Metformin and Vitamin B12 Deficiency: Where Do We Stand?

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ABSTRACT - The association between metformin use and low vitamin B12 levels in type 2 diabetes mellitus patients is well-established. However, many aspects of the topic remain to be elucidated. There is still controversy on the current diagnostic approaches to vitamin B12 deficiency. It is now believed that measuring the serum levels of the vitamin may not reflect its metabolic status. Moreover, there were conflicting results from studies attempting to quantify and explore metformin-associated vitamin B12 deficiency and its clinical impacts. This article reviews the cellular functions of vitamin B12, the biomarkers utilized to define the vitamin deficiency and metformin-induced vitamin B12 deficiency with an emphasis on its prevalence and clinical impacts.

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder that is increasingly becoming a pandemic in developed and developing worlds (1). In 2010, 285 million people, representing about 6% of the world's adult population, were T2DM patients (2). This number is expected to reach 439 million by 2030 (3). The disease is associated with various systemic macrovascular and microvascular complications. T2DM can lower the quality of life and result in heavy social and economic burdens, making the disease a public health concern. T2DM absorbs 5-10% of healthcare budget in many countries (4).

Both the European and American guidelines recommend the use of metformin as a first-line pharmacological therapy in T2DM (5). Findings from clinical studies confirmed that the medication improves cardiovascular outcomes in T2DM patients (6). Due to its proven effectiveness, relative safety and potential for use with other anti-diabetic medications, metformin is currently the most widely prescribed oral anti-diabetic agent (7). It is estimated that the medication is routinely prescribed to 120 million patients with diabetes around the world (8).

In 1971, vitamin B12 malabsorption was reported in metformin-treated diabetic patients (9). Since then, the association between metformin use and low vitamin B12 levels has been supported by different levels of evidence. Several aspects of the topic, however, still await clarifications. The reported prevalence of vitamin B12 deficiency among metformin-treated patients has shown great variation and ranged between 5.8% and 52% (10-20). A substantial part of the problem is the yet incompletely defined term of vitamin B12 deficiency. The diagnostic criteria of vitamin B12 deficiency are controversial and not agreed upon. Consensus is still lacking on which biomarkers are most indicative of the deficiency and what their ideal cut-offs are. The mere measurement of serum vitamin B12 levels is not considered sufficient to reflect the vitamin metabolic status (21). Moreover, research on the clinical implications of metformin-induced low vitamin B12 has yielded conflicting results.

In this review, vitamin B12 kinetics and intracellular functions are described. Tests for defining vitamin B12 deficiency, their diagnostic values, cut-off points and limitations are discussed. Proposed mechanisms and prevalence estimates of metformin-induced vitamin B12 deficiency are reviewed. Factors possibly affecting such estimates are also explained.

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The impact of metformin use on serum vitamin B12 levels and on other vitamin status-assessing biomarkers as well as the clinical consequences of vitamin B12 deficiency in metformin-treated T2DM patients are reviewed.

VITAMIN B12

Overview of vitamin B12

Vitamin B12, also known as cobalamin, is a water-soluble cobalt-containing vitamin that serves as a co-factor for metabolically significant enzymes. Vitamin B12 is a general term for all forms of cobalamins active in humans, including cyanocobalamin, hydroxocobalamin, methylcobalamin and 5-deoxyadenosyl cobalamin (adenosyl-Cbl). The first three forms are available as commercial products in different dosage forms. All forms of vitamin B12 are converted intracellularly into adenosyl-Cbl and methylcobalamin, the biologically active forms at the cellular level (22). As a co-factor, vitamin B12 plays a crucial role in intracellular enzymatic reactions related to DNA synthesis as well as amino and fatty acid metabolism. Such reactions are essential for the central nervous system functioning and erythropoiesis.

Vitamin B12 from diet to target cells

Vitamin B12 reaches its target cells through a complex course that involves several proteins and receptors (figure 1). Comprehending this multistep course is essential for understanding the multifaceted nature of vitamin B12 deficiency and the controversies associated with its diagnosis.

Dietary vitamin B12 is normally bound to proteins. Food-bound vitamin B12 is released in the stomach under the effect of gastric acid and pepsin. Therefore, proton pump inhibitors (PPIs) and histamine-2 receptor antagonists (H2RAs) may cause vitamin B12 deficiency by suppressing gastric acid secretion (23). The free vitamin is then bound to R-binder, a glycoprotein in gastric fluid and saliva that protects vitamin B12 from the highly acidic stomach environment. Pancreatic proteases degrade R-binder in the duodenum and liberate vitamin B12. The free vitamin is then bound by the intrinsic factor (IF) – a glycosylated protein secreted by gastric parietal cells – forming IF-vitamin B12 complex (24). IF resists proteolysis and serves as a carrier for vitamin B12 to the terminal ileum where the IF-vitamin B12 complex undergoes a receptor-mediated endocytosis. Pernicious anemia is an autoimmune disease characterized by the production of antibodies that target IF or the gastric parietal cells, resulting in vitamin B12 malabsorption and deficiency.

IF-vitamin B12 complex binds to the ileal cubilin receptor. Cubilin is a glycosylated protein expressed on the apical side of ileal enterocytes (25,26). IF-vitamin B12 complex binds to specific cubilin domains (27). Such interaction requires calcium cations, which may strengthen the functional affinity of the complex to the receptor (24). The complex of IF-vitamin B12-cubilin receptor is then endocytosed by the ileal enterocyte. Following the internalization, the IF-vitamin B12 complex detaches from cubilin. The complex reaches the lysosome where IF is degraded and vitamin B12 passes the lysosomal membrane to the cytoplasm.

The vitamin then appears in circulation bound to transcobalamin-I (TC-I) or transcobalamin-II (TC-II). It is estimated that 20-30% of the total circulating vitamin B12 is bound to TC-II protein (28). The protein binds newly absorbed vitamin and transports it to the target tissues where its absorption occurs through a receptor-mediated internalization process (28). Measuring vitamin B12-bound TC-II (holo-TC-II) is utilized as a diagnostic tool to evaluate vitamin B12 status. TC-I binds 70-80% of circulating vitamin B12, preventing the loss of the free unneeded portion (28).

The liver and, to a lesser extent, the kidneys represent the main stores of vitamin B12. The human liver stores 1-1.5mg of vitamin B12 (28). Vitamin B12 is known to undergo enterohepatic circulation involving its excretion in bile and reabsorption in the distal ileum (29). It is estimated that 4mcg of vitamin B12 is secreted daily in bile in a form bound to R-binder (29). Enterohepatic circulation may result in the reabsorption of more than half of the biliary vitamin B12 (29).

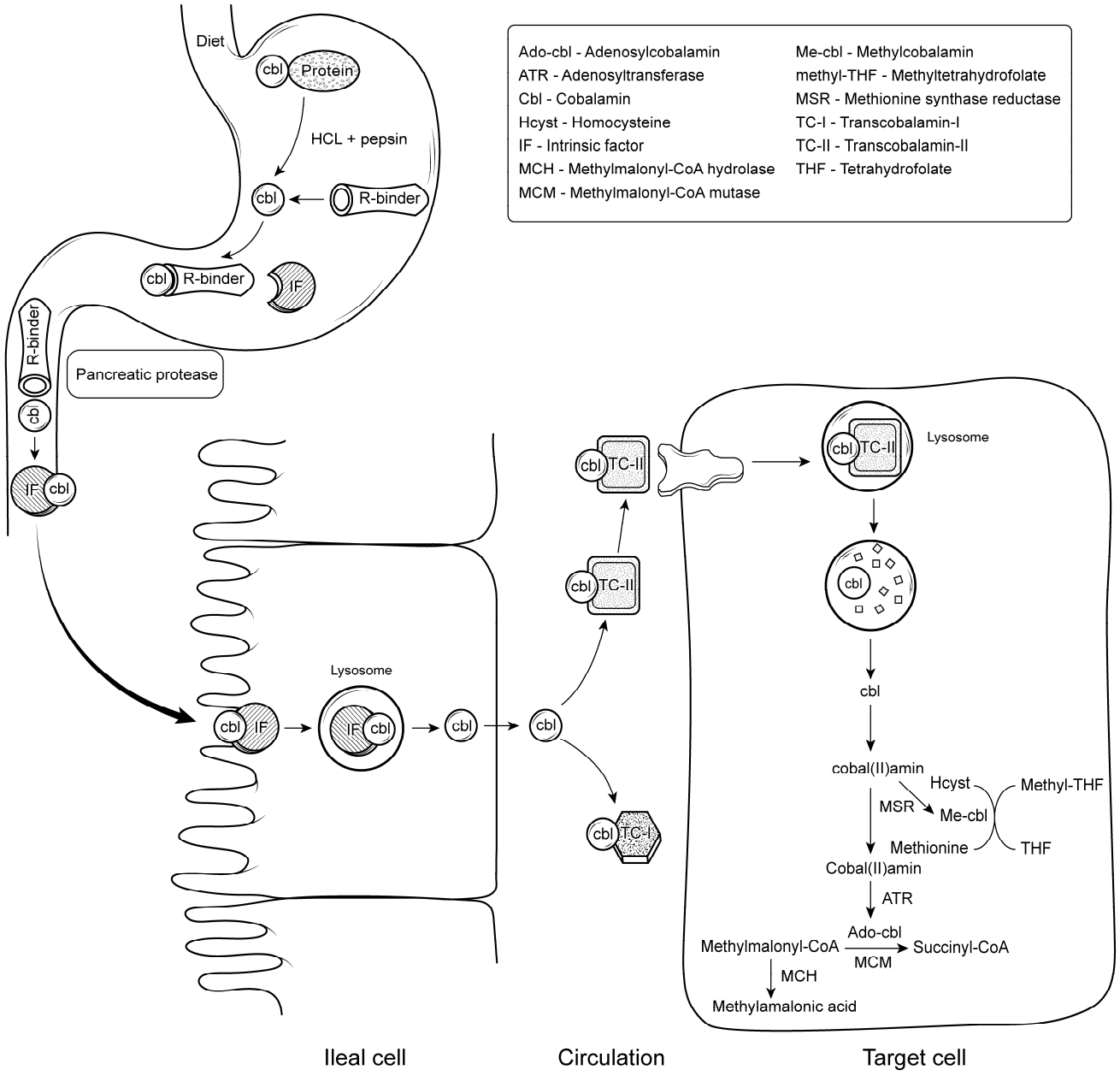

Figure 1 Vitamin B12 digestion, absorption, transport and intracellular function. See text for detailed explanation.

Intracellular kinetics and functions of vitamin B12

Different forms of vitamin B12 are converted to cobal(II)amin divalent cation in the target cells by processes that involve heterolytic and homolytic cleavage mechanisms (22). Cobal(II)amin is then converted to adenosyl-Cbl or methylcobalamin, which enter the methylmalonyl-CoA mutase (MCM) and methionine synthase (MS) pathways, respectively (figure 1).

1. Methionine synthase pathway

Homocysteine accepts a methyl group from methylcobalamin, resulting in the formation of methionine and cobal(I)amin, a monovalent super-nucleophilic intermediate (22). Cobal(I)amin then removes a methyl group from methyl-tetrahydrofolate, giving tetrahydrofolate and re-forming methylcobalamin. The reaction is catalyzed by the MS enzyme encoded in humans by CblG locus (30,31). Overall, MS catalyzes the transfer of

a methyl group from methyl-tetrahydrofolate to homocysteine to form tetrahydrofolate and methionine, using methylcobalamin as a co-factor. Vitamin B12 deficiency can prevent the ultimate methyl transfer from methyl-tetrahydrofolate to form tetrahydrofolate. Folate is hence "trapped" in the metabolically inactive form, methyl-tetrahydrofolate. Cytoplasmic conversion of homocysteine to methionine is also suppressed and the plasma levels of the homocysteine are elevated under vitamin B12 deficiency. Plasma homocysteine is utilized in diagnosing cellular vitamin B12 deficiency. As methyl-tetrahydrofolate is the methyl group donor in the methylation of homocysteine to methionine, folate deficiency can also result in elevated plasma homocysteine levels.

The MS pathway can explain the pathogenesis of a part of the clinical manifestations of vitamin B12 deficiency. Reduced synthesis of tetrahydrofolate results in an impaired DNA synthesis that may cause megaloblastic anemia and other hematologic signs of vitamin B12 deficiency. Low intracellular availability of methionine also reduces the formation of s-adenosylmethionine (SAM), which is an essential methyl group donor in DNA synthesis reactions (32).

2. Methylmalonyl-CoA mutase pathway

Adenosyl-Cbl is synthesized in the mitochondria under the catalysis of ATP-dependent cobalamin adenosyl transferase enzyme. Adenosyl-Cbl serves as a co-factor in the isomerization reaction catalyzed by MCM enzyme and involves the conversion of methylmalonyl-CoA to the Krebs cycle intermediate succinyl-CoA (22). The mechanism by which adenosyl-Cbl acts is radical-based and involves the formation of a free radical and the migration of a hydrogen atom to synthesize succinyl-CoA (22). Deficiency in vitamin B12 blocks the conversion of methylmalonyl-CoA to succinyl-CoA. Accumulated methylmalonyl-CoA is hydrolyzed to methylmalonic acid (MMA) in a reaction catalyzed by methylmalonyl-CoA hydrolase (MCH) enzyme. Vitamin B12 deficiency results in elevated plasma levels of MMA. MMA is thus used as a diagnostic test to evaluate the cellular status of vitamin B12.

VITAMIN B12 DEFICIENCY

Overview

The complex and multistep nature of vitamin B12 absorption in the gastrointestinal tract increases the possibility of malabsorption when the process is interrupted at any point during the course. Therefore, malabsorption is the main cause of vitamin B12 deficiency. As animal products represent the main source of the vitamin for humans, dietary insufficiency is a potential cause of deficiency in cases of strict veganism or vegetarianism (33).

Age-related low vitamin B12 status is believed to be attributed to chronic poor absorption and low dietary intake (34). In older individuals, most deficiency cases are attributed to "food-vitamin B12 malabsorption" (35,36) . Those with the disorder are unable to release vitamin B12 from its carrier in food. Reduced gastric acid secretion and gastric dysfunction are the main causes of the disorder (36). Pernicious anemia is also a known cause of vitamin B12 deficiency. Other stomach-related clinical conditions, including achlorohydria, gastric atrophy, gastrectomy, gastric surgery and, possibly, *helicobacter pylori* persistent infection, can interfere with the absorption of vitamin B12 and result in deficiency (24). Pancreatitis (37), ileal resection, Crohn's disease and parasite infections can also lead to vitamin B12 deficiency.

Medication-associated vitamin B12 deficiency is also well described. The association between the use of H2RAs or PPIs and low vitamin B12 was reported in several clinical studies (23,38-40). Metformin-induced vitamin B12 deficiency is currently well-known. The clinical advantages of metformin as a first-line medication in T2DM along with the increasing incidence and prevalence of the disease uncovered the real volume of the problem. The debate continues on whether metformin-associated low vitamin B12 has the potential for clinical implications.

Clinical manifestations of vitamin B12 deficiency

The clinical picture of vitamin B12 deficiency consists mainly of neurological and hematological manifestations. Hematological manifestations include macrocytosis and megaloblastic anemia which may be associated with other signs and symptoms of deficiency, such as pancytopenia, glossitis, gastrointestinal dysfunction, psychosis or neurological disorders (33). Neurological signs and

symptoms may take many forms, including peripheral neuropathy (PN) which generally manifests as numbness and paresthesia (41), optic neuropathy (33), and neuropsychiatric disorders such as chronic fatigue syndrome, psychosis, mood disorders (42) or depressive symptoms (43).

Vitamin B12 deficiency may also result in disordered bowel motility, manifested as mild constipation or diarrhea, and loss of bowel or bladder control may develop (33). The deficiency may impair immune response (44-46) and lower bone mineral density (47).

Diagnosis of vitamin B12 deficiency

The diagnosis of vitamin B12 deficiency has always been controversial. Diagnostic tests depend on the direct measurement of the circulating vitamin or on measuring the levels of other biomarkers that accumulate as a result of cellular deficiency. Serum vitamin B12 and holo-TC-II tests measure the circulating part of the vitamin. Homocysteine and MMA are the biomarkers of metabolic vitamin B12 deficiency that show elevated levels when the vitamin is deficient at the cellular level.

It has always been difficult to determine the specificities and sensitivities of the tests used in assessing vitamin B12 status due to the absence of a gold standard comparator. Researchers currently compare biomarkers against other biomarkers that are believed to have better diagnostic accuracy (48). However, even the more accurate biomarkers have their own sensitivity and specificity limitations (48). In diagnostic research, the true positive and true negative cases are determined by a reference test: the gold standard. The absence of such a reference test in the diagnosis of vitamin B12 deficiency renders any claimed specificity or sensitivity liable to criticism and uncertainty.

1. Serum vitamin B12 test

The sensitivity of the serum vitamin B12 test in assessing the vitamin's status is generally considered high. Studies have showed that vitamin B12 levels <148 pmol/L have a sensitivity that exceeds 95% in patients with megaloblastic anemia (49,50). Bolann *et al.* used >50% post-therapy decline in MMA as a gold standard to define vitamin B12 deficiency, and reported 90% sensitivity of <148 pmol/L cut-off point of serum vitamin B12 (51). Contrarily, the specificity of serum vitamin B12 test is low. Clarke *et al.* applied strict MMA criteria of >450 and >750 nmol/L as reference tests, and found that serum vitamin B12 <200 pmol/L had specificities of 72% and 75%, respectively (52).

In the 1950s, the serum vitamin B12 concentration cut-off point of ≤90 pmol/L was used to define vitamin B12 deficiency (53). Currently, the cut-off point of < 148 pmol/L is more commonly used in research and clinical fields (48). A roundtable convention revised the relevance and accepted the cut-off points of 148 pmol/L and 200 pmol/L currently used by scientists and clinicians (21). The cut-off point of 148 pmol/L misses 3-5% of deficient cases, while the cut-off of 200 pmol/L identifies all deficient cases but results in higher false positive rates (21). With the discovery of the biomarkers that describe vitamin B12 metabolic status and its subclinical deficiency, suggestions to change the cut-offs to <221 or <258 appeared in an attempt to capture more deficient cases (54,55).

Falsely low serum vitamin B12 levels were reported in pregnancy and folate deficiency (53). TC-I protein carries 80% of circulating vitamin B12 and can thus affect its serum levels. Carmel *et al*. reported that 15% of cases of low serum vitamin B12 levels may be attributed to TC-I deficiency (56). Elevated TC-I concentrations can also result in falsely raised serum vitamin B12 levels. Myelogenous leukemia and some types of cancer represent the known causes that may result in elevated TC-I. Jeffery *et al*. reported that high TC-I levels account for 8% of cases with elevated serum vitamin B12 levels (57). People of black ethnicity tend to show higher circulatory levels of TC-I and vitamin B12 (58). The concentrations of vitamin B12 are also elevated in individuals with impaired renal function (59).

2. MMA test

Vitamin B12, under the catalysis of the enzyme methylmalonyl-CoA mutase, synthesizes succinyl-CoA from methylmalonyl-CoA in the mitochondria. Deficiency of vitamin B12 thus results in elevated MMA levels. Therefore, it can be said that measuring MMA levels provides a more accurate estimation of the cellular status of vitamin B12 compared with the vitamin's serum levels. That is, testing for MMA can better reflect the metabolic or the functional status of vitamin B12.

Elevated MMA test has >95% sensitivity to vitamin B12 deficiency in patients with pernicious anemia (60). In such overt deficiencies, sensitivity

of MMA elevation is slightly better than that of low vitamin B12 levels (48).

MMA test cut-offs ranging between 210 and 480 nmol/L are used to define vitamin B12 deficiency. Hence, the prevalence of the deficiency as defined by MMA elevation can vary according to the cut-off points used. The cut-off of >270nmol/L is currently the most commonly used. Pfeiffer *et al*. used the low cut-off point of 210 nmol/L as a physiologic choice based on MMA levels in vitamin B12-repleted individuals (61). That point represented the maximal inhibition of MMA levels by administering vitamin B12. However, such low cut-offs carry greater risks of overdiagnosis when MMA is the only tool used to define vitamin B12 deficiency (48).

A large epidemiologic study in Norway found that MMA levels were affected by creatinine concentrations, age and sex (62). It was also suggested that contracting plasma volume plays a role in some cases of unexplainable high MMA levels (50). The antibiotics' ability to reduce MMA levels suggests a role for the gut bacteria that produce propionic acid, the precursor of MMA (60,63). Therefore, the specificity of the MMA test is uncertain and the test is not qualified for use as a gold standard for defining vitamin B12 deficiency.

3. Holo-TC-II test

Vitamin B12 circulates in plasma bound to TC-I and TC-II carrier proteins. The portion attached to TC-II protein is known as holo-TC-II. Holo-TC-II attaches 20-30% of total plasma vitamin B12, and the remainder is attached to TC-I, forming a metabolically inert complex (28). Measuring holo-TC-II is believed to reflect the bioavailable vitamin B12 as the protein is responsible for the immediate transfer of the newly absorbed vitamin from the ileal enterocytes to the target cells. Chen *et al*. found that the metabolic status of vitamin B12 was a major determinant of holo-TC-II serum levels (64). Furthermore, they concluded that the absorption status of vitamin B12 also influenced serum holo-TC-II levels.

The diagnostic accuracy of holo-TC-II remains controversial. The test is thought to have sensitivities and specificities comparable to that of serum vitamin B12 when compared to MMA elevations (48). Several studies suggested that holo-TC-II slightly outperformed the serum vitamin B12 test (65,66). The specificity of the holo-TC-II test remains unclear.

The used cut-offs of holo-TC-II show great variation, perhaps because of the relatively limited utilization of the test. Laboratories use cut-offs that range between 11 and 41 pmol/L (66). Relationships between chosen cut-offs and resultant outcomes warrant additional research.

Renal failure is associated with elevated levels of holo-TC-II (67). Mild renal insufficiency has a modest impact on serum vitamin B12 and holo-TC-II levels, unlike its effect on MMA and homocyteine concentrations (59). Several studies suggested that the levels of holo-TC-II are affected by folate disorders, use of oral contraceptives, myelodysplasia, certain hematologic disorders and alcoholism (66,68,69). Having the half-life of just six minutes and being dependent on the absorbed vitamin B12, holo-TC-II levels may fluctuate following dietary perturbations (70). The undefined sites of synthesis and kinetics, relatively limited utilization in practice and research, lack of systematic investigations on accuracy, and fluctuations reflecting dietary absorption, are all limitations against the use of holo-TC-II as a reliable test for assessing vitamin B12 status.

4. Homocystein test

The MS enzyme catalyzes the transfer of a methyl group from methyl-tetrahydrofolate to homocysteine to result in the formation of tetrahydrofolate and methionine, utilizing vitamin B12 as a co-factor. Therefore, homocysteine elevated concentrations are associated with vitamin B12 deficiency, and homocysteine may be used as a test to assess the metabolic status of vitamin B12.

An expert panel recommended setting cut-offs for homocysteine levels considering age and folate fortification status (71). In folate-fortified communities, the panel recommended 12 micromol/L and 16 micromol/L for those aged 15-65 years and >65 years, respectively. For communities where folic acid fortification is not implemented, cut-offs of 15 and 20 micromol/L for those aged 15-65 years and >65 years, respectively, are recommended.

The plasma homocysteine test has a sensitivity comparable to that of MMA (72). However, confounders limit the value of the test and reduce its specificity in the diagnosis of vitamin B12 deficiency. Folate deficiency elevates homocysteine levels. In populations where folic acid fortification is implemented, vitamin B12 deficiency is the main cause of high homocysteine levels (73). Renal

failure and old age are other major causes of elevated plasma homocysteine levels (74,75). Vitamin B6 and vitamin B2 (riboflavin) deficiencies can also increase homocysteine concentrations (76,77).

5. Response to treatment

The response of homocysteine and MMA to therapeutic doses of vitamin B12 can be diagnostically informative (48). However, the holo-TC-II response to therapy carries no diagnostic significance as pharmacologic administration of vitamin B12 will increase its blood level, but not necessarily reverse the deficiency (48). Using biochemical response as a diagnostic tool is impractical. In addition, the observed response can be a mere representation of "regression to the mean" phenomenon (48). It can also be argued that responsive MMA and homocysteine do not prove their clinical impact.

New concepts in vitamin B12 deficiency

Subclinical (marginal, borderline or subtle) vitamin B12 deficiency and functional vitamin B12 deficiency are recently introduced terms. Subclinical vitamin B12 deficiency is defined by low-normal vitamin B12 levels with elevated concentrations of metabolic biomarkers in the absence of clinical symptoms and signs (78). The cut-off points used to define the elevated metabolic biomarkers and the low-normal vitamin B12 levels are controversial. The most commonly accepted low-normal definition for serum vitamin B12 levels ranges between 150 and 220 pmol/L (79). Malabsorption of food-bound vitamin B12 is a possible etiology of the subclinical deficiency (48,80). The significance of subclinical deficiency is yet to be studied thoroughly. The follow-up of individuals with subclinical deficiency showed that they may regress to the normal status, progress to overt deficiency, or remain asymptomatic for years (48,80,81). The condition may be associated with neurologic or cognitive manifestations (21). Clinical research has not proved whether the early detection and treatment of subclinical vitamin B12 deficiency inhibits the progression to the overt deficiency (48).

Functional vitamin B12 deficiency, often referred to as vitamin B12 resistance, describes the presence of elevated levels of MMA, despite the normal serum concentrations of the vitamin. Studies reported that 7-30% of the elderly have elevated MMA levels despite normal vitamin B12 concentrations (82,83). Pharmacological doses of the vitamin reduced the MMA levels in most elderly individuals with high MMA (84,85). The condition has been linked to anemia, decreased cognitive function and neuropathy (86,87). Functional vitamin B12 deficiency is believed to be more common in T2DM patients (88,89). Vitamin B12 therapy was reported to reduce MMA and improve neuropathic symptoms in diabetes patients with functional vitamin B12 deficiency (78).

METFORMIN AND VITAMIN B12 DEFICIENCY

Overview

Tomkin *et al*. were the first to describe metformin-associated vitamin B12 malabsorption in 1971 (9). Currently, there is a consensus on the medication's ability to lower vitamin B12 serum levels. However, the debate continues on metformin's ability to cause cellular vitamin B12 deficiency and result in clinical consequences. The absence of a gold standard diagnostic test for vitamin B12 status generated controversies over the accuracy of tests currently used to assess the deficiency, adding complexity to the topic of metformin-induced vitamin B12 deficiency.

The usual metformin prescribed dose ranges between 1000mg and 3400mg per day. The dose is quite high in terms of milligrams. Therefore, interference with dietary vitamin B12 absorption is plausible, considering it is recommended that the medication be taken with or immediately after food.

The effect of metformin on vitamin B12 levels

The relationship between metformin use and low vitamin B12 was described in many observational studies (11,12,15-17,20,90). Randomized clinical trials have proved that receiving the medication for a few months can significantly lower vitamin B12 levels (91-93). The percentage of reduction in vitamin B12 levels attributable to metformin use ranged from 17.8% to 26.8% in cross-sectional studies (11,12,15,16) and from 6.3 % to 18.7% in clinical trials with 6-16-week durations (91-93).

De Jager *et al*. provided the strongest evidence of metformin-associated low vitamin B12 levels by conducting a 4.3 years duration randomized controlled trial (10). The trial reported a 19% metformin-associated reduction in vitamin B12 levels. The study was the first to show the

progressive decrease in vitamin B12 levels in patients on metformin over time, and the first to report the medication's potential to lower the vitamin to levels that usually require pharmacological substitution. Following the trial's publication, more epidemiological studies targeted the investigation of the possible clinical consequences of metformin-induced vitamin B12 deficiency.

A recent meta-analysis also confirmed that metformin induces a reduction in vitamin B12 levels (94). The study reported the positive association between the metformin dose and the lowering of the vitamin concentrations.

Mechanism of metformin-induced malabsorption of vitamin B12

Many mechanisms were proposed to explain how metformin interferes with the absorption of vitamin B12. Intestinal bacteria overgrowth resulting in the binding of IF-vitamin B12 complex to bacteria instead of being absorbed was an early suggested mechanism (95). It was also proposed that metformin reduces the vitamin absorption by altering the intestinal motility (96).

The most currently accepted mechanism suggests that metformin antagonizes the calcium cation and interferes with the calcium-dependent IF-vitamin B12 complex binding to the ileal cubilin receptor (97). The reversal of metformin-associated vitamin B12 malabsorption by calcium supplementation greatly supported the latter mechanism. The study of Bauman *et al*. proposed the mechanism that describes the malabsorption of vitamin B12 by metformin (97). Type-2 diabetic participants were divided into two groups: the first group was given metformin, and the second (control) group was given a sulfonylurea. The metformin group, but not the control group, showed a statistically significant gradual decrease in serum vitamin B12 and holo-TC-II levels over the first three months. Oral calcium supplementation was then introduced to the metformin group for one month. At the end of that month, holo-TC-II levels increased in the metformin group by 53%. The absence of bacterial overgrowth was confirmed by hydrogen breath tests and by similar baseline and study end concentrations of serum vitamin B12 analogs. The authors built on the previously reported ability of biguanides to give a positive charge to the membrane's surface (98) and on the essential role the calcium plays in the binding of IF-vitamin B12 complex to ileal receptors (99) to introduce the theory of the mechanism by which metformin inhibits vitamin B12 absorption. They proposed that the protonated metformin molecule directs itself towards the hydrocarbon core of the ileal cell membrane and positively charges the membrane surface, displacing the divalent calcium cations by repulsion forces (figure 2). Such displacement impairs the calcium-dependent binding of IF-vitamin B12 complex to the ileal cubilin receptor and malabsorption of the vitamin ensues.

Since bile is secreted into the duodenum, the above theory connotes that metformin may inhibit the absorption of bile vitamin B12 later in the distal ileum. Therefore, the medication has the theoretical potential to inhibit both dietary and enterohepatic vitamin B12 absorption.

Impact of metformin on the biomarkers of cellular vitamin B12 deficiency

1. Impact on homocysteine levels

The results of a 16-week randomized controlled trial showed that, when compared to the control group, metformin use resulted in a significant 14% decrease in vitamin B12 and a 4% increase in homocysteine (but folate was also reduced) (92). Sato *et al*. reported a negative correlation between vitamin B12 and homocysteine levels among metformin-treated T2DM patients (79). A positive correlation between the cumulative dose of metformin and the levels of homocysteine was also reported (100). Metformin-users were found to have slightly higher homocysteine levels than non-users (101). In the randomized controlled trial of De Jager *et al*., the 4.3-year treatment with metformin resulted in a minor statistically significant increase in homocysteine concentrations (10). However, homocysteine levels did not show a progressive increase with time similar to that reported with vitamin B12 levels.

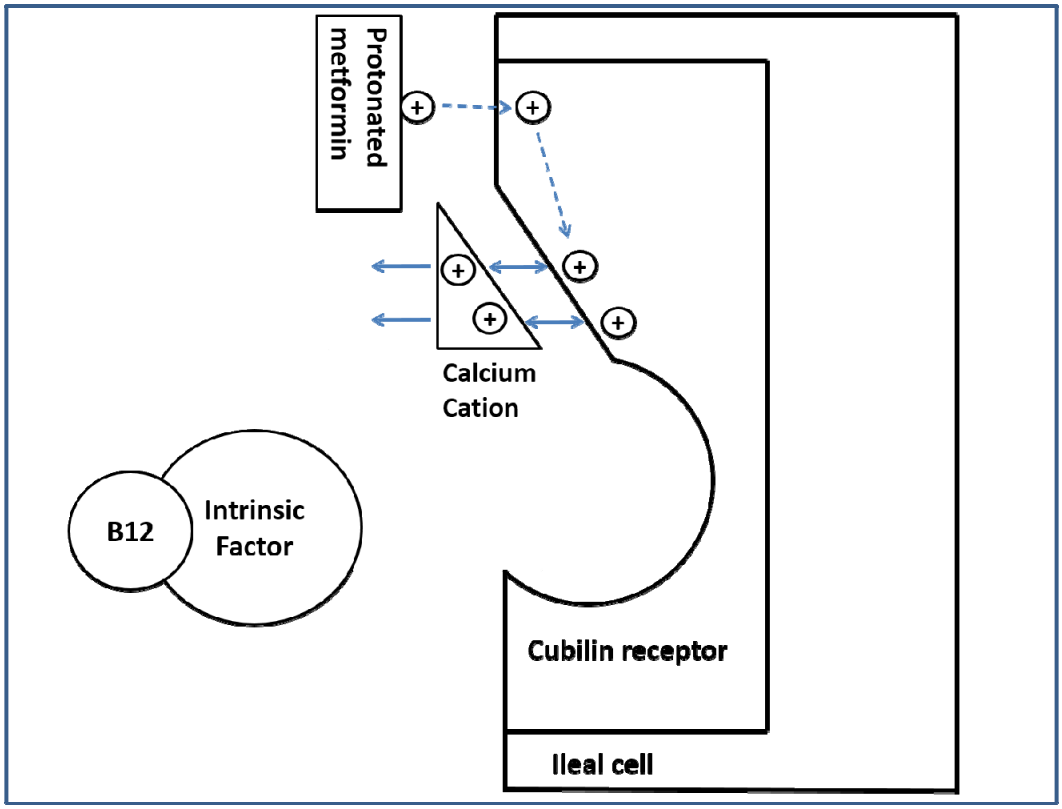

Figure 2 Mechanism of inhibition of vitamin B12 absorption by metformin. See text for detailed explanation.

A clinical trial in Norway proved that a 12-week folic acid supplement in patients with T2DM using metformin significantly reduced homocysteine levels (102). This finding raised the question whether the metformin-induced elevated homocysteine mentioned above was mediated by low vitamin B12 and not by low folate. Several studies have also concluded that metformin did not elevate homocysteine levels, like the cross-sectional study in Thailand which showed that homocysteine concentrations were not significantly affected by metformin use (103). A randomized trial showed that taking metformin for 16 weeks did not affect the levels of homocysteine in women with polycystic ovary syndrome (93). Reinstatler *et al*. also found no statistically significant difference in the mean levels of homocysteine when metformin users and non-users were compared (11).

2. Impact on MMA levels

Unlike other tests of vitamin B12 status, testing for MMA is a costly process that uses gas chromatography-mass spectrometry (GC-MS) techniques and requires special equipment. This may be the reason behind the relatively low utilization of MMA tests in the investigation of metformin's impact on the functional status of vitamin B12.

A case-control study reported higher MMA levels in T2DM patients who were taking metformin compared to the group not taking metformin (100). The study also reported a correlation between the cumulative dose of the medication and the MMA levels for the first time. However, a British cross-sectional study found no statistically significant differences in MMA concentrations between the users and non-users of metformin (104). A randomized controlled trial in Norway also reported no metformin effect on MMA in women with polycystic ovary syndrome treated with the medication for 16 weeks (93). Similar results were obtained by Greibe *et al*. after treating women with polycystic ovary syndrome with metformin for six months (105).

Prevalence of metformin-induced vitamin B12 deficiency

Comparing the prevalence estimates of metformin-associated vitamin B12 deficiency obtained from different epidemiological studies is not straightforward and requires judicious considerations of certain factors. Most importantly, the biomarkers used to define the deficiency, together with their cut-offs, can greatly affect the value of the prevalence estimate. However, most of the studies used serum vitamin B12 as a marker and the cut-off points of <148 pmol/L or <150 pmol/L. Attention should also be paid to the mean ages in different studies as vitamin B12 levels decrease with age. Variations in doses and durations of metformin use can also impact the final prevalence values. Other variables, such as the study settings and whether the renally-impaired patients were excluded, should also be considered. Table 1 shows the studies that measured the prevalence of metformin-induced vitamin B12 deficiency, their diagnostic cut-off points and other sample and study characteristics.

Clinical consequence of metformin-induced low vitamin-B12

Following the establishment of the association between long-term metformin use and low vitamin B12 levels by observational and interventional studies, researchers moved to the next step and investigated the clinical implications of such an association.

1. Peripheral neuropathy

PN is a primary complication of T2DM and a direct manifestation of vitamin B12 deficiency. Examining the anticipated relationship between vitamin B12 and PN in metformin users became essential following the clinical studies that proved the lowering effect of vitamin B12.

Both randomized controlled trials, the gold standard in clinical research, and cohort studies lack the practicality to give immediate answers to the question of the association of metformin-induced vitamin B12 deficiency with PN due to the anticipated insidiousness of such neuropathy. Case-control and cross-sectional studies are perhaps the most convenient designs to examine the possible association. This explains the fact that all the current evidence comes from such epidemiological studies. Unfortunately, case-control and cross-sectional designs have more weaknesses relative to randomized controlled trials and cohort studies, and their results are generally less reliable.

PN as a clinical consequence of metformin-induced vitamin B12 deficiency was recently investigated by five observational studies with conflicting results. Three studies reported no association, two reported increased neuropathy among metformin-exposed patients, and one study revealed that non-users of the medication had more severe neuropathy. The studies showed substantial variation in designs and settings and, more importantly, used PN-assessing tools with different degrees of subjectivity. Table 2 shows the settings, designs and results of studies investigating the impact of metformin-induced low vitamin B12 on PN in T2DM patients.

2. Neuropsychiatric manifestations

Low vitamin B12 levels were previously linked to depressive symptoms (43,108) and cognitive impairment (109). Two recent studies reported that the vitamin deficiency among metformin-treated patients was associated with worsened cognitive performance and increased risk of depression (107,110).

3. Hematological manifestations

Studies on metformin-associated low vitamin B12 have not reported any significant impact of medication use on the hematological findings – hemoglobin concentrations, prevalence of anemia, mean corpuscular volume or macrocytosis (11-13,15,16,90). It is worth mentioning that none of these studies had the investigation of hematological findings as a primary objective. The results were part of comparing the clinical and laboratory variables in metformin exposed and non-exposed patients.

CONCLUSIONS

There is almost a current consensus on metformin's potential to lower vitamin B12 levels. Whether the medication can cause cellular vitamin B12 deficiency remains controversial. The reported prevalence of the deficiency has shown great variation due to differences in the utilized diagnostic biomarkers, their cut-off points as well as sample- and study-related factors. Controversy over proper and practical vitamin B12 deficiency diagnostic approaches is probably a factor behind the relatively slow advancement of research on

metformin-induced vitamin deficiency. The yet undefined clinical implications of marginal and functional vitamin B12 deficiency further complicated the topic.

Several studies have recently investigated metformin-induced vitamin B12 deficiency's ability to cause or worsen PN in T2DM patients with conflicting results. Exploiting large electronic record databases and using more objective assessing tools to quantify the outcomes may be beneficial for the progression of research on the clinical consequences of metformin-induced vitamin B12 deficiency.

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Table 1. Clinical studies that measured the prevalence of metformin-induced vitamin B12 deficiency, their diagnostic cut-off points and other sample and study characteristics

Study	Obtained prevalence	Cut-off point of vitamin B12 (pmol/L)	Mean age (years)	Mean metformin dose (mg)	Mean metformin duration (years)	Study settings	Exclusion of renally-impaired patients
De Jager *et al.* (10)	9.9% 28.1%	<150 <220	64	2050	4.3	Outpatient clinics, the Netherlands	Yes
Reinstatler *et al.* (11)	5.8% 22%	≤148 ≤221	63.4	-	5*	NHANES, United States	Yes
Hermann *et al.* (12)	8% 23%	<150 <200	58.2	2200	5.2	Outpatient clinic, Sweden	Yes
Liu *et al.* (13)	29% 52%	<150 <220	79.7	-	-	Geriatric outpatient clinic, Hong Kong	No
Nervo *et al.* (14)	6.9% 43.7%	<125 <250	63.7	2550*	4*	Internal medicine clinic, Brazil	No
Iftikhar *et al.* (15)	31%	<111	56	1740	1.8	Outpatient internal medicine clinic, Pakistan	Yes
Calvo Romero and Ramiro Lozano (18)	8.6%	<146	71.6	1770	3.6	Internal medicine clinic, Spain	No
Kang *et al.* (19)	14.2%	≤222	59.4	1305	6.9	Hospital diabetes center, South Korea	Yes
Beulens *et al.* (20)	28.1%	≤148	61.6	1306	5.3	Primary care center, the Netherlands	No
De Groot-Kamphuis *et al.* (17)	14.1%	<150	62.6	-	4.9*	Outpatient clinic, the Netherlands	No
Singh *et al.* (16)	7.1% 28.5%	<111 <163	53	-	-	Patients referred to department of internal medicine of a tertiary hospital, India	Yes
Ahmed *et al.*(106)	28.1%	<150	58.5	2400	9.6	Outpatient diabetes clinics of two tertiary hospitals, South Africa	Yes

*median values, all units of vitamin B12 levels were converted to pmol/L; NHANES: National Health and Nutrition Examination Survey.

Table 2. Settings, designs and results of studies investigating the impact of metformin-induced low vitamin B12 on peripheral neuropathy in T2DM patients

Study	Setting	Design	Results
Wile and Toth (100)	Neuromuscular clinic at a university hospital, Canada	Case-control study. Cases were T2DM patients on metformin with primary diagnosis of PN. Controls were T2DM patients not taking metformin with primary diagnosis of PN.	Metformin group had more severe PN (assessed by TCSS and NIS). Electrophysiological markers showed no significant difference between the two groups. Cumulative metformin dose showed a significant positive correlation with TCSS scores (rho = 0.80) and NIS scores (rho = 0.79).
Singh *et al.* (16)	Internal medicine clinic in a tertiary hospital, India	Cross-sectional study. Randomly selected T2DM patients were divided into metformin users and non-users.	Metformin group had more severe PN (assessed by TCSS). Cumulative metformin dose revealed a significant positive correlation with TCSS (rho= 0.53).
De Groot-Kamphuis *et al.* (17)	Secondary care outpatient diabetes clinic, the Netherlands	Cross-sectional study. Randomly selected T2DM patients were divided into metformin users and non-users.	Prevalence of neuropathy (obtained from records) was significantly lower in metformin group.
Chen *et al.* (104)	Diabetes clinic of a tertiary hospital, UK	Cross-sectional study. Randomly selected T2DM patients were divided into metformin users and non-users.	All PN-assessing tools (monofilament, neurothesiometry, NTSS-6 and s-LANSS) showed no significant differences between the two groups.
Biemans *et al.* (107)	Four primary care centers, the Netherlands	Cross-sectional study. Metformin-treated T2DM patients were divided into vitamin B12-deficient and normal groups.	There were no significant differences in PN (assessed by MNSI and extracted from records) between the two groups.
Ahmed *et al.* (106)	Diabetes clinics of two tertiary hospitals, South Africa	Cross-sectional study. Metformin-treated T2DM patients were divided into vitamin B12-deficient and normal groups.	There was no difference in presence of PN (assessed by NTSS-6) between the two groups. Levels of vitamin B12 and NTSS-6 scores were not correlated.

MNSI: Michigan Neuropathy Screening Instrument; NIS: Neuropathy Impairment Score; NTSS-6: Neuropathy Total Symptom Score-6; rho: Spearman's rank correlation coefficient; s-LANSS: Self-administered Leeds Assessment of Neuropathic Symptoms and Signs; TCSS: Toronto Clinical Scoring System.