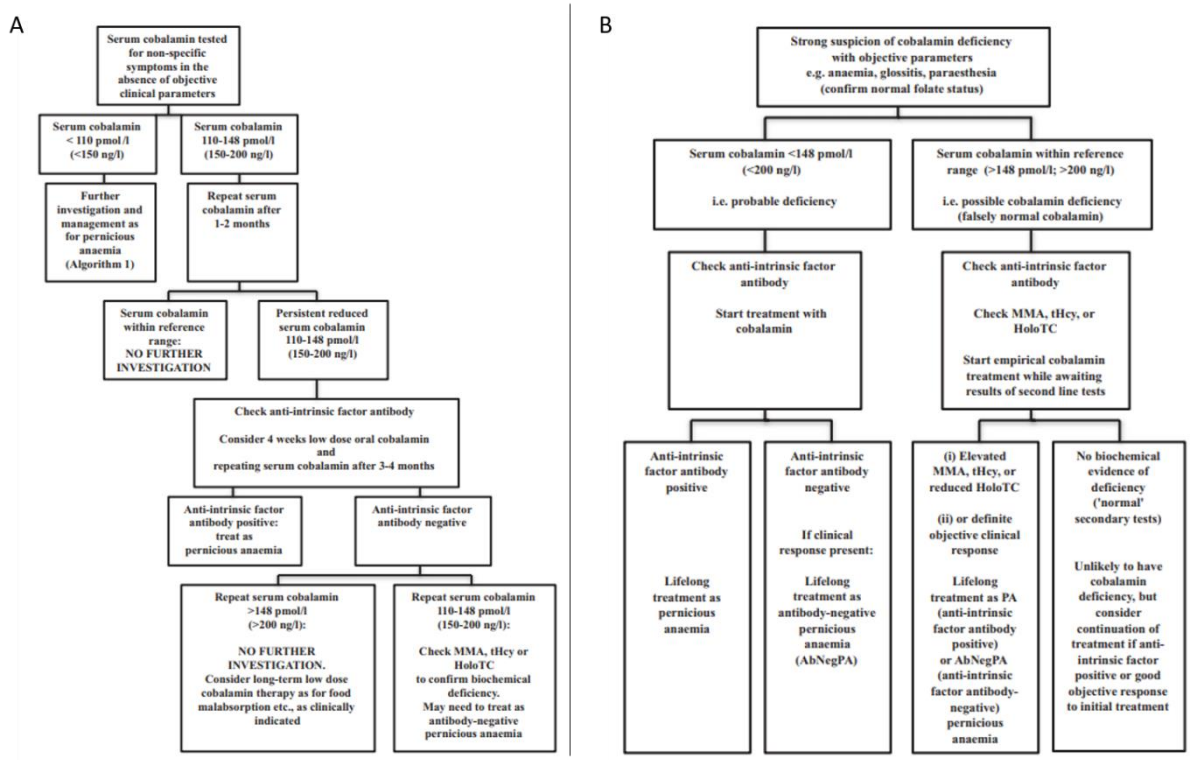

Figure 5: Algorithms suggested by Devalia et al. [7]**.** (A) An algorithm developed to investigate low serum cobalamin in patients without objective clinical parameters. (B) An algorithm developed to investigate patients presenting with a strong clinical suspicion of cobalamin deficiency and objective parameters to support this.

- *Herrmann and Obeid (Germany)*

In 2008, Herrmann and Obeid (review article) published a guideline which was widely recommended in Germany for the diagnosis of B12 deficiency in risk groups (Fig. 6) [50]. By combining holoTC as the earliest lab marker of B12 deficiency and MMA as the marker of functional deficiency, a negative B12 balance (low holoTC and normal MMA) can be distinguished from a B12 deficiency (low to moderate holoTC and high MMA) or a B12-repleted individual (moderate holoTC and normal MMA). In 2013, the same authors published a two-step algorithm for the diagnosis of B12 deficiency in adults making use of the same two biochemical markers, but slightly different cut-offs [51]. Based on their own findings using 1359 samples, they observed that the area under the ROC curve was higher for holoTC (measured by the Abbott AxSYM analyzer) than for total B12 (immunoassay on the ADVIA Centaur System) to detect MMA levels > 300 nmol/L. Therefore, they suggested to use holoTC as a first-line test and to additionally test MMA when holoTC is between 23 and 75 pmol/L (i.e. the range extending from the 90% diagnostic sensitivity to the 90% diagnostic specificity) in patients with normal renal function. Besides, decreasing MMA levels after B12 treatment may confirm the diagnosis of B12 deficiency in individuals with an impaired renal function.

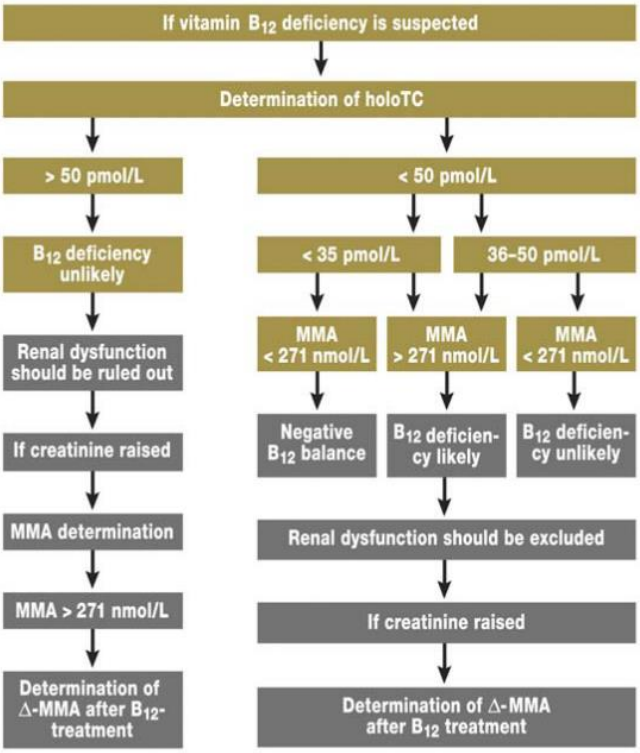

Figure 6: Algorithm published by Herrmann and Obeid [50]**.**

- *cB12, the combined indicator of vitamin B12 status*

In 2015, Fedosov et al. introduced the combined indicator of the vitamin B12 status, defined by the following formula: "cB12 = log10((holoTC*B12)/(MMA*tHcy))-(age factor)" (Table 6) [52, 53]. This indicator combines the four biochemical serum markers for B12 deficiency together with age into a single index reflecting B12 status, which differs from the classic "if → then" structure of most diagnostic algorithms. Since routine measurements usually include only 2-3 markers, the authors suggested a method to calculate the cB12 when one or two biomarkers are not determined and provided easy-to-use spreadsheets in the Supplemental Materials of the publication [53]. Of note, an adjustment of the formula is required if homocysteine is included and folate is below 10 nmol/L or 4.4 µg/L. As the cB12 score was shown to have a stronger association with hemoglobin and cognitive function than the single markers, some researchers already used the cB12 score as a reference to assess the diagnostic accuracy of the individual markers [54–56]. When the cB12 score was used to define subclinical B12 deficiency, holoTC performed better than total B12 in recognizing subclinical B12 deficiency in a standard cohort, reaching a similar diagnostic accuracy as MMA [56]. This was mainly driven by the outstanding performance of HoloTC in women of 50 years or older [56]. Therefore, the cB12 score seem to be an asset in unravelling the biochemical patterns of B12 deficiency, as it is considered diagnostic more reliable than any single biomarker, and can be employed as a "gold standard" for B12 deficiency next to MMA levels. However, the reliability of this promising marker in a clinical context has to be further investigated before it can be used as a universally accepted reference tool.

Table 6: The classification of the vitamin B12 status based on the combined indicator cB12. Adapted from Fedosov et al. [53].

Classification	Equivalence to single cut-off points*	Biological interpretation	Guidelines
Elevated B12 **cB12 > 1.5**	B12>881 holoTC>190 tHcy<8.0 MMA<0.11	The pathogenesis of high B12 is not fully understood	Consider potential causes of high B12 levels such as liver disease or current or recent supplementation or treatment
B12 adequacy **cB12: –0.5 - 1.5**	252<B12<881 37<holoTC<190 8.0<tHcy<13.6 0.11<MMA<0.35	Expected to accomplish all B12 status dependent functions	No action advised unless signs/symptoms present
Low B12 **cB12: –1.5 - –0.5**	161<B12<252 20<holoTC<37 13.6<tHcy<19.2 0.35<MMA<0.84	Potential subclinical manifestations of B12 deficiency i.e., absence of hematological changes, but subclinical neurological impairment	Consider recommending oral supplements
Possible **B12 deficiency** **cB12: –2.5 - –1.5**	157<B12<161 8.4<holoTC<20 19.2<tHcy<51 0.84<MMA<1.7	Potential manifestations of B12 deficiency	Potentially prescribe oral supplements, assess again in 3–6 months
Probable **B12 deficiency** **cB12: < –2.5**	B12<157 holoTC<8.4 tHcy>51 MMA>1.7	It is possible to observe clinical manifestations of B12 deficiency. Clinical outcomes are needed to confirm potential clinical deficiency	Consider immediate treatment with IM injections, determine cause with primary consideration for the possibility of pernicious anemia

*B12 is expressed as ng/L, holoTC as pmol/L, tHcy and MMA as µmol/L.

6. Conclusion literature study

This literature study demonstrates that all four biochemical markers (serum cobalamin, holotranscobalamin, MMA and homocysteine) have their own strengths and weaknesses. Therefore, it is generally acknowledged that the combination of at least two markers is the safest option for diagnosing clinical and subclinical B12 deficiency, while reasonably keeping in mind that reference values should not be strictly interpreted in all patients. The recently developed cB12 score seems to be a promising tool and future studies using this score as a reference for (sub)clinical deficiency will shed new light on the diagnostic accuracy of the individual biomarkers. Based on the current knowledge, MMA seems to be the most reliable, sensitive and specific marker for diagnosing B12 deficiency and is considered the gold standard in many publications, provided that non-B12-related modifiers (a.o. age and renal function) of MMA are taken into account. Currently, only two biochemical markers for B12 deficiency (total serum cobalamin and homocysteine) are available in the clinical laboratory of AZ Groeninge. Based on this literature study, MMA was selected to extend the repertoire of B12 biomarkers in our laboratory in an attempt to improve the diagnostic process of cobalamin deficiency. The following chapter describes the method verification for MMA in serum and the measurement of MMA in a selected hospital-specific patient population.

Question II: Is it possible to improve the diagnostic process of cobalamin deficiency by implementing the analysis of methylmalonic acid in the clinical laboratory of AZ Groeninge?

1. Method verification of the MMA MassChrom reagent kit (Chromsystems)

The MMA MassChrom reagent kit from Chromsystems was implemented according to the manufacturers specifications using LC-MS/MS (Acquity H Class plus – Xevo TQ-XS (Waters)). Sample preparation comprises addition of the deuterated internal standard to the serum sample followed by a simple filtration step using the so-called Clean-Up Tubes. The sample volume required is only 50-100 µL (depending on whether a ½ dilution is used). A method verification was performed according to the EMA guideline on bioanalytical method validation [57]. The linearity, repeatability, reproducibility, accuracy, total error, selectivity, carry-over, freeze-thaw stability, stability at room temperature and at 4°C was evaluated. All set acceptance criteria were fulfilled and the method was, therefore, approved for clinical use. The repeatability was 1.1% for the low QC level L1 (MMA=174 nmol/L) and 1.8% for the high QC level L2 (MMA=576 nmol/L) (acceptance criteria < 15%). The reproducibility was 14.3% for the LLOQ (MMA= 43.5 nmol/L), 6.5% for L1 and 7.1% for L2 (acceptance criteria < 15%; <20% at the LLOQ level). The bias varied from -5.7 to 4.4% for the between-run conditions (including the LLOQ) (acceptance criteria < 15%). The total error for L1 was 13.6% and for L2 17.4% (acceptance criteria set at < 30%). MMA was stable during maximum 2 freeze/thaw cycles, for minimum 7 days at room temperature and for minimum 7 days at 4°C.

2. Verification of reference values

The study was approved by the local Ethics Committee at AZ Groeninge. The reference ranges stated in the MassChrom reagent kit insert are derived from Allen et al. [46], but it is recommended to establish lab-specific reference ranges. Therefore, reference values for MMA reported in literature were verified according to the CLSI EP28-A3C guidelines. These guidelines prescribe that reference values reported in the literature may be transferred if the analytical system and the test subject population are comparable. To assess the acceptability of the transference of a reference interval, three approaches can be used: (1) a subjective assessment; (2) a statistical test on a relatively small number of reference individuals (e.g., *n*=20); or (3) an evaluation of a larger number of reference individuals (> 20 but < 120 individuals). We selected the second option (which is based on the binomial test) and sampled 20 healthy volunteers older than 18 years (male/female= 10/10). The chosen reference values are acceptable to transfer if no more than 2 of the 20 tested subjects fall outside these limits. The inclusion criteria of the reference persons were as follows: (1) older than 18 years and not pregnant; (2) no known B12 deficiency during the past year; (3) no known impaired renal function; and (4) no recent intake of vitamin supplements containing vitamin B12. In addition to MMA, other parameters were tested to avoid the inclusion of B12 deficient volunteers or individuals with impaired renal function (eGFR < 60 ml/min/1.73m²). As such, total serum cobalamin, holotranscobalamin, folate, creatinine, eGFR, homocysteine, haemoglobin, the number of red blood cells and the mean corpuscular volume (MCV) of the red blood cells were measured. The results of the healthy volunteers are presented in Table 7 and Figure 7.

Most upper reference limits for MMA reported in literature are between 250 and 400 nmol/L in the middle-aged healthy population (Table 4). Moreover, well-established reference labs use similar upper reference limits (e.g., ARUP laboratories and Mayo Clinic: 0.00-0.40 µmol/L). The reference values we chose to transfer are the values reported by Erdogan et al. in 2010: 60 - 360 nmol/L [36]. These reference values were established in 40 apparently healthy adult subjects using an LC-MS/MS method [36]. Only one reference person in our population has a MMA value slightly higher than 360 nmol/L (i.e. 363 nmol/L). Therefore, the published reference values (60 – 360 nmol/L) may be adequately transferred to the analytical system in the clinical laboratory of AZ Groeninge. As recommended in literature [16, 17, 44, 45], a grey zone for MMA will be additionally applied (360 – 800 nmol/L) to consider mildly increased MMA levels caused by renal impairment, age or other unknown factors. If

a test result for MMA is within this grey zone, a comment will be added to the patient's lab report to alert the clinician that cautious interpretation is required.

Table 7: Overview of the results of the healthy volunteers (*n*=20). Data are presented as median (range).

	Healthy volunteers	Reference values
Ratio male:female	10:10	n.a.
Age (years)	33.5 (22-64)	n.a.
MMA (nmol/L)	208 (141-363)	n.a.
Total serum cobalamin (ng/L)	429 (209-590)	> 200
Holotranscobalamin (pmol/L)	66.4 (33.8- > 150)	Kit insert: 37.5 – 188 pmol/L
Homocysteine (µmol/L)	10.4 (6.22-14.7)	< 15
Folate (µg/L)	5.3 (2.2-9.2)	> 3.88
Creatinine (mg/dL)	0.80 (0.66-1.18)	Age-dependent: cf. lab tests guide AZG
eGFR (ml/min/1.73m²)	> 90 (76 - > 90)	n.a.
Haemoglobin (g/dL)	m: 15.8 (14.4-17.3) f: 13.3 (12.3-14.2)	m: 13.7-17.1 f: 11.8-15.5
Number of red blood cells (*10^12/L)	m: 5.1 (4.7-6.0) f: 4.5 (4.1-4.9)	m: 4.3-5.71 f: 3.75-5.11
MCV (fL)	90.2 (80.5-96.7)	84.0-98.3

Furthermore, the correlations between (i) MMA and age, (ii) MMA and holoTC, (iii) holoTC and total cobalamin, and (iv) total cobalamin and MMA were explored in this apparently healthy population using Spearman's rank correlation coefficient (Fig. 7). A significant correlation ($p < 0.001$; $\alpha=0.05$) was observed between total cobalamin and holoTC (active B12), suggesting that there is no added value of measuring holoTC in an apparently healthy population tested for total cobalamin. The other investigated correlations were statistically not significant.

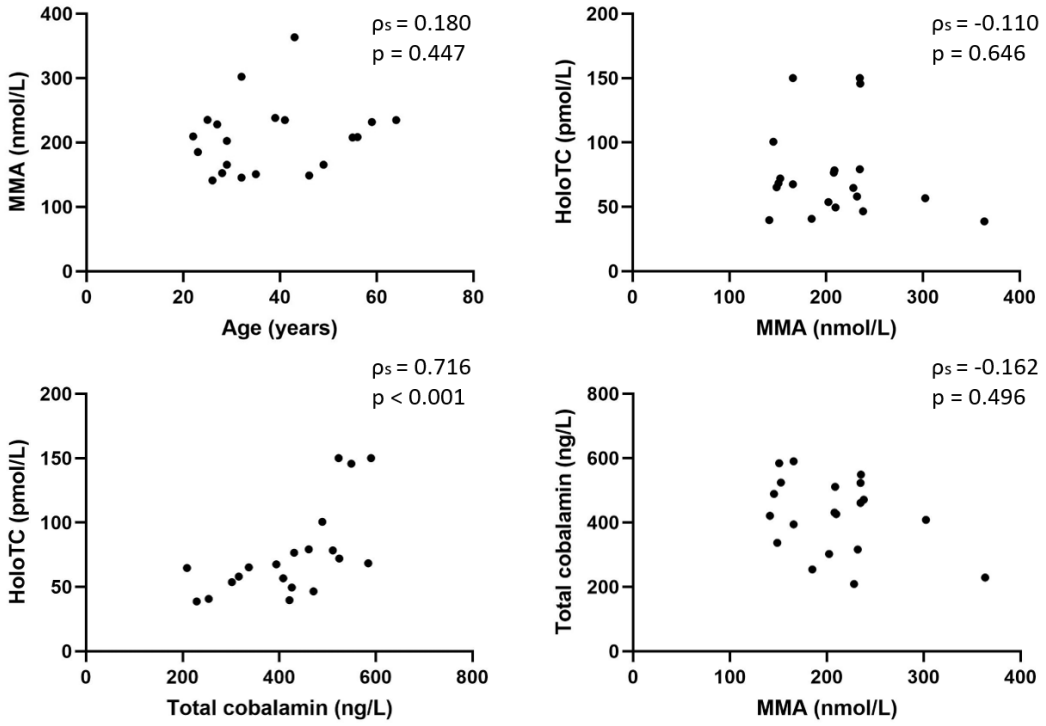

Figure 7: Spearman's rho (ρ_s) correlation analysis of age, MMA, holoTC and total cobalamin in the healthy volunteers (n=20).

3. Prevalence of B12 deficiency in the patient population of AZ Groeninge using different total B12 cut-offs

To estimate the prevalence of B12 deficiency based on total serum cobalamin in the adult patient population (> 18 years) of AZ Groeninge, a retrospective analysis was performed. Over a period of 6 months (1/1/2022 – 31/5/2022), 4916 patients were analysed for total serum cobalamin with a median age of 74 years (range 19-100 years). When applying the cut-off values used in NHANES in the United States (1999-2004) of < 200 ng/L, < 271 ng/L and < 347 ng/L, the prevalence of B12 deficiency in our laboratory is 2.1%, 10.1% and 26.7%, respectively, which is comparable to the prevalence estimated in NHANES (2.9%, 10.6% and 25.7%). Thus, the prevalence of B12 deficiency based on different serum B12 cut-offs in the Belgian adult population is comparable to the US adult population.

4. Measurement of B12 biochemical markers in a hospital-specific patient population

- *Total cobalamin and MMA in a hospital specific patient population*

The study was approved by the local Ethics Committee at AZ Groeninge. In total, 80 leftover samples from adult patients were included in this study. The samples were selected according to their B12 result and divided into three groups: B12 < 200 ng/L, 200-300 ng/L B12 and 300-400 ng/L B12. In addition, the patients were stratified according to their renal function (eGFR calculated using the CKD-EPI equation). MMA was measured in all samples. The results are summarized in Figure 8. When the serum B12 concentration was lower than 200 ng/L, only 20% (if eGFR > 90 mL/min/1.73m²) to 27% (if eGFR < 90 mL/min/1.73m²) of the patients had an MMA concentration > 800 nmol/L, which is indicative of B12 deficiency. Within the same group of patients (B12 < 200 ng/L), 18% (if eGFR < 90 mL/min/1.73m²) to 53% (if eGFR > 90 mL/min/1.73m²) of the patients had a normal MMA (and thus a low total cobalamin without functional deficiency), and 27% (if eGFR > 90 mL/min/1.73m²) to 55% (if eGFR < 90 mL/min/1.73m²) of the patients had an MMA concentration within the grey zone (360-800 nmol/L). The latter clearly demonstrates that patients with impaired renal function show increased MMA levels. Obviously, the fraction of patients with normal MMA levels was higher in the patient groups with a B12 concentration of 200-400 ng/L as compared to the patient group with a B12 level < 200 ng/L.

Next, we corrected the MMA concentrations for the eGFR (adjusted MMA) using the formula developed by Van Loon et al. [15] (cf. supra) in the patient group with impaired renal function (eGFR < 90 mL/min/1.73m²). After correction, 36% of the patients with a B12 < 200 ng/L had a normal MMA level instead of 18% of the patients without correction (Fig. 8). Interestingly, 21% (5/24) of the samples with an MMA_{obs} > 360 nmol/L are reclassified as B12 non-deficient (MMA_{adj} < 360 nmol/L) after correction. This is in line with the confirmatory study of Nielsen et al. in which 19.7% of the B12 deficient samples (MMA > 430 nmol/L) were reclassified as non-deficient after correction for eGFR [26].

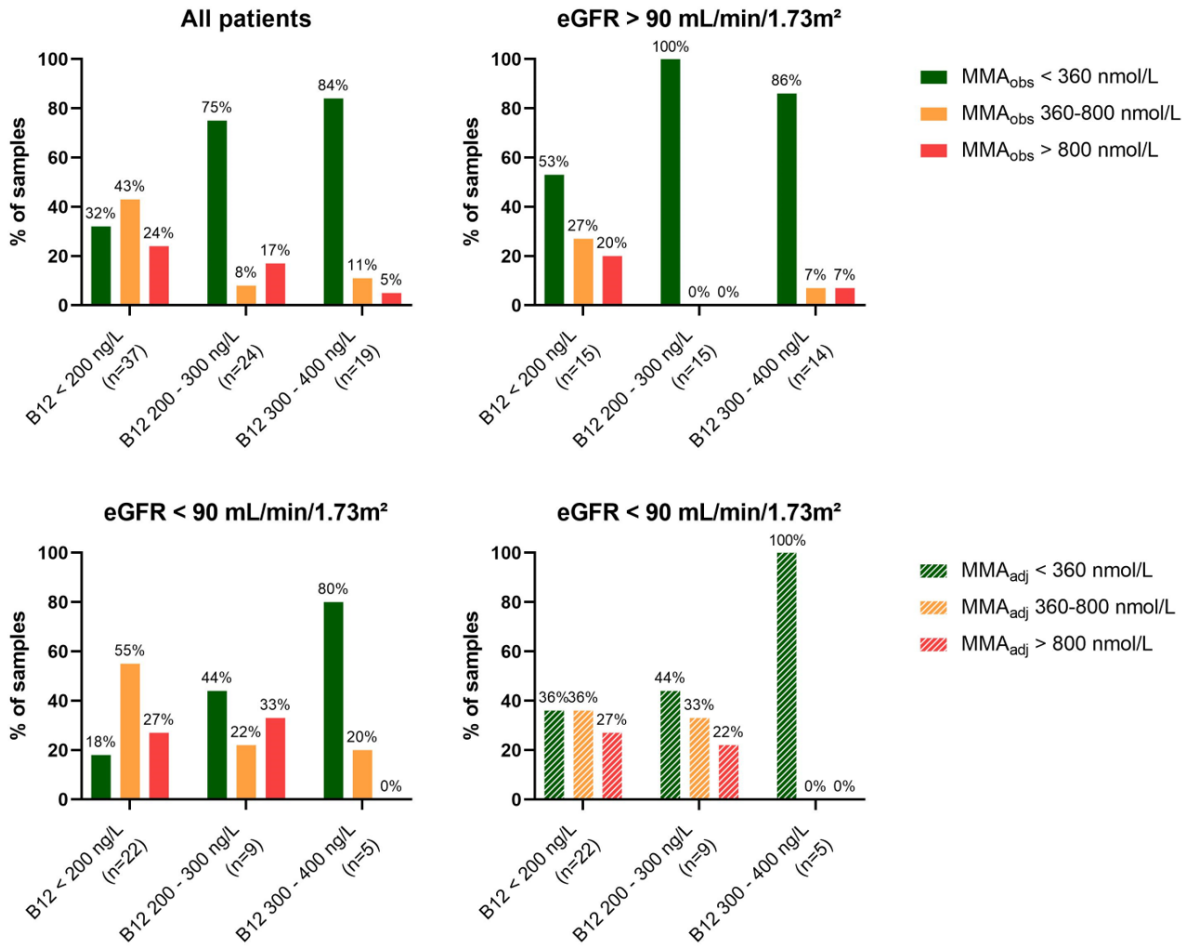

Figure 8: Serum B12 and MMA concentrations in a hospital-specific patient population.

- *Correlation analysis of the B12 biochemical markers*

To investigate the relationship between the B12 biochemical markers, the correlations between (i) MMA and eGFR, (ii) MMA and holoTC, (iii) total cobalamin and MMA, and (iv) total cobalamin and holoTC were explored in the hospital-specific patient population using Spearman's rank correlation coefficient (α=0.05) (Fig. 9). In this population, a significant correlation was observed between renal function expressed as eGFR and MMA. This is in line with multiple other studies showing a correlation between MMA and renal function as described in the literature study. Furthermore, total cobalamin significantly correlated with MMA. HoloTC did not significantly correlate with MMA, suggesting that holoTC is a poor predictor of functional B12 deficiency in our patient population. Moreover, holoTC did not significantly correlate with total cobalamin, which contrasts the significant correlation of serum cobalamin and holoTC in the healthy volunteers. When only considering patients with an eGFR > 90 mL/min/1.73m², similar results were observed, but no significant correlation was observed between MMA and total cobalamin (Fig. 10). The latter suggests that the significant correlation between MMA and total cobalamin in all patients not stratified for renal function (Fig. 9) is mainly driven by the mildly increased MMA levels within the grey zone in patients with impaired renal function.

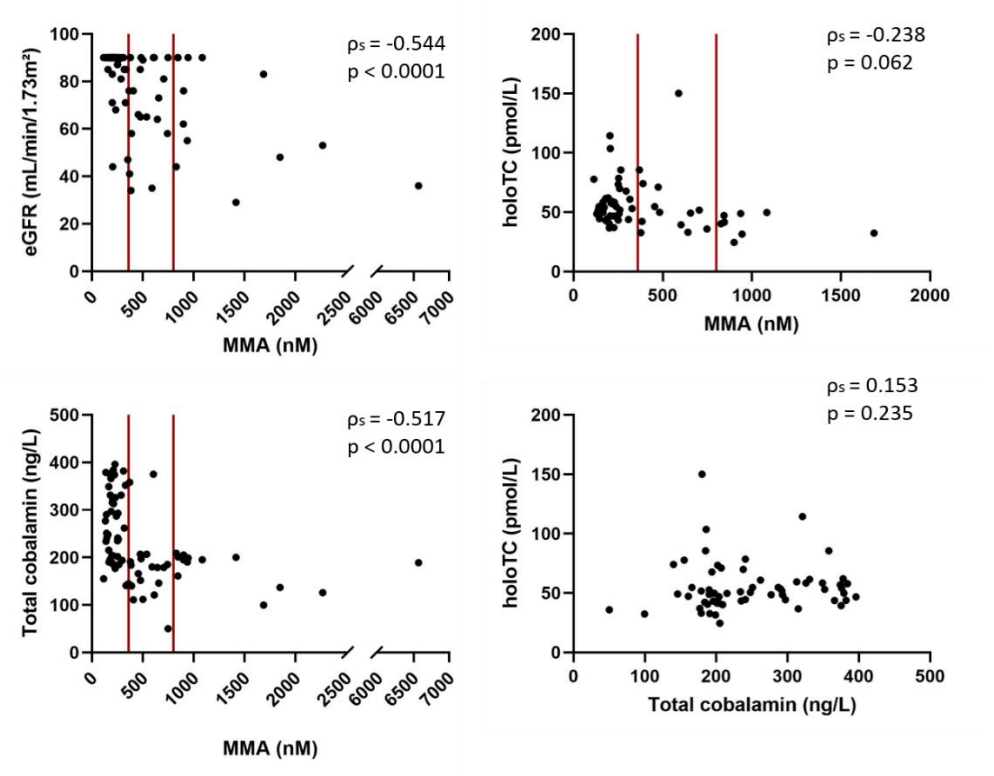

Figure 9: Spearman's rho (ρ_s) correlation analysis of MMA, total cobalamin, holoTC and eGFR in all patients. The grey zone for MMA (360 – 800 nmol/L) is indicated by red lines.

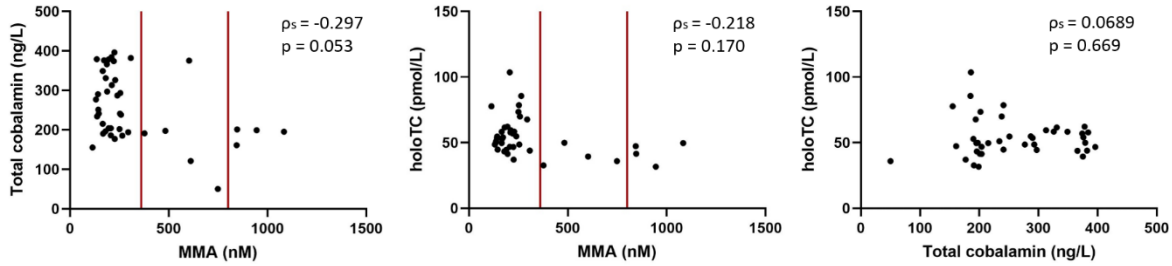

Figure 10: Spearman's rho (ρ_s) correlation analysis of MMA, total cobalamin, holoTC and eGFR in patients without renal impairment (eGFR > 90 mL/min/1.73m²). The grey zone for MMA (360 – 800 nmol/L) is indicated by red lines.

- *The cB12 score as a reference for B12 deficiency*

The cB12 score, as defined by Fedosov et al. [52, 53], using three (B12+MMA+holoTC or Hcy) or four markers corrected for folate was calculated in 64 samples. Furthermore, the 2cB12 score based on two markers (MMA and B12) was calculated in all samples (*n*=80). The results are displayed in Figure 11. In the group of patients with normal MMA (< 360 nmol/L), our data show that 97% (eGFR > 90 mL/min/1.73m²) to 98% (all patients) of the patients had a 3/4cB12s score compatible with B12 adequacy and 91% (all patients) to 94% (eGFR > 90 mL/min/1.73m²) of the patients had a 2cB12score compatible with B12 adequacy. The remainder of patients in this group were classified as subclinical B12 defiency, whereas none were classified as clinical B12 deficient. In the group of patients with increased MMA (> 360 nmol/L), 78% (all patients) to 89% (eGFR > 90 mL/min/1.73m²) of the patients have a 3/4cB12 score compatible with (sub)clinical B12 deficiency and 98% (all patients) to 100% (eGFR > 90 mL/min/1.73m²) of the patients have a 2cB12s core compatible with (sub)clinical B12 deficiency. Furthermore, when using the 3/4cB12 score to distinguish B12 (sub)clinical deficiency from adequacy, ROC

analysis revealed an optimal MMA cut-off of 343 nmol/L (eGFR > 90 mL/min/1.73m²) or 373 nmol/L (all patients), which closely allies to the transferred cut-off value of 360 nmol/L (Tabel 8). Moreover, the MMA level at which 100% specificity is reached in our small population is 622 nmol/L (all patients) or 676 nmol/L (eGFR > 90 mL/min/1.73m²), closely resembling the upper limit of the grey zone applied in our laboratory (800 nmol/L) [16, 17, 44, 45]. In conclusion, when the combined indicator cB12 based on three or four markers is used to define B12 deficiency, the MMA cut-off level of 360 nmol/L is able to adequately distinguish B12 (sub)clinical deficiency from B12 adequacy in most of the patients and closely resembles the optimal cut-off level revealed by ROC analysis.

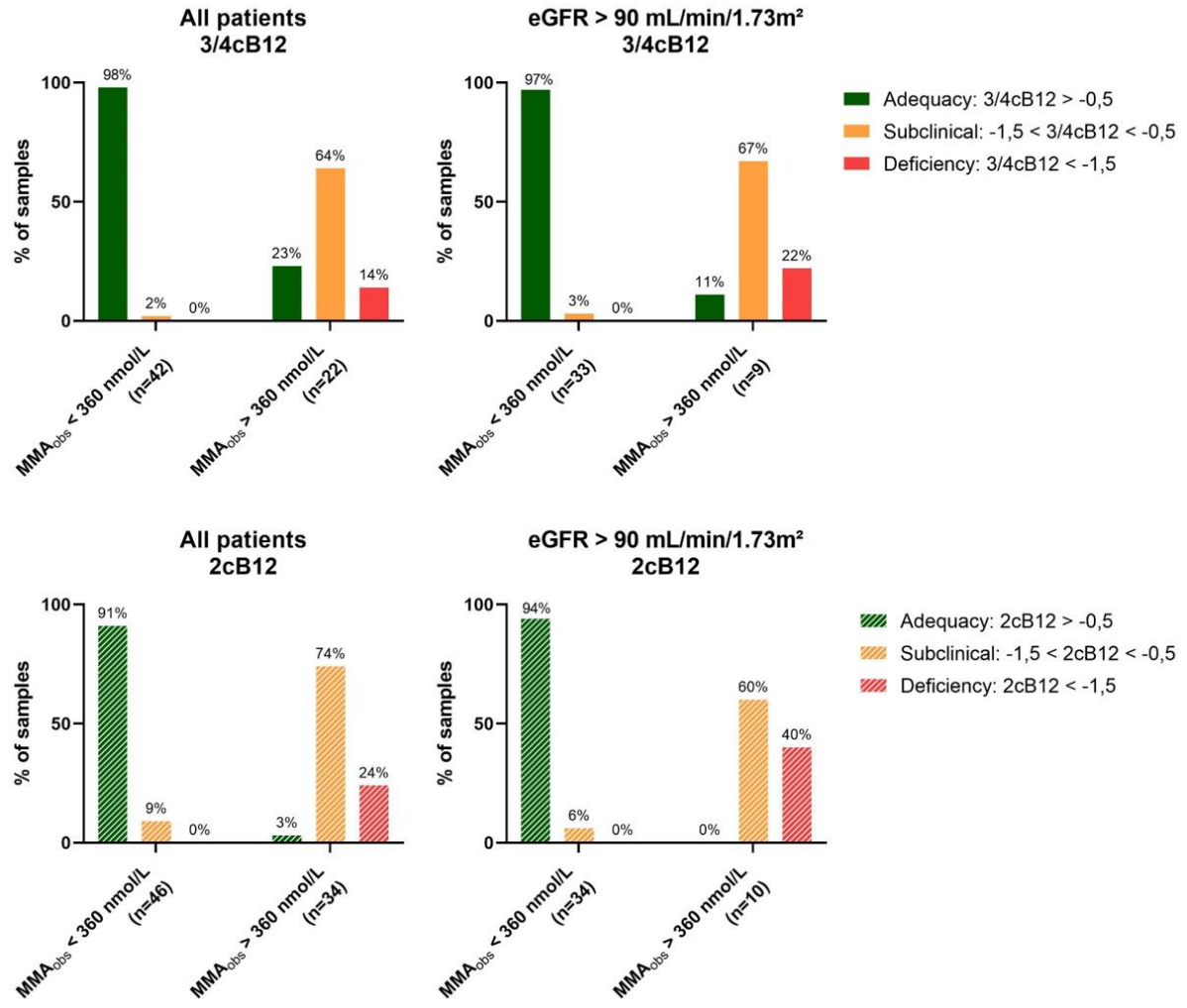

Figure 11: Evaluation of the MMA cut-off level when the cB12 score is used as a reference for B12 deficiency.

Table 8: ROC analysis of MMA when the 3/4cB12 score is used as a reference for B12 deficiency. B12 deficiency comprises a clinical and subclinical deficiency (3/4cB12 score < -0.5) and B12 adequacy includes patients with a 3/4cB12 score > -0.5.

	Number of patients with B12 deficiency	Number of patients with B12 adequacy	Optimal cut-off	100% sensitivity	100% specificity	AUC
All patients	18	46	373 (94% sens; 91% spec)	> 228	> 622	0.9626
eGFR > 90	9	33	343 (89% sens; 97% spec)	> 228	> 676	0.9630

5. Study limitations

The major limitation of this study is the rather small number of patients (*n*=80) with approximately half of the patients having a normal renal function. Therefore, the patient groups with impaired and normal renal function should be extended to perform a thorough clinical validation of the eGFR-corrected MMA, on the one hand, and the cB12 score, on the other hand. Furthermore, the presence or absence of clinical symptoms of B12 deficiency was not investigated in this study. This could help to better evaluate the accuracy of the cB12 score to define B12 (sub)clinical deficiency in our patient population.

6. Conclusions

First of all, the MassChrom reagent kit from Chromsystems was successfully implemented according to the manufacturers specifications using LC-MS/MS (Acquity H Class plus – Xevo TQ-XS (Waters)). Afterwards, the reference values for MMA reported by Erdogan et al. (60 - 360 nmol/L) [36] were verified using 20 healthy volunteers. Next, MMA was measured in a delineated hospital-specific patient population with low serum cobalamin levels (< 400 ng/L). Our data show that only 20% (if eGFR > 90 mL/min/1.73m²) to 27% (if eGFR < 90 mL/min/1.73m²) of the patients with a serum B12 concentration less than 200 ng/L had an MMA concentration indicative of a functional B12 deficiency (> 800 nmol/L). Moreover, no significant correlation was observed between MMA and total cobalamin in patients without renal impairment. Therefore, MMA may elucidate the actual B12 status of patients with a B12 concentration below the presumed cut-off level in the absence of functional deficiency, and prevent overtreatment of B12 deficiency in a non-deficient population. Furthermore, (subtle) neurological and haematological symptoms of B12 deficiency are shared with other diseases. Therefore, the measurement of MMA may prevent misdiagnosis of B12 deficiency in patients with low serum cobalamin concentrations, alerting the clinicians to extensively explore the differential diagnoses. Furthermore, our data reveal a significant correlation between MMA and eGFR, confirming that in patients with impaired renal function MMA concentrations should be interpreted with caution, which is partially coped with the implementation of a grey zone for MMA (360-800 nmol/L) and a comment on the lab report. Besides, the eGFR-adjusted MMA may be useful, but requires further investigation in our lab-specific patient population. Finally, our data demonstrate that 5 to 17% of the patients with an apparently normal cobalamin concentration (200-400 ng/L) had an MMA level indicative of B12 deficiency. Therefore, the measurement of MMA in patients with cobalamin levels above the presumed cut-off of 200 ng/L is useful to reliably confirm the diagnosis of B12 deficiency in patients with clinical symptoms, thereby preventing a delayed treatment, irreversible neurological damage, a potential never-ending quest for other causes and needless worrying of the patient and their families. In conclusion, to answer the question raised in this critically appraised topic: the implementation of an analytical method for MMA in serum will considerably improve the diagnostics of cobalamin deficiency in the clinical laboratory of AZ Groeninge.

TO DO/ACTIONS

1. Informing the clinicians (e.g., hematologists, neurologists) on the implementation of an analytical method for MMA.
2. Follow-up study of correlations between MMA levels and total cobalamin concentrations to further explore the actual prevalence of B12 deficiency in our lab-specific patient population.
3. Extending the evaluation of the implemented MMA reference ranges and grey zone, the cB12 score and eGFR-corrected MMA levels by including additional patients with normal and impaired renal function, and by correlating MMA or the cB12 score to the presence or absence of clinical symptoms.

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