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Status of Vitamin B12 Level Among Type 2 Diabetes Mellitus Patients on Metformin Treatment Attending Diabetic Clinic of The Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia.

BY

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This is to certify that the thesis prepared by Genet Kefeany entitled "**Status of Vitamin B12 Level Among Type 2 Diabetes Mellitus Patients on Metformin Treatment Attending Diabetic Clinic of The Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia**" submitted in partial fulfillment of the requirements of the Degree of Masters of Sciences in Clinical Laboratory Sciences (Clinical chemistry) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Abstract

Background: Metformin is a standard therapy, most commonly prescribed oral anti-hyperglycemic agent for individuals with type-2 diabetes (T2DM). Many studies also documented the association between long-term metformin use and low vitamin B12 levels among individuals with T2DM; however, metformin-induced vitamin B12 deficiency(VBD) prevalence estimates showed large variation among studies. To the best of our knowledge, studies on the association between long term metformin use and low vitamin B12 levels among individuals with T2DM are not found in Ethiopia.

Objective: To determine the status of vitamin B12 level and risk factors associated with its deficiency in patients with type 2 diabetes mellitus on metformin treatment attending diabetes clinic of the Tikur Anbesa Specialized Hospital, Addis Ababa, Ethiopia.

Methods: Institutional based cross-sectional study was conducted from March 12/ 2017 to May 15/2017 at diabetes clinic of TASH. Convenient sampling method was used to include a total of 121 T2DM patients with and without metformin treatment. Blood samples were collected and Vitamin B12 levels were determined by Cobas e411 analyzer by electro chemiluminescence immunoassay. Neuropathy Total Symptom Score-6 questionnaire (NTSS-6 scores) was used to compare severity of Peripheral neuropathy (PN) in both groups. VBD was defined as serum concentration of <200 pg/dl. NTSS-6 scores >6 is considered to have PN. Finally data was entered and analyzed through SPSS version 20 computer software packages.

Results: From the total of 121 study participants serum B12 levels were low(<200pg/ml) in 15(21.1%) T2DM patients with metformin (n=71) as compared to 2 patients (4.0%) without metformin treatment(n=50). Mean B12 level with metformin was found to be 331.58 pg/ml(± 134.48) as compare to those without metformin 482.23pg/ml(± 235.24), the difference was stastically significant with p value <0.001.The factors that interfered with serum B12 levels were duration of diabetes, dose and duration of metformin use. Vitamin B12 levels and NTSS-6 scores were not correlated (Spearman's rho =0.021, P=0.819).

Conclusion: Among patients with T2DM treated with metformin had low serum B12 levels than patients not treated by metformin with significant effect of metformin dose and duration on B12 levels. The deficiency was not associated with peripheral neuropathy.

Keywords: Type 2 Diabetes Mellitus**,** Status, Vitamin B12 deficiency, Metformin, neuropathy

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List of Abbreviation

Ado-Cbl	Adenosyl-Cobalamin
AMP	Adenosine mono phosphate
AMPK	AMP-activated protein kinase
ATP	Adenosine tri phosphate
Cbl	Cobalamin
DM	Diabetes Mellitus
DNA	Deoxyribonucleic acid
EPHIL	Ethiopian public health institute laboratory
Hcy	Homocysteine
IF	Intrinsic factor
MMA	Methylmalonic acid
NTSS-6	Neuropathy Total Symptom Score-6
PN	Peripheral neuropathy
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TASH	Tikur Anbesa Specialized Hospital
VBD	Vitamin B12 deficiency
BMI	Body mass index
FBS	Fasting Blood Sugar

1. Introduction

1.1 Background

1.1.1 Diabetes Mellitus

Diabetes mellitus (DM) is a group of metabolic diseases characterized by elevated blood sugars (hyperglycemia) with impaired metabolism of carbohydrate, fat and protein resulting from defects in insulin secretion, insulin action, or both [1]. DM is classified into different types:- Type 1diabetes mellitus (T1DM), Type 2 diabetes mellitus (T2DM), Other specific types of diabetes and Gestational diabetes mellitus (GDM) [2]. All forms of diabetes mellitus appear to involve disruption of pancreatic beta-cell function. In T2DM (previously called adult-onset or Non insulin dependent diabetes mellitus (NIDDM)), the pancreas is able to produce insulin, nevertheless the produced insulin is either not sufficient or the body is unable to respond to its effects (also known as insulin resistance), leading to a build-up of glucose in the blood. T2DM usually occurs in adults, but is increasingly seen in children and adolescents as well [3]. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels [1].

1.1.2 Metformin

The care for diabetic patients is a complex process and involves managing disease complications as well as drug related side effects in addition to glycemic control. Metformin is generally considered a drug of choice and the most frequently prescribed first line therapy to treat hyperglycemia in individuals with T2DM. In obese patients apart from glycemic control metformin have shown additive effects in controlling the lipids and exhibits cardio-protective features. Thus metformin treatments is important in improving cardiovascular morbidity and mortality, which is a major cause of death in patients with T2DM. European Association for the Study of Diabetes and American Diabetes Association guidelines has recommend metformin in T2DM patient as a first line drug concurrent with life style modification (diet, weight control and physical activity) [4-6]. Metformin is effective anti-hyperglycemic agent and its pharmacologic mechanism of actions is different from other classes of oral anti hyperglycemic agents. For example anti-diabetic agents like insulin and Glibenclamide (class sulfonylurea's) lower blood sugar level by activating tyrosine kinase receptor pathway and by stimulating insulin release,

respectively [7,8]. But metformin acts by inhibiting production of hepatic glucose, decreasing intestinal absorption of glucose, and improving insulin sensitivity and increasing peripheral glucose uptake and utilization [4,9]. However, metformin acts primarily at the liver by reducing glucose output through a mild inhibition of the mitochondrial respiratory-chain complex 1, resulting in reduced ATP levels and accumulation of AMP. Gluconeogenesis is reduced as a result of ATP deficit limiting glucose synthesis, increased AMP levels leading to reduced activity of the key gluconeogenic enzyme Fructose-1,6-bisphosphatase(FBPase), inhibition of adenylate cyclase and cyclic AMP- protein kinase A (cAMP-PKA) signaling, and inhibition of Mitochondrial glycerophosphate dehydrogenase(mGPD) contributing to altered redox state and reduced conversion of glycerol to glucose. Metformin-induced change in AMP/ATP ratio also activates AMP-activated protein kinase (AMPK). The activated AMPK decreases flux of free fatty acids and inhibits lipolysis, which may indirectly improve insulin sensitivity through reduced lipotoxicity (reduces hepatic lipogenesis) and exert an indirect effect on hepatic insulin sensitivity to control hepatic glucose output (Figure 1) [10-13].

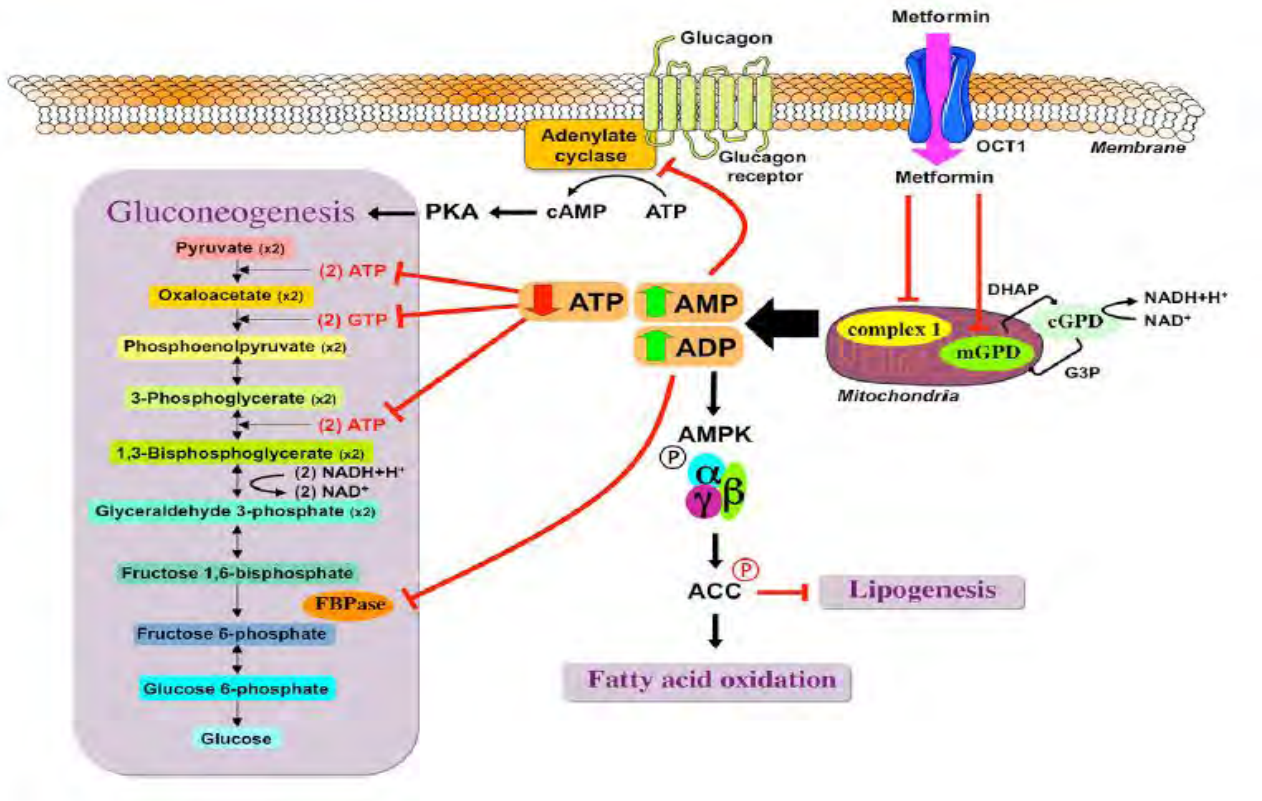

Figure 1. Molecular Mechanisms of Metformin-Induced Inhibition of Hepatic Glucose Output

Source; Cell Metabolism 2014 Elsevier Inc.

Drug side effects of metformin are mild and usually include gastrointestinal symptoms, such as abdominal distress, soft stools, and diarrhea. These side effects appear shortly after the initiation of metformin and promptly disappear after discontinuation [14,15]. However, it may also have side effects with potential adverse health outcome associated with its use such as vitamin B12 deficiency [15].

1.1.3 Vitamin B12

Vitamin B12, also called cobalamin (a tetra pyrrole structure that chelates an atom of cobalt in the center of the structure), is a water-soluble vitamin that plays a crucial role in many chemical reactions and affects many mechanisms in the body. It is involved in the formation of red blood cells, nucleic acid metabolism, transference of methyl groups, synthesis and repair of myelin sheaths [16,17]. Vitamin B12 cannot be synthesized in the body and must be obtained from main dietary sources of B12 such as dairy products, meat (especially liver) and eggs. Individuals consume about 2.4 µg vitamin B12 per day, of which only 50-60% is absorbed [18].

The acidic environment of the stomach enables the release of B12 that is bound to food. Free vitamin B12 then binds to a protein called haptocorrin. In the duodenum, vitamin B12 is released from haptocorrin by pancreatic proteases and then binds to intrinsic factor (IF), a glycoprotein secreted by the gastric parietal cells. Absorption of vitamin B12 occurs in the terminal ileum and is aided by binding the complex to the IF receptor on the mucosal surface. Once absorbed, majority of vitamin B12 (70-80%) is bound to haptocorrin (named as holo-haptocorrin) and is not biologically active. Only less than 30% of the B12 is bound to trascobalamin II (named as holo-transcobalamin (HoloTC)) which is the active fraction that enters cells for metabolic reactions. The interruption of one or any combination of these steps places a person at risk of developing VBD. VBD can lead to a variety of disorders, including anemia, neuropathy, memory impairment, changes in the function of the spinal cord, dementia, depression, and even brain atrophy [18-21].

The exact mechanism of vitamin B12 deficiency due to metformin is not known, however, studies showed that high prevalence of VBD in T2DM patients on metformin treatment. Usually, the established clinical benefits of metformin use in patients with T2DM make its side effects to be unnoticed and VBD, may not be easily detected without close attention [15]. The present study was planned to find out the serum vitamin B12 status in T2DM patients on metformin treatment, to investigate the relationship between vitamin B12 and peripheral neuropathy and to identify the risk factors for the vitamin deficiency in these patients.

1.2 Statement of the problem

The incidence and prevalence of diabetes is increasing globally, in 2015, an estimated 415 million adults were living with diabetes. This number is expected to rise to 642 million by 2040[22]. T2DM accounts for about 90% to 95% of all diagnosed cases of diabetes in adults. It is increasingly becoming a pandemic in developed and developing worlds [23,24]. In 2010, 285 million people, representing about 6% of the world's adult population, were T2DM patients and this number is expected to reach 439 million by 2030 [25,26]. According to IDF estimates, DM prevalence in Africa was 3.2% and 2.9% in Ethiopia [22]. Local study conducted in 2012 in Ethiopia, Addis Ababa found 6.5 % prevalence of DM[27]. This figure was higher than prevalence reported by IDF. 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 [28].

Metformin is the most prescribed anti-diabetic drug in patients with T2DM [4]. An estimated of 48 million adults have VBD [29] and Many patients on metformin develop VBD, consequently developing neuropathy and anaemia which is falsely attributed to underlying DM by physicians and so never addressed [30]. However, the established clinical benefits of metformin use in patients with T2DM make its side effects to be overlooked and no routine screening for it [15]. The prevalence of VBD among metformin-treated patients has shown great variation among the studies and ranged between 5.8% and 36% [31-34]. To the best of our knowledge, studies on the association between long term metformin use and low vitamin B12 levels among individuals with T2DM are scarcely found in Ethiopia.

Neuropathy is one of the most common and debilitating long term complications of diabetes affecting up to 50% of patients, which is a major cause of morbidity and associated with increased mortality [35-38]. The major potential health problem from VBD is neuropathy, and the symptoms of diabetic neuropathy overlap with the paresthesias, impaired vibration sense, and impaired proprioception associated with VBD as a result, VBD –induced nerve damage may be confused with or contribute to diabetic PN, which can lead to unnecessary drug use and misdiagnosis as other demyelinating nerve diseases [39-41]. And also potential of VBD to cause or worsen PN in T2DM patients has been investigated with conflicting results. Therefore , this study was designed to evaluate the serum levels of vitamin B12, to identify risk factors for VBD and to identify whether VBD associated with neuropathy among T2DM patients on metformin treatment.

1.3 Significance of the study

As far as the investigators' knowledge goes, researches conducted on the Status of Vitamin B12 deficiency in type 2 diabetes mellitus patients on metformin treatment on Ethiopian patients, are not available. Thus, findings of the present study may indicate possible vitamin B12 deficiency that follows long term therapy of metformin treatment, and also may help to determine whether physicians should consider screening for B12 deficiency in T2DM patients on metformin therapy that may help in improving the patients health.

Furthermore, the risk factors identified have implications for planning to screen as well as prepare prevention strategies in metformin-treated patients and identifying the correct cause or contributing factors for the peripheral neuropathy is crucial because simple vitamin B12 replacement may reverse neurologic symptoms inappropriately attributed to hyperglycemia.

The result of this study will help the Policy makers /Administers as one input to establish a system for screening serum vitamin B12 level in T2DM patients on metformin treatment and formulation of guidelines regarding vitamin B12 monitoring and supplementation in such patients.

Moreover, this study may help to establish baseline information for other similar researches, conducted in the future. It will also help the clinician to manage type 2 diabetic patients optimally.

2. Literature Review

2.1 Vitamin B12 deficiency

Vitamin deficiencies are common problems worldwide, and of them vitamin B12 deficiency being recognized as a health concern nearly 100 years ago. The definition of VBD varies with the assay used [42]. The complex and multistep nature of vitamin B12 absorption in the gastrointestinal track (GIT) increases the possibility of malabsorption, by which VBD can develop. Causes of VBD include: Insufficient dietary intake, Gastric abnormalities, Small bowel disease, Pancreatic insufficiency and Medications [43,44].

Methylcobalamin and deoxyadenosylcobalamin are the two active form of cobalamin (Cbl) found in the body and both are essential cofactors for two enzymatic pathways of intracellular metabolism. The methyl form attaches to methionine synthase and the adenosyl form attaches to methymalonyl CoA mutase. Cbl removes a methyl group from methyl-tetrahydrofolate to regenerate tetrahydrofolate and forming methylcobalamin. Homocysteine (Hcy) accepts a methyl group from methylcobalamin, resulting in the formation of methionine by the enzyme methionine synthase (Figure 2). This pathway is closely linked to the generation of thymidine which is vital for deoxyribonucleic acid(DNA) synthesis. A deficiency in vitamin B12 can lead to increased Hcy (hyperhomocysteinemia) levels in plasma, which has been shown to have potentially toxic effects on neurons and the vascular endothelium. In addition, VBD can result in disruption of DNA synthesis caused by thymidine lack and resulting in megaloblastic anaemia, as well as other adverse effects on the nervous system and other organs [44, 45].

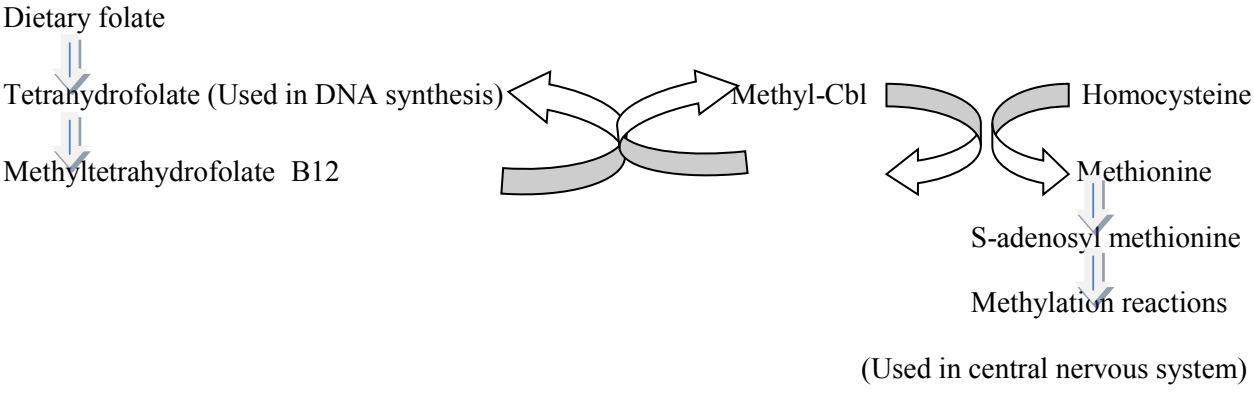

Figure 2. Synthesis of methionine from homocysteine (methionine synthetase)

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 methylmalonyl-mutase enzyme and involves the conversion of methylmalonyl-CoA to the Krebs cycle intermediate succinyl-CoA (Figure 3). VBD thus results in elevated methylmalonic acid(MMA) levels followed by defective fatty acid synthesis of the neuronal membranes [46,47].

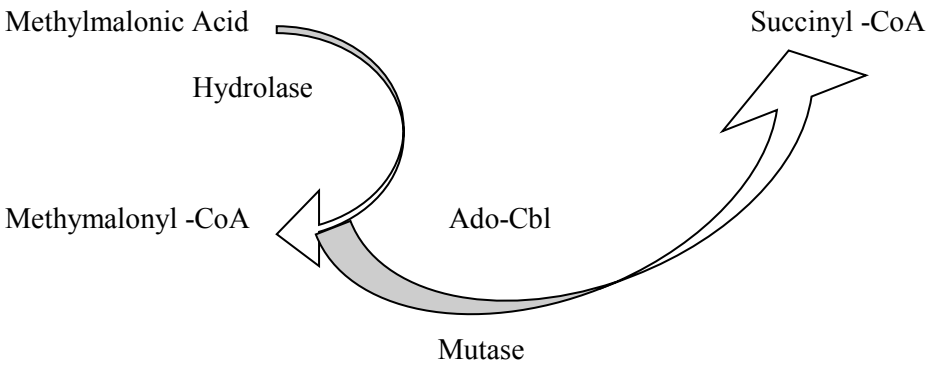

Figure 3. Conversation of methymalonyl-coenzyme A(CoA) to succinyl-CoA.

2.2 Mechanism of metformin-induced malabsorption of vitamin B12

Vitamin B12 malabsorption associated with metformin were described in 1971[48] . Since then many observational studies and randomized trials have been shown that metformin significantly lower vitamin B12 levels in T2DM patients [49-52]. The mechanism through which metformin induce VBD in patients with T2DM is presently unclear. The proposed mechanism include alteration in small bowel motility, which stimulate bacterial overgrowth and consequential vitamin B12 deficiency, competitive inhibition or inactivation of vitamin B12 absorption, alteration in IF levels and interaction with the cubulin endocytic receptor and Inhibition of the calcium dependent absorption of vitamin B12–IF complex at the terminal ileum has been the most suggested mechanisms [9, 53-55]. A study conducted in 2003 have shown that metformin modulation of calcium-dependent membrane channels can reduce vitamin B12 levels. Because absorption of B12-IF complex uptake by ileal cell membrane receptors is calcium-dependent, and metformin affects calcium dependent membrane action, thereby resulting in B12 deficiency [9]. This study support the theory of ability of biguanides to give a positive charge to the membrane's surface and role of calcium in the binding of IF vitamin B12 complex to ileal

receptors and also to introduce the theory of the mechanism by which metformin inhibits vitamin B12 absorption. The hydrophobic tail of biguanides such as metformin, extends into the hydrocarbon core of membranes. The protonated biguanide group gives a positive charge to the surface of the membrane, which displaces divalent cations (figure 4). Thus, the biguanides alter membrane potentials and affect their calcium-dependent binding of IF-vitamin B12 complex to the ileal cubilin receptor and malabsorption of the vitamin ensues [56].

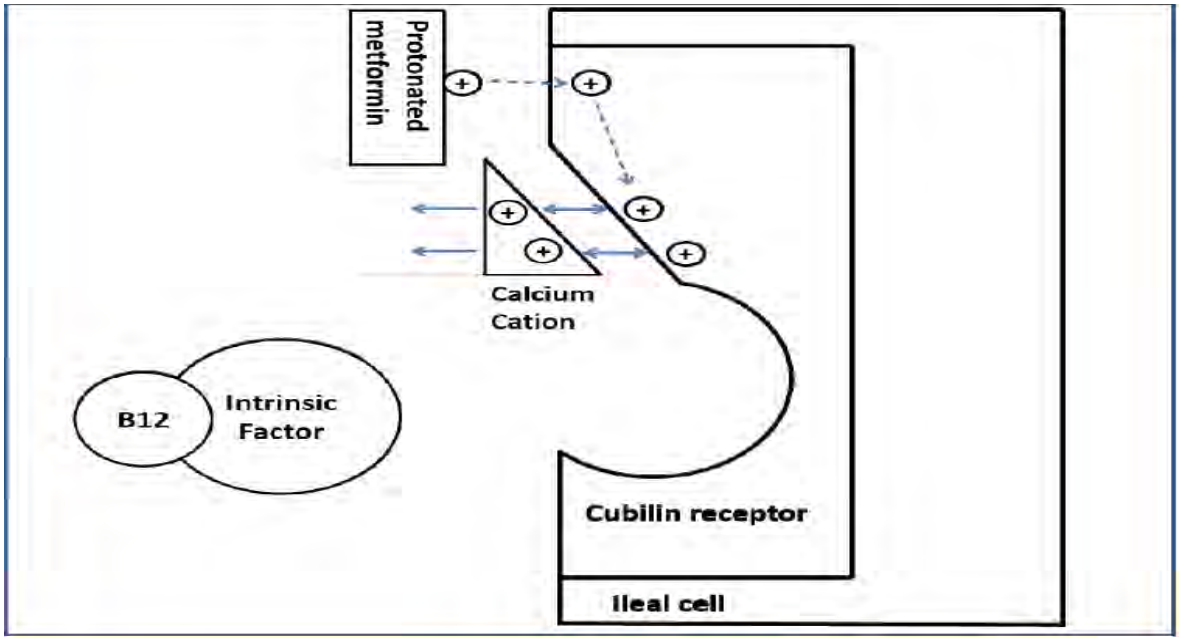

Figure 4. Mechanism of inhibition of vitamin B12 absorption by metformin. *Source:* J Pharm Pharm Sci (www.cspsCanada.org) 19(3) 382 - 398, 2016

2.3 Metformin associated vitamin B12 deficiency

Several studies and case reports have been documented an increased frequency of vitamin B12 deficiency among T2DM, due to metformin treatment. National Health and Nutrition Examination survey(NHANS) conducted during 1999-2006, showed that metformin therapy is associated with a higher prevalence of biochemical B12 deficiency. From those diabetic patients using metformin 5.8% were vitamin B12 deficient compared with 2.4% of those not using metformin and 3.3% of those without diabetes. Consumption of any supplement containing B12 showed that a two-thirds reduction among those without diabetes but no reduction among those with T2DM. The amount of B12 recommended by the Institute of Medicine (2.4 mg/day) and the

amount available in general multivitamins (6 mg) may not be enough to correct this deficiency among those with diabetes [31].

Increased risk of vitamin B 12 deficiency associated with current dose and duration of metformin use, despite adjustment for many potential confounders have been studied, a study conducted in Hong Kong (Ting RZ etal in 2006), showed that increased risk of VBD associated with current dose and duration of metformin. Each gram metformin dose increment conferred an odds ratio of 2.88. Among those using metformin for 3years or more, the adjusted odds ratio was 2.39 compared with those receiving metformin for less than 3years [57]. Similarly, a case control study from Pakistan (Raheel Iftikhar etal in 2013), demonstrated significantly high prevalence of VBD (31%) in patients treated with metformin as compared to 8.6% among controls with significant effect of dose and duration of metformin use on B12 levels [58]. Also a cross-sectional study conducted in 2006 found a 22% prevalence of metabolically confirmed B12 deficiency in the primary care type 2 diabetic population [32].

Another cross-sectional study was conducted at a public university hospital in Brazil (Damião PC etal, in 2015), showed that B12 deficiency was more frequent in the T2DM-met group (22.5%) as compared to control groups not using metformin (7.4%). The conclusion from this study was among T2DM patients, treatment with metformin and concomitant use of PPI/H2-antagonists are associated with a higher chance of developing B12 deficiency than among non-diabetics [59].

A case-control type of analytical study conducted in India, (Priti Agarwal etal in 2016) including a total of 50 patients with metformin use and 50 control groups demonstrated metformin significantly associated with decrease in vitamin B12, with mean serum vitamin B12 levels in the study group of 431.84±265.76 and 744.76±271.927 in the controls[60].

A cross-sectional study in Ireland (Omar Marar etal in 2011) was conducted in a total of 216 T2DM Irish patients on metformin and 70 T2DM patients not on metformin. The study showed that high prevalence of VBD (33%) in patients on metformin treatment compared to only 7.5% in the control group. They also demonstrated that an inverse relationship exists between vitamin B12 levels and the dose and duration of metformin use. Therefore, they suggest that the measurement of vitamin B12 should become an essential part of the annual review in all patients with T2DM on metformin therapy [33].

Another case control study compared the prevalence of VBD in T2DM aged between 30-96 years. VBD was recorded in 36% of T2DM patients on metformin and most of them were females [34].

K.S. Akinlade etal in 2015 conducted a cross-sectional study in Nigeria to determine the serum level of vitamin B12 in Nigerian patients with T2DM on metformin. And showed that low serum vitamin B12 level is associated with longer duration and higher dose of metformin use. From their findings VBD and borderline deficiency were recorded in 8.6% and 26.0% of the patients respectively. Vitamin B12 level was significantly lower in patients who have been on metformin for>10 years compared with patients with <10 years history of metformin use. Similarly, patients who were on metformin at a dose of >1000 mg/day had significantly lower vitamin B12 level when compared with patients on<1000 mg/day [61].

In contrast, another study (Norbert Shtaynberg etal in 2012, Island) showed that no difference in the prevalence of vitamin B12 deficiency in metformin users compared with non-users. There were 47 T2DM patients taking metformin treatment and 41 control subjects not taking metformin. The result showed that vitamin B12 deficiency between groups taking metformin vs. not taking were 17.1% and 12.8%, respectively. And based on their daily dosage, rate of deficiency were 14.8%(for 1000mg or less) and 10.0% (for more than 1000mg), which was not significantly different [62].

2.4 Metformin-induced low vitamin-B12 and Peripheral neuropathy

Both diabetes mellitus and Vitamin B12 deficiency are associated with development of peripheral neuropathy. PN by DM and vitamin B12 deficiency may produce overlapping clinical pictures. A prospective case-control study conducted during 2002-07 Compared patients with T2DM on metformin treatment (n= 59) and those without metformin exposure (n=63) with concurrent symptomatic PN. The study showed that metformin-treated patients had depressed Cbl levels,-elevated fasting MMA and Hcy levels and also more severe peripheral neuropathy The investigators suggested that Interval screening for Cbl deficiency upon initiation as well as during metformin therapy to detect potential secondary causes of worsening peripheral neuropathy and systemic Cbl therapy should be considered when indicated[30].

Contrarily, Observational cross-sectional study conducted in Bangladesh (Kamrul-Hasan ABM etal in 2016), encompassing 50 newly diagnosed T2DM and 50 controls and showed that vitamin B12 level was statistically similar in patients with or without clinically evident PN in newly diagnosed Bangladeshi T2DM patients [63].

Another study conducted in Egypt (Hassna Osama etal, 2016) intended to screen vitamin B12 and homocysteine levels in patients with T2DM on metformin therapy. Their study demonstrated that the prevalence of vitamin B12 deficiency of all patients with T2DM in study was 28.4% (25/88); 20 (33.3%) patients of them were patients taking metformin. Also there was a significant correlation between metformin dose and vitamin B12 levels,also between VBD and hyperhomocysteinemia. There was significant relation between homocysteine and macrovascular complications and between vitamin B12 and PN [64].

Similarly, another cross-sectional study in South Africa (Marwan A. Ahmed etal in 2013,) was conducted to investigate the prevalence of vitamin B12 deficiency in T2DM patients on metformin; the association between vitamin B12 and peripheral neuropathy. Among 121 participants, the prevalence of vitamin B12 deficiency was 28.1 % but does not appear to be any significant effect on PN in those receiving metformin [65].

Literature search shows that paucity of studies on the status of vitamin B12 deficiency in patients with T2DM on metformin therapy in Ethiopian population. Published Studies have documented the rate of prevalence of metformin-induced VBD in T2DM patients to be widely variable depending on the study population. And also the potential causation or worsen of peripheral neuropathy (PN) by VBD in T2DM patients has been investigated with conflicting results. Therefore an assessment of Vitamin B12 levels in the Ethiopian metformin-treated diabetic population will cast a light on the status of VBD and factors that may be contributing to the development of the deficiency.

3. Objective

3.1 General Objective

- To determine the status of vitamin B12 level and risk factors associated with its deficiency in patients with type 2 diabetes mellitus on metformin treatment attending diabetes clinic of the Tikur Anbesa Specialized Hospital, Addis Ababa, Ethiopia.

3.2 Specific Objective

The specific objectives of this research are to:

- ✓ Determine the status of vitamin B12 level in patients with type 2 diabetes mellitus
- ✓ Assess the association between vitamin B12 deficiency and Metformin dose
- ✓ Assess the association between vitamin B12 deficiency and Metformin duration of treatment
- ✓ Determine the burden of peripheral neuropathy among patients with vitamin B12 deficiency.

4. Materials and Methods

4.1 Study Area

This study was conducted at Tikur Anbessa Specialized Hospital which largest referral public and University Hospitals in the country, located in the nation's capital Addis Ababa, Ethiopia. TASH is selected by purposive sampling method. The hospital provides a tertiary level referral treatment and open for 24 hours for emergency services. TASH offers diagnosis and treatment for approximately 370,000- 400,000 patients a year [66]. There are different units and clinics that provide specialized services for their clients. Among these is found the diabetes & endocrinology referral clinics. In these diabetes unit approximately 150 type 2 diabetic patients seen weekly.

4.2 Study Design and period

Institutional based cross- sectional study was conducted from March 12/2017 to May 15/2017.

4.3. Population

4.3 1. Source Population

All Patients with diabetes mellitus having follow up at diabetic clinic of TASH during the study period.

4.3.2 Study population

T2DM patients with and without metformin treatment who visit diabetic clinic during the study period and only selected ones who meet study criteria.

4.4 Inclusion and Exclusion criteria

4.4.1 Inclusion criteria

- **For metformin group:** A diagnosis of T2DM and have been using metformin.
- **For non metformin group:** A diagnosis of T2DM and no history of metformin use.
- For both groups:-
    - ✓ Base line record data
    - ✓ Aged 40 years and more
    - ✓ Provide consent to participate in the study

4.4.2 Exclusion criteria

Patients who have a history of partial or total gastrectomy, a diagnosis of pernicious anaemia, T1DM, patients on histamine 2 receptor blocker , pregnant or lactating women, liver disease and those who use B12 supplementation were excluded.

4.5 Study Variables

4.5.1 Independent variable

- Age
- Sex
- Dose of metformin treatment
- Duration of metformin treatment
- Body mass index (BMI)
- Duration of T2DM, Peripheral neuropathy

4.5.2 Dependent (outcome) variables

- Vitamin B12 deficiency

4.6 Sample size Determination and Sampling Strategy

4.6.1 Sample Size Determination

The sample size is calculated based on single sample size estimation. The value of p is taken considering 95% confidence interval, 5% margin of error and; the value of p taken was 8.6% from the previous study conducted in Nigeria (2015) on vitamin B12 levels in patients with T2DM on metformin [61]. The calculated sample size using the following formula is 121 T2DM on metformin treatment.

The sample size $n = z(\alpha 2)^2 p(1-p)/d^2$

Where n = Sample size

z (α2)2 =At 95% confidence interval Z value (α = 0.05) = 1.96

p = Proportion of occurrence of the event to be studied

d= Margin of error at (5%) (0.05)

$n = (1.96)^2 .0.086(1- 0.086)/(0.05)^2$,

n=121

4.6.2 Sampling procedures

Convenient sampling technique was employed to include study participants who meet the inclusion criteria to address the stated objectives during the study period.

4.7 Operational Definitions

- Vitamin B12 deficiency:- Serum concentration of <200 pg/ml.
- Neuropathy Total Symptom Score-6 (NTSS-6) is a questionnaire which will be used to grade peripheral neuropathy. Those with NTSS-6 scores >6 is considered to have peripheral neuropathy.

4.8. Data collection procedures

4.8.1. Demographic data

Demographic characteristics and exposure to risk factors

Data collectors or nurses were identified, trained and informed to collect the data as per the pre-structured questionnaire. During a regular scheduled visit day from the outpatient diabetic clinic of TASH, in total 121 T2DM : 71 with metformin and 50 without metformin were selected through convenient sampling according to inclusion and exclusion criteria. Before collecting data, the purpose of the study was explained to the participants accordingly, written informed consent was collected from each eligible study participants. Characteristics of patients including age, gender, cigarette smoking, alcohol consumption, Fasting blood sugar(FBS), BMI, T2DM duration, metformin use (duration and dose) and others were collected from patients' medical records and questionnaires. (Annex III).

4.8.2 Laboratory analysis

- **Blood Sample collection and Storage**

During the study, fasting blood samples were collected from each participant using aseptic measures. Following collection, specimens was transported carefully to TASH clinical chemistry laboratory within 30 minutes, centrifuged and separated serum stored in refrigerator at -20^{o}c.

❖ **Transportation of specimens**

Collected serum sample was transported carefully to Ethiopian public health institute (EPHI) clinical chemistry laboratory using cooler boxes with ice packs as temporary storage. The serum sample was stored in deep freezer at -80^{o}c until serum vitamin B12 analysis performed.

❖ **Sample analysis**

Vitamin B12 levels was measured by Cobas e401 systems analyzer (Roche Diagnostics) which is a fully automatic run-oriented analyzer system for the determination of immunological tests using the electrochemiluminescence immunoassay "ECLIA" process. All components and reagents for routine analysis are integrated in or on the analyzer. The Elecsys vitamin B12 assay employs a competitive test principle using intrinsic factor specific for vitamin B12. The results were-verified by a laboratory technologist. Serum levels of vitamin B12 more than 200pg/ml was considered to be normal.

❖ **Vitamin B12 determination**

Method

❖ Electro chemiluminescence immunoassay method

Reaction Principle (Competition principle)

- Vitamin B12 + Ruthenium labeled intrinsic factor + Biotin labeled Vitamin B12 → Ruthenium labeled intrinsic factor Vitamin B12 biotin complex
- Vitamin B12 in the sample competes with the added vitamin B12 labeled with biotin for the binding sites on the ruthenium-labelled intrinsic factor complex.

Reagent used:- pretreatment reagents (PT1 and PT2), Streptavidin-coated microparticles (M), Intrinsic factor(R1) and Vitamin B12 -biotin(R2)

Quality control solutions:- PreciControl Varia(1 and 2) solution was used for quality control. (Annex VI)

❖ **Neuropathy Total Symptom Score-6**

PN was assessed by NTSS-6 questionnaire. The examiner interviews each study participant and completes the questionnaire. The frequency and intensity of symptoms were graded based on Bastyr et al. definition [72].

4.9. Data Quality Assurance

Data quality was ensured through standardized data collection materials, questionnaires were thoroughly checked for completeness and consistency by study advisors and principal investigator. For laboratory analysis to evaluate and monitor the quality of the test and to maintain high standard of accuracy of the result, Pre-analytical, analytical and post analytical stages of quality assurance that is incorporated in Standard operating procedures (SOPs) of the EPHI clinical chemistry laboratory was strictly followed. In addition, well trained and experienced laboratory professionals participated in the laboratory analysis procedure.

4.9.1. Pre-Analytical

To collect the demographic data, the questioner were carefully prepared by investigator, and it was translated to local language Amharic to make sure that the participants can understand the questions at the time of interview. Before the actual data collection, Proper training was given to the data collectors prior to Pretesting the questionnaires, and intensive supervision was made by principal investigator during data collection.

Concerning sample collection, blood sample was collected using aseptic measures from each patients by seiner laboratory professionals and principal investigator to minimize discomfort of the patients and to improve the quality of sample. In addition, standard Operating procedure (SOP) were carefully followed during blood collection.

4.9.2 Analytical

EPHI clinical chemistry laboratory, Standard operating procedures (SOPs) prepared for serum vitamin B12 were strictly followed carefully by the laboratory technologist and principal investigator to perform the vitamin B12 analysis. Before analysis started, Instrument was calibrated and two level control materials were run and the results were evaluated using Levey-jenning (LJ) chart.

4.9.3 Post-Analytical

The result obtained was collected by data collection format that can be understand and correctly interpreted by the laboratory technologist and principal investigator.

4.10. Data Management and Analysis

All laboratory and clinical data was entered in computer software for statistical calculation in SPSS version 20. Descriptive statistics was applied to summarize the data. Mean and Standard deviation (±SD) was calculated for age, years with diabetes, dosage of metformin, duration of metformin intake, FBS and serum vitamin B12 levels. Frequency and percentages were calculated for qualitative variables i.e. gender, vitamin b12 deficiency. Associations between B12 deficiency and categorical variables was determined using the chi-square test. Mann-Whitney U test was applied for data which was skewed. The Spearman rank correlation coefficient (rs) was used to evaluate the correlation between numerical variables. multivariate analysis using logistic regression was performed to identify factors independently associated with B12 deficiency. In all cases P-value less than 0.05 was considered as statistically significant. Finally, the results were presented using figures, and tables.

4.11. Ethical consideration

Before running the study, ethical clearance was sought and obtained from the Departmental Research and Ethics Review Committee of Addis Ababa University, College of Health Sciences, School of Allied Health Sciences, Department of Laboratory Sciences. Moreover, permission from DM clinic of TASH was granted before starting this project. Written informed consent was collected from each study participant before joining the study. The information gathered from each participant kept confidential. Only intended volume of blood samples for the study was drawn and after analysis the left-over is discarded. The study results shall be disseminated to health care providers to aid in patient care.

5. Result of the Study

5.1 Patient characteristics

A total of 121 subjects were included from March 2017 to May 2017, Among these 71 T2DM patients who were on metformin therapy (41 males , 30 females) and 50 patients (24 males , 26 females) without metformin therapy. Table 1 shows T2DM patients demography and clinical characteristics. Out of the total, 99 (81.8%) patients were resident of Addis Ababa while 22(18.2%) patients were from outside Addis Ababa.

Table 1: Demographic data and clinical characteristics of T2DM patients attending diabetes clinic of the TASH, Addis Ababa, Ethiopia, 2017

Characteristics	**Metformin group (n=71)**	**Non metformin group (n=50)**	**P-value**
Age(Year, mean ±SD)	59.31 ± 9.7	59.00 ± 9.1	0.850
Male n(%)	41 (42.3%)	24(48.0%)	
Female n(%)	30 (57.7%)	26(52.0%)	0.290
Alcohol Consumption n(%)	14(19.7%)	8(16.0%)	0.602
History of CVD n(%)	4(5.6%)	6(12.0%)	0.210
History of hypertension n(%)	37(52.1%)	26(52.0%)	0.990
Duration of DM (Year, mean ±SD)	12.11± 5.874	14.80 ± 9.91	0.211
Daily dose of MTF(mg/day)	1508.45 ± 515.7	-	-
Duration of MTF use (Year, mean ±SD)	6.61 ± 4.32	-	-
BMI (Kg/m2)	27.28 ± 4.02	27.20 ± 3.61	0.887
NTSS-6 (median)	5.0	3.3	0.398
FBS (mg/dl **,**median**)**	160.0	138.5	0.016*

The data are shown as the median, means ± SD or n (%), * P value <0.05 SD-Standard Deviation, BMI-Body-Mass Index, NTSS-6-Neuropathy Total Symptom Score, FBS- Fasting Blood Sugar, MTF-Metformin, DM- Diabetes mellitus, CVD-Cardio- Vascular Disease.

Mean age among metformin group was 59.31(±9.70)years, while it was 59.10±(9.10) in those without metformin therapy, which was statistically non-significant. Similarly, BMI among patients on metformin group and without metformin were not stastically significant with a mean of 27.28 (±4.02) verses 27.20 (±3.61), respectively. Fasting plasma glucose concentration (mg/dl) in the metformin group (median=160) was statically higher than those without metformin treatment(median=138), (U=1336, p=0.016). The mean duration of diabetes in the metformin treatment group was 12.11(±5.87)years while in those without metformin was 14.80(±9.91)years. The difference between the two groups was not stastistically significant with p value 0.211. The mean of metformin treatment duration and total daily dose were 6.61(± 4.32) years and 1508.45(±515.7)mg, respectively. The treatment modality of the study participants were 38(31.4%) Insulin alone, 28(23.1%), Metformin plus Glibenclamide, 23(19.0%) Metformin alone, 20(16.5%) Metformin plus Insulin, 6(5.0%) Glibenclamide alone and 6(5.0%) on diet.

5.2 Vitamin B12 Status

The mean serum vitamin B12 level among patients taking metformin were found to be 331.58 pg/ml(± 134.48) as compared to those without metformin with a mean 482.23pg/ml(± 235.24), the difference between the two groups was statistically significant with p value <0.001(Figure5). Fifteen (21.1%) patients on metformin had a vitamin B12 deficiency compared to only two (4.0%) without metformin treatment (Table 2).

Table 2: Status of Vitamin B12 level between T2DM patients with and without metformin treatment attending diabetes clinic of the TASH, Addis Ababa, Ethiopia, 2017

T2DM Patients	Vitamin B12 level(pg/ml)		P-value
	<200pg/ml (Deficient)	≥200pg/ml (Normal)	
T2DM with metformin n(%)	15 (21.1%)	56(78.9%)	0.008*
T2DM without metformin n(%)	2(4.0%)	48(96.0%)	

The data are shown as n(%), *P value <0.05, T2DM- Type 2 Diabetes Mellitus

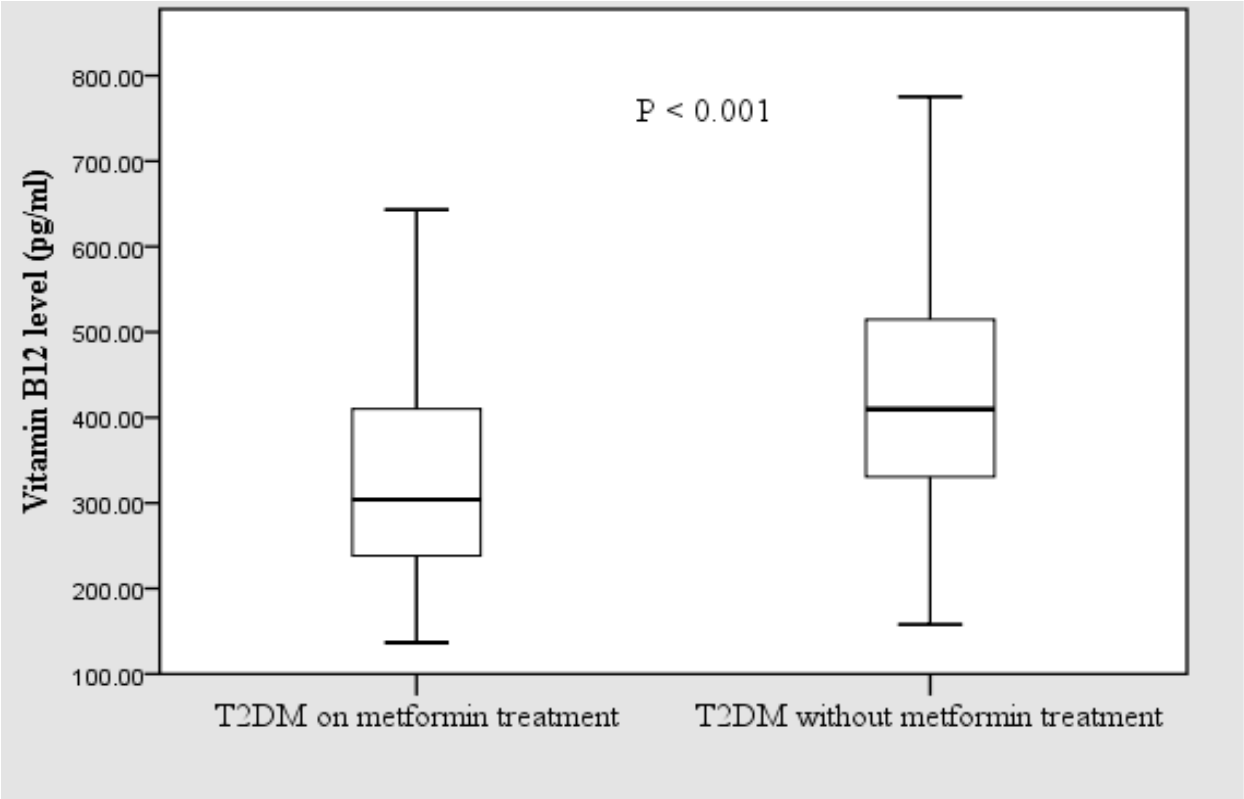

Figure 5. Vitamin B12 serum levels in diabetic patients on metformin and without metformin treatment attending diabetes clinic of the TASH Addis Ababa Ethiopia, 2017.

5.3 Factors associated with vitamin B12 deficiency

Table 3 shows key comparisons of variables between patients with and without B12 deficiency among T2DM patients with metformin treatment. Among patients on metformin with and without vitamin B12 deficiency, there was no significant difference in relation to age, weight, BMI, Neuropathy and NTSS-6 score. Even though, mean dose of metformin among B12 deficient (<200pg/ml) patient was 1793mg (± 356) compare to patients with normal B12 levels (≥200pg/ml) where the mean dose was 1432mg(± 527) and the difference was found to be statistically significant (U=257, p=0.025). Likewise, duration of metformin therapy was also found to influence vitamin B12 levels and mean duration of metformin treatment among B12 deficient (<200pg/ml) patient was 12.3(± 4.0) years while in patients with normal B12 levels (≥200pg/ml) the duration of therapy with metformin was 5.1(± 3.0) years and the difference was found to be statistically significant (U=60, p <0.001).

Table 3: Demographic characteristics and vitamin B12 level among T2DM patients on metformin attending diabetes clinic of the TASH, Addis Ababa, Ethiopia, 2017

Variables	Vitamin B12 levels (pg/ml)		P value
	Deficient <200pg/ml	**Normal >200pg/ml**	
Number of patients N (%)	15(21.1%)	56 (78.9%)	0.051
Male n(%)	8(19.5%)	33(80.5%)	0.697
Female n(%)	7(23.3%)	23(76.7%)	
Age (Year, mean, SD)	63.5(±7.5)	58.2(±10.0)	0.076
BMI (Kg/m2, mean, SD)	28.28(±5.24)	27.50(±3.63)	0.278
Dose of metformin (mg/day, mean, SD)	1793.33(±357.5)	1432.14(±527.1)	0.008*
Duration of metformin use (Year, mean, SD)	12.27(±3.95)	5.09(±2.94)	<0.001*
Duration of diabetes (year ,mean, SD)	16.80(±5.89)	10.86(±5.24)	0.001*
NTSS-6 score n(%)			
≤6 (neuropathy absent)	9(23.1%)	30(76.9)	0.657
>6 (neuropathy present)	6(18.8%)	26(81.2%)	

The data are shown as the median, means ± SD or n (%), * P value <0.05 SD-Standard Deviation, BMI-Body-Mass Index, NTSS-6-Neuropathy Total Symptom Score, FBS- Fasting Blood Sugar.

We also correlated effects of duration of diabetes, metformin dose and treatment duration with vitamin B12 level among T2DM patients with metformin treatment and a significant negative correlation existed between the vitamin B12 level and duration of diabetes (r=-0.391, p= 0.001) by using Spearman's correlation coefficient, stating that as duration of diabetes increased the Vitamin B12 levels decreased and vice-versa. Likewise, vitamin B12 levels had a negative correlation with duration of metformin use (r= -0.608, P < 0.000) and daily dose of metformin (r= -0.268, P < 0.024).

When daily dose of metformin was classified as ≤ 1000mg/day or >1000mg/day, the prevalence of vitamin B12 deficiency was higher in the T2DM patients on metformin dose of>1000 mg/day (32.5% versus 6.5%; P = 0.008). Similarly, duration of metformin use was categorized as ≤5years or >5 years, the prevalence of vitamin B12 deficiency was higher in the T2DM-metformin group with>5 years of metformin use (38.9% versus 2.9%; P< 0.001).

Table 4: Dose and duration of Metformin versus Vitamin B12 level of T2DM patients on metformin attending diabetes clinic of the TASH, Addis Ababa, Ethiopia, 2017

Daily dose of MTF(mg/day)	**Vitamin B12 level (pg/ml)**		**P-value**
	Deficient (<200pg/ml)	**Normal(≥200pg/ml)**	
≤ 1000mg/day n(%)	2(6.5%)	29(93.5%)	0.008*
> 1000mg/day n(%)	13(32.5%)	27(67.5%)	
Duration of MTF (Years)			
≤ 5 years n(%)	1(2.9%)	34(97.1%)	<0.001*
>5 years n(%)	14(38.9%)	22(61.1%)	

The data are shown as n (%), * P value <0.05, MTF-Metformin

Multiple logistic regression was used to assess the risk factors for vitamin B12 deficiency in T2DM patients with metformin treatment (Table 5). The factors that significantly interfered with serum B12 levels after multiple regressions were duration of diabetes, dose and duration of metformin. Each mg increase in metformin dose increases odds for vitamin B12 deficiency by 1.004 times with significant p value (95 % CI: 1.000 to 1.007, P=0.023). Likewise, duration of metformin use increases odds for B12 deficiency by 1.865 times(95% CI: 1.265-2.749, p= 0.002). Duration of diabetes was also associated with vitamin B12 deficiency (OR=1.306, 95 % CI: 1.008 to 1.691, P=0.043).

Table 5: Risk factors for vitamin B12 deficiency analyzed with multiple logistic regression among T2DM patients on metformin attending diabetes clinic of the TASH, Addis Ababa, Ethiopia, 2017. OR >1 Indicates greater risk for VBD

Variables	**Odds ratio (95% CI)**	**p-value**
Age	1.036(0.893 to 1.203)	.637
Duration DM	1.306(1.008 to1.691)	.043*
DDMTF	1.004(1.000 to 1.007)	.023*
DMTF	1.865(1.265 to 2.749)	.002*

*P value <0.05, DM:- Duration of Diabetes, DDMTF:-Daily Dose of Metformin, DMTF:- Duration of Metformin

5.4 Relationship between vitamin B12 and peripheral neuropathy

Among T2DM patients only 6(35.3%) of vitamin B12 deficient patients had neuropathy compared to 43(41.3%) of those with normal vitamin B12 levels (Table 6). The mean NTSS-6 scores in metformin group was 5.8 (± 4.6) while in those without metformin was 5.5 (± 5.53). The median value in metformin group (median=5) was higher than median value of those without metformin (median =3) but the difference was not stastically significant, (U=60, p=0.396). The value of Spearman's rank correlation coefficient (rho) was 0.021 with a P value of 0.819, indicating that there was no sufficient evidence of association between vitamin B12 levels and NTSS-6 scores.

Table 6: Relationship between of vitamin B12 and peripheral neuropathy among T2DM patients attending diabetes clinic of the TASH, Addis Ababa, Ethiopia, 2017

Vitamin B12 Status	Peripheral Neuropathy		P-value
	Absent	Present	
Normal	61(58.7%)	43(41.3%)	0.637
Deficient	11(64.7%)	6(35.3%)	

6. Discussion

The present study has found that serum levels of vitamin B12 was significantly lower among T2DM patients on metformin therapy compared to T2DM patients without metformin therapy. We also found a significant association between metformin daily dose, duration of therapy and duration of diabetes with lower serum B12 levels among T2DM patients on metformin.

Metformin is the most commonly prescribed oral anti-hyperglycemic agent in T2DM patients and apart from gastrointestinal adverse effects ,it is a very safe drug which is recommended by most recent guidelines [4-5]. However, metformin is also known to cause vitamin B12 deficiency[15]. Considering the high prevalence of diabetes and its complications in Ethiopia and widespread use of metformin, underlying vitamin B12 deficiency in such patients may represent a significant problem. The magnitude of this problem in our population was unknown. Thus our study assessed the prevalence of B12 deficiency in Type 2 diabetes mellitus patients on metformin and demonstrates a significant association between metformin use and decreasing B12 levels.

In current study the serum vitamin B12 levels were found to be deficient (<200pg/ml) in 15 (21.1%) patients on metformin treatment while only 2 patients in those without metformin treatment were deficient. The prevalence of vitamin B12 deficiency shown in previous studies ranged from 5.8 to 33% in patients taking metformin [31-33,50]. The reason for the wide variation in the reported prevalence is not straightforward. This variation in the reports could at least be partially explained by methodological differences among the studies that include the variability of measurement methods of vitamin B12 levels in the laboratories (HPLC, radio assay etc.) and difference in definitions of vitamin B12 deficiency (cut-off point).

Our reported prevalence(21.1%) was high relative to previously reported studies conducted in USA, Korea and Nigeria with prevalence of 5.8%, 9.5% and 8.6%, respectively [15,31,61] and lower than prevalence previously reported from studies conducted in Canada, Pakistan, Ireland, and South Africa with prevalence of 31%, 31%, 33% and 28% respectively[30,33,58,65]. It is in-line with findings seen in cross-sectional study by Pflipsen et al. in USA (2009) which found that 22% of their patients with T2DM had a vitamin B12 deficiency[32]. Other Cross-sectional study at a public university hospital in Brazil [59] also showed a biochemical B12 deficiency more frequent in the T2DM metformin group than control group (22.5% versus 7.4%).

In contrast to our study Nobert et al., in Afghanistan (2011) showed that there was no differences in prevalence of vitamin B12 deficiency in metformin users compared to non-users [62].The study used different parameter for the determination of vitamin B12 deficiency (homocysteine, methylmalonic acid) and defined B12 deficiency as serum B12 <180pg/ml or 180-300pg/ml with methylmalonic acid >380 or only elevated methylmalonic acid >318. Besides, patients enrolled in this study were those who were on short duration of metformin treatment (only for 30 days) and aged 20-80years, which is differ from our study. It is known that the risk of developing metformin associated vitamin B12 deficiency is greatly influenced by increasing age, metformin dose and duration of use[51,57,58].

Our study results showed a statically significant inverse relationship between, duration of diabetes, duration and dose of metformin treatment with vitamin B12 levels (r= -0.391, r= -0.608, r=−0.268, respectively). Thus, there is an increased risk of developing vitamin B12 deficiency in patients with T2DM on metformin therapy for long duration diabetes, metformin use and higher dose of metformin. Also odd ratio showed that total daily dose of metformin (mg) (OR= 1.004; 95 % CI: 1.000 to 1.007, P=0.023), duration of metformin use (OR = 1.865; 95% CI: 1.265-2.749, p= 0.002) and duration of diabetes (OR=1.306, 95 % CI: 1.008 to 1.691, P=0.043) increased the odds of vitamin B12 deficiency with significant p value. This result is in-line with a study conducted in Hong Kong by Ting RZ *et al* in 2006.Ting et al. showed that increased risk of vitamin B12 deficiency is associated with current dose and duration of metformin [57], in which there was a two-fold increase in risk of developing vitamin B12 deficiency with each 1g/day dose increment. Damio et al. in Brazil (2015) also reported stastically significant correlation between the dose of metformin and vitamin B12 deficiency – the higher the dose, the lower the average vitamin B12 level [59].

Concerning the duration of metformin use, there was a non-significant trend towards an association between B12 deficiency and longer duration of metformin use. There have been reports of decrease in vitamin B12 absorption and level following metformin use typically starting as early as 4th month [9,68]. However, according to most reports, clinically evident features of vitamin B12deficiency manifests by 5-10 years owing to the large body stores mainly in the liver that are not quickly depleted [18,30,54,57]. According to our findings patients with metformin use of >5 years (38.9%) and daily dosage >1000mg/day (32.5%) showed higher rate of vitamin B12 deficiency compared to those with metformin use of ≤5 years (2.9%) and daily

dosage of ≤ 1,000 mg (6.5%) with p value < 0.05 which corroborates with earlier studies [51,60,61].

Our study found no stastically significant difference in presence of neuropathy, only 6(35.3%) of vitamin B12 deficient patients had neuropathy compared to 43 (41.3%) of those with normal vitamin B12 levels.(p value 0.637). The levels of vitamin B12 and the NTSS-6 scores were not correlated (Spearman's rho= 0.021, P=0.819). Our result is similar with a cross sectional study conducted in South Africa by Marwan A. Ahmed1et al (2016) which reported that no statically significant difference in the presence of neuropathy between those with normal and deficient vitamin B12 levels among patients with T2DM on metformin therapy [65]. This study also used NTSS-6 scores questionnaire to assess neuropathy,-a similar tools with our study. Similarly, our findings are also in concordance with the results of the cross-sectional study of Chen *et al*. which revealed no significant differences between metformin users and non-users when neuropathy status was assessed by both objective (monofilament and neurothesiometry) and relatively subjective (questionnaires) measures [69].In addition to those studies our results are in-line with recently published study which reported that metformin use was not associated with the presence of diabetic peripheral neuropathy in T2DM patients [70, 71].

Our study finding is differ from the case–control study conducted in Canada by Wile and Toth (2010) who reported more severe neuropathy among T2DM patients on metformin compared to non-metformin group with p value of<0.001[30].This might be due to the method used by this study where clinical (Toronto Clinical Scoring System and Neuropathy Impairment Score) and electrophysiological measures (nerve conduction studies) were used. Therefore, In addition to using multiple diagnostic methods, to assess neuropathy it also included patients who have diagnosed peripheral neuropathy in contrast the study we conducted where we used only Neuropathy Total Symptom Score-6 questioner for assessment and included patients with both asymptomatic and symptomatic neuropathies to make it more representative. This might have led to the difference in the results of our study. Singh et al. also reported more severe neuropathy as assessed by TCSS in metformin-treated T2DM patients [50].

7. Strength and Limitation of the study

7.1 Strength of the study

- ✓ Up to the knowledge of the investigators, this is the first in its kind on the assessment of vitamin B12 level among T2DM patients having metformin treatment.
- ✓ This study did not exclusively relied on the laboratory result but incorporated major clinical data from medical records and using questioners.

7.2 Limitations of the study

- ✓ Vitamin B12 status in this study is only assessed by measuring serum vitamin B12 level. Current recommendations suggest adding methylmalonic acid or homocysteine tests to better assess the intracellular status of the vitamin B12 and these are helpful biomarkers when serum B12 levels are borderline [72].
- ✓ We also did not do electro diagnostic tests for neuropathy diagnosis, instead screened for neuropathy only by NTSS-6 questionnaire, which is relatively subjective and symptom history-dependent. However, the questionnaire has been validated and was found suitable for evaluating peripheral neuropathy in clinical trials [73].
- ✓ Due to lack of resource we did not perform glycated hemoglobin (HgA1c) test which is actually used for glycemic control.

8. Conclusion

This cross-sectional study demonstrated that patients with T2DM treated with metformin had low serum B12 levels than those patients not treated with metformin treatment.

The risk factors for vitamin B12 deficiency among T2DM patients with metformin treatment were duration of diabetes, increased dose and duration of metformin treatment. The prevalence of vitamin B12 deficiency was higher in the T2DM patients with increased daily dose of metformin treatment (>1000mg/day), increased duration of metformin treatment use(>5 years) and also long term diabetes.

NTSS-6 scores result for assessment neuropathy showed that 35.3% of T2DM patients had neuropathy and vitamin B12 deficient compared to 41.3% T2DM patients with normal vitamin B12 level, the difference was not statically significant, indicated that there was no sufficient evidence of association between vitamin B12 levels and NTSS-6 scores.

9. Recommendation

Vitamin B12 deficiency has known clinical complications, including anemia, neuropathy, memory impairment, changes in the function of the spinal cord, dementia, depression, and even brain atrophy. Monitoring of vitamin B12level as well as proper supplementation of vitamin B12 will reduce those complications and improve management of diabetes mellitus. Therefore, we recommend that:-

- Vitamin B12 should be measured prior to initiation of metformin treatment to know the base line status and regularly monitored in patients with type 2 diabetes who are on metformin treatment.
- Further research that judiciously considers study design issues is warranted to clarify the possible impact of metformin-induced vitamin B12 deficiency on peripheral neuropathy in T2DM patients and evaluate effect of B12 replacement in these patients towards reducing B12 deficiency and associated symptoms.
- Even though we did got association of fasting blood sugar between patients with metformin and without metformin treatment groups, we cannot get published documents to justify it, as a result of this we recommended that further studies should be done.

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Annexes

Annex I. Participant information sheet

Department of Medical Laboratory Science, Collage of Allied Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia.

Title of the Research Project: Status of Vitamin B12 deficiency in type 2 diabetes mellitus patients on metformin treatment at Tikur Anbesa Specialized Hospital , Addis Ababa, Ethiopia. First of all we would like to thank you in advance for your cooperation and consent in participation in this study. Please read or listen when it is read for you about the general information of the study . If you have any question regarding the study please ask freely.

Background information

My name is Genet Kefeany , a Master's degree(M.Sc.) student in Medical Laboratory science specializing in clinical chemistry at Addis Ababa University . I am planning to conduct a medical research study, which I invite you to take part in.

Background: Diabetes Mellitus is the most common endocrine disorder and metformin is the most frequently prescribed first-line therapy for individuals with type-2 diabetes. However, long term use of metformin lead to vitamin B12 deficiency. Therefore the knowledge of status of vitamin B12 deficiency in T2DM patients on metformin and identifying associated risk factors will be helpful for the Prevention and management of disease complication on T2DM patients caused due to vitamin B12 deficiency.

Aim of the study

- The purpose of this study is to assess the status of vitamin B12 deficiency and associated risk factors in patients with type 2 diabetes mellitus taking metformin treatment at Tikur Anbesa Specialized Teaching Hospital , Addis Ababa, Ethiopia.

Benefits for participants

Study participants will not have any financial incentives or other inducements from participating on this study. However, based on the diagnosis result you will be treated accordingly. Most importantly, the result of the study will be beneficial for planning to screen or prevention

strategies in metformin- treated T2DM patients. hence, you are indirectly benefiting other patients and the society in this respect.

Risks and complication

There are no anticipated risks to your participation. Venus blood sample will be collected once. During collection of sample you may feel some discomfort but this does not produce serious pain.

Confidentiality

On the request paper your name or your identities will not be mentioned and any information given to me will remain confidential and your privacy will be respected. Samples and information given by the participants will serve only for this research not for any other purpose. Participation in this study is exclusively voluntarily. If you are not interested to participate, there will be no consequences.

Person to contact

If you have any questions about this study you should feel free to ask now or anytime throughout the study by contacting:

PI Address: Genet Kefeany : Department of Medical Laboratory Sciences, Collage of Allied Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia

Cell phone: +251-0911 75 22 70

Email: genetkefeni@gmail.com

Participant information sheet (Amharic version)

በአዲስ አበባ ዩኒቨርሲቲ፤ የጤና ሳይንስ ኮሌጅ

የህክምና ላቦራቶሪ ት/ክፍል

የጥናቱ ርዕስ፡- ሜትፎርሚን መድሀኒት በሚወስዱ የስኳር ህመምተኞች ላይ የቫይታሚን ቢ 12 ሁኔታ ምን ይመስላል የሚል ጥናት በጥቁር አንበሳ ስፔሻላይዝድ ሆስፒታል አዲስ አበባ ኢትዮጵያ፡፡ በቅድሚያ በዚህ ጥናት ላይ ለመሳተፍ ፍቃደኛ በመሆንዎ እናመሰግናለን፡፡እባክዎ ስለጥናቱ ጠቅላላ መረጃ በጥሞና ያንብቡ ወይም ሲነበብሎት ይስሙ፤ ማንኛዉም ፤ ግልፅ ያልሆነ ነገር ካጋጠመዎት በነፃነት ይጠይቁ፡፡

ስለጥናቱ መግቢያና አላማው

እኔ ገነት ከፈኒ እባላለሁኝ፡፡ በአዲስ አበባ ዩኒቨርሲቲ በህክምና ለቦራቶሪ ሳይንስ (በክሊኒካል ኬሚስትሪ ስፔሻሊቲ) በመደበኛው ፕሮግራም የማስተርስ /የሁለተኛ/ ድግሪ እየተማርኩ ሲሆን ለመመረቂያ ማሚያ የሚሆነኝን ጥናት ሜትፎርሚን መድሀኒት በሚወስዱ የስኳር ህመምተኞች ላይ የቫይታሚን ቢ 12 ሁኔታ ምን ይመስላል የሚል ጥናት በጥቁር አንበሳ ስፔሻላይዝድ ሆስፒታል ውስጥ በማጥናት ላይ እገኛለው፡፡

የስኳር በሽታ የደማቻንን የስከኳር መጠንን የሚቆጣጠር ሆርሞን መዛባት ምክንያት የሚመጣ በሽታ ነዉ፡፡ ለ ታይፕ ቱ የስከኳር ህሙማን አዘዎትሮ የሚሰጠዉና የመጀመሪያ ተመራጭ የመድሀኒት ዓይነት ሜትፎርሚን ይባላል፡፡ነገር ግን ይህን መድሀኒት ለረጅም ጊዜ በሚወስዱት ህሙማን ላይ የቫይታሚን ቢ 12 ዕጥረትን ይከሰታል፡፡ ስለዚህ የቫይታሚን ቢ 12 ሁኔታን ማወቁና ለዚህ እጥረት አጋላጭ የሆኑትን ምክንያቶችን መለየት ይህን መድሀኒት ለሚወስዱ ህሙማን በ የቫይታሚኑ እጥረት ምክንያት የሚመጣዉን ተጨማሪ በሽታን ለመከላከልና አስፈላጊዉን ቅድመ ጥንቃቄ ምርመራ እንዲደረግ ይረዳል፡፡

በዚህ ጥናት መሳተፍ የሚያስገኛቸው ጥቅሞች

በዚህ ጥናት ስለተሳተፉ ምንም ዓይነት ክፍያ/ጉቦ አይከፈልዎትም ፡፡ ቢሆንም በምርመራዉ ዉጤት መሰረት ህክምና ያገኛሉ፡፡ በጣም አስፈላጊዉ ነገር የዚህ ጥናት ዉጤት ሜትፎርሚን መድሀኒት ለሚወስዱ ህሙማን የቫይታሚን ቢ 12 ዕጥረትን የመከላከልና አስፈላጊዉን ቅድመ ጥንቃቄ ምርመራ እቅድ እንዲኖር ይረዳል፡፡ ስለዚህ በጥናቱ በመሳተፍዎ በተዘዋዋሪ መንገድ ለሌሎች ህሙማን ብሎም ለህብረተሰቡ ይጠቅማሉ ማለት ነዉ፡፡

የጥናቱ ተሳታፊ በመሆንዎ የሚገጥሞት ጉዳት/ ችግር

የጥናቱ ተሳታፊ በመሆንዎ ምንም ዓይነት ጉዳት አይጠበቅም፡፡ የደም ናሙና የሚወስደዉ አንዴ ነዉ ፡፡ የደም ናሙና በሚስጡበት ወቅት ትንሽ ያለመመቸት ስሜት ሊሰማዎት ይችላል ግን ያ ምንም የከፍ ጉዳት የሚያደርስ አይደለም፡፡

ሚስጥራዊነቱ

በመጠየቂያው ወረቀት ላይ የተሳታፊዎች ስም ወይም ማንነት አይገለጽም። የሚሰጡት መረጃ ሚስጥራዊነቱ የተጠበቀ ነው፡፡ በተሳታፊዎች የሚሰጥ ናሙና ለዚህ ጥናት ጥቅም ብቻ የሚያገለግል ይሆናል። በዚህ ጥናት ዉስጥ ላለመሳተፍ ከወሰኑ በዚህ የህክምና ቦታ ዉስጥ የሚሰጥዎት አገልግሎት አይቋረጥም፡፡ በጥናቱ ለመሳተፍ የሚስማሙ ከሆነ የስምምነት ቅጹ ላይ በጹሑፍ ወይም በጣት ፊርማዎትን ማስቀመጥ ይጠበቅቦታል፡፡

ጥያቄ ካሎዎት

ይህን ጥናት በተመለከተ ወይም ከዚህ ጋራ በተዛመደ መልኩ ስለሚያጋጥሙ ድንገተኛ ችግሮች ወይም ጥያቄ ካሎት በሚከተለዉ አድራሻ ይጠቀሙ፡፡

ገነት ከፈኒ

የሕክምና ላብራቶሪ ሳይንስ ት/ክፍል፤ የጤና ሳይንስ ኮሌጅ ፤ አዲስ አበባ ዩኒቨርሲቲ

ሞባይል: +251- 09 11 75 22 70

ኢ-ሚይል: genetkefeni@gmail.com

Annex II Informed consent form

I have been informed about the objective of the study entitled "status of vitamin B12 deficiency and associated risk factors in patients with type 2 diabetes mellitus on metformin treatment at Tikur Anbesa Specialized Hospital , Addis Ababa. I am also informed that the blood sample that I gave is going to be used only for this study and all information contained within the questionnaire, including the result is to be kept confidential. Moreover, I have been well informed of my right to refuse information, decline to cooperate and drop out of the study if I want and none of my actions will have any bearing at all on my overall health care. I understand that I will not get any money for my participation and all the information is explained by phlebotomist and principal investigator.

Therefore, with full understanding of the situations I agree to give the entire necessary information for laboratory analysis. I have had the opportunity to ask questions about the project and received clarification to my satisfaction in a language I understand. I was also told that results for the vitamin B12 test will be given to the health facility and that I may ask the information if I want.

Participant code:_____ Signature: _____

Address of the participant: _____

Date: _____

Informed consent form (Amharic version)

የስምምነት መጠየቅ ያቅጽ

የዚህ ጥናት ርዕስ "ሜትፎርሚን መድሀኒት በሚወስዱ የስኳር ህመምተኞች ላይ የቫይታሚን ቢ 12 ሁኔታ ምን ይመስላል የሚል ጥናት በጥቁር አንበሳ ስፔሻላይዝድ ሆስፒታል ይሆናል፡፡ በቅድሚያ በዚህ ጥናት ላይ ለመሳተፍ ፍቃደኛ በመሆንዎ እናመሰግናለን፡፡ እባክዎትን ከዚህ በታች የተዘረዘሩ ነጥቦች በጥሞና ያንቡቡ እና በመጨረሻ በተሰጠው ክፍት ቦታ ይፈርሙ::

- በጥቁር አንበሳ ስፔሻላይዝድ ሆስፒታል"ሜትፎርሚን መድሀኒት በሚወስዱ የስኳር ህመምተኞች ላይ የቫይታሚን ቢ 12 ሁኔታ ምን ይመስላል " የሚካየሄደውን ጥናት ዓላማ ተረድቻለሁ።
- የ ምሰጠው ናሙና ለዚህ ጥናት ብቻ እንደሚዉል አውቂያለሁ።
- ለጥናቱ የምሰጠው ናሙና እንዲሁም ውጤቱ በሚስጥር እንደሚያዝ ተረድቻለሁ።
- በ ጥናቱ በመሳተፌ የሚከፈለኝ ክፍያ እንደሌለ አውቂያለሁ።
- ሁሉም የሚያስፈልገው ነገር በተመራመረሪው ይብራራልኛል።

ስለዚህ ከላይ የተጠቀሱትን ነጥቦች በመረዳት ናሙና (ደም) ለመስጠት ተስማምቻለሁ።

የጥናቱ ተሳታፊ መለያ ቁጥር፡_____

የተሳታፊ ፌርማ፡_____

ቀን፡_____

Annex III Questioner

Addis Ababa University Collage of Health Sciences, School of Allied Health Science Department of Medical Laboratory Science. Questionnaire on the status of vitamin B12 deficiency in type 2 diabetes mellitus patients on metformin treatment at Tikur Anbesa Specialized Hospital.

Date_____

Study participant code_____

Address_____

A. Socio Demographic Characteristics of the Study participants.

Gender 1. Male_____ 2. Female_____

Age_____

B. Medical History

I. Diagnosis

1. Hypertension Yes_____ No_____
2. Smoking Status Current smoker ____ Past smoker ___ Non smoker_____
3. Alcohol consumption Yes_____ No_____
4. Presence of neuropathy Yes_____ No_____
5. other (specify)_____

II. Duration of illness (for T2DM, years)_____

III. Medication Currently on

1. Do you take metformin drug ? Yes_____ No_____
2. Total daily dose of metformin (gram/day) _____
3. Duration of metformin use (for how long?)_____
4. other (specify)_____

V. Neuropathy Total Symptom Score-6 (NTSS-6) Questioners Sheet)

SN	Questions	Never	Frequency Score			Intensity Score		
			Occasional	Often	Almost	Mild	Moderate	Sever
1	"Do you experience a deep, aching, tightness, boring, pulling, or squeezing pain in your feet or legs?"							
2	"Do you experience unusual sensitivity or tenderness when your feet							
3	"Do you experience burning pain in your feet or legs?"							
4	Do you experience sharp, stabbing, or shooting pain, electrical shock–like pain, or surges of pain that last seconds to minutes in your feet or legs?"							
5	"Do you experience numbness, lost sensation, or a 'dead feeling' like an anesthetic, without prickling in your feet or legs?"							
6	"Do you experience a prickling or tingling feeling, with or without an 'asleep' feeling, in your feet or legs?"							

Total Score_____

Questioner (Amharic) ,የጥናት ጥያቄዎች

በአዲስ አበባ ዩኒቨርሲቲ፤ የጤና ሳይንስ ኮሌጅ

የህክምና ላቦራቶሪ ት/ክፍል

ሜትፎርሚን መድሀኒት በሚወስዱ የስኳር ህመምተኞች ላይ የቫይታሚን ቢ 12 ሁኔታ ምን ይመስላል የሚል ጥናት በጥቁር አንበሳ ስፔሻላይዝድ ሆስፒታል አዲስ አበባ ኢትዮጵያ፡፡

ቀን_____

የጥናቱ ተሳታፊ መለያ ቁጥር_____

የመጡበት አድራሻ_____

1. ማህበራዊ ዳሰሳ

ፆታ ወንድ ☐ ሴት ☐

እድሜ_____

2. የጤናዎ ታሪክ

2.1 ምርመራ

2.1.1 የደም ግፊት አለቦት? አዎ ☐ አይደለም ☐

2.1.2 ሲጋራ አጭሰዉ ያዉቃሉ? አዎ ☐ አይደለም ☐ አቁሚያለሁ ☐

2.1.3 አልኮል ይጠጣሉ? አዎ ☐ አይደለም ☐ አቁሚያለሁ ☐

2.1.3 የነርቭ ችግር አለቦት? አዎ ☐ አይደለም ☐

3.የበሽታዎቆይታ(ለ ዓይነት II የስኳር ህመም)_____

4. በአሁኑ ጊዜ ለ ዓይነት II የስኳር ህመም የሚወስዱት መድሀኒት ምንድነዉ?_____

4.1 ሜትፎርሚን ከሆነ፡ በቀን የሚወስዱት የመድሀኒቱ መጠን(ሚሊግራም)_____ ለምን ያህል ጊዜ ሜትፎረሚንን ወሰዱ (በዓመት)_____

4.2 ሌላ ካለ/ያብራሩ/ _____

5. ለ ስኳር ህመምተኞች የነርቭ ህመም ምልክቶችን ለመለየት የሚረዱ ጥያቄዎች

ተ.ቁ	ጥያቄዎች	አላጋጠመኝም	ካጋጠመዎት በቀን ለምን ያህል ጊዜ ህመሙ ይመላለስቦታል?			የህመሙ ስሜት ጥንካሬ		
			አልፎአልፎ	ብዙ ጊዜ	ያለማቋረጥ	መጠነኛ	መካከለኛ	ሀይለኛ
1	እግርዎን የመቆርጠም፤ የመወጠር /አጥብቆ መያዝ ስሜት፤ መገትገት ፤ መጎተት /መንጠቅ ወይም የመጭመቅ /መጫን ህመም ስሜት አጋጥመዎታል?							
2	እግርዎ ሲነካ ወይም ሲሄዱበት ያልተለመደ የህመም ስሜት ወይም ቁስለት አጋጥመዎታል?							
3	እግርዎን የማቃጠል ስሜት ይሰማዎታል ?							
4	ለደቂቃወች የመሚቆይ ሀይለኛ፤ ፈጣን ከአንድ ቦታ ጀምሮ ወደ ሌላኛዉ የሰዉነት ክፍል የሚስፈነጠር/ የሚሄድ ዉጋት ፤ እንደ ኮሬንቲ / ኤሌክትሪክ ንዝረት ዓይነት የህመም ስሜት እግርዎን ተሰምቶዎት ያዉቃል?							
5	እግርዎን ዉጋት የሌለዉ የመደንዘዝ፤በድን - መሆን ዓይነት ስሜት ተሰምቶዎት ያዉቃል							
6	በእንቅልፍ ሰዓት ወይም ያለእንቅልፍ እግርዎን በሹል ነገር የመወጋት ዓይነት ወይም የመውረር የህመም ስሜት ተሰምቶዎት ያዉቃል?							

ድምር፡_____

Annex IV Checklists

Checklist to gather medical history and current medication from medical records

No	Parameter	Recoeds	Remark
1	Diabetic Complications	Peripheral neuropathy 1. present 2 Absent	
2	Medication history of T2DM	2.1 Diet alone	
		2.2 Metformin alone	
		2.3 Metformin +other(specify)	
		2.4 Insulin alone	
		2.5 Insulin +other(specify)	
		2.6 Daonil alone	
3	For T2DM on metformin	3.1 Duration of metformin use(years)_____	
		3.2 Daily dose of metformin use(mg/ml)_____	

Checklist to record anthropometric data and blood pressure

No	Parameter	Value	Remark
1	Body weight		
2	Height		
3	BMI		
4	Blood pressure		

Checklist to record laboratory findings and Neuropathy Score

Date of Specimen Collection_____

No	Laboratory findings	Result	Normal Value
1	FBS		
2	Serum vitamin B12 level		
3	NTSS-6		

Comments__

_____

_____

Name of Investigator _____Signature _____ Date _____

Annex VI Standard Operating Procedure for vitamin B12

Title:- Vitamin B12 Serum by Roche e 411 analyzer "ECLIA" procedure

Method:-Electrochemiluminescence immunoassay method "ECLI"

Test summary

Vitamin B12 is an essential cofactor for two enzymes involved in one-carbon metabolism: methylmalonyl CoA mutase (reduced function of this enzyme results in increased serum MMA levels) and methionine synthetase (this enzyme catalyzes the remethylmation of homocysteine to methionine) [1]. A serum B12 level below the normal expected range may indicate B12 deficiency. However, a B12 level within the low normal range does not exclude B12 deficiency; symptomatic patients need to be further evaluated with MMA, folic acid, and homocysteine [2,3]. A chronic dietary deficiency of either folate or vitamin B12 causes macrocytic anemia, although strict dietary deficiencies are rare. Most people who develop a vitamin B12 deficiency have an underlying stomach or intestinal disorder that limits the absorption of vitamin B12. Subtly reduced cognitive function resulting from early vitamin B12 deficiency is sometimes the only symptom of these intestinal disorders. Untreated deficiencies will lead to megaloblastic anemia and vitamin B12 deficiency results in irreversible central nervous system degeneration. Hematologic signs, however, are not always present in vitamin B12 deficiency and hematologic signs and neurologic abnormalities can be inversely correlated [4].

Principle

Vitamin B12 is measured using cobas e 411 systems analyzer by electro chemiluminescence immunoassay method, which employs a competitive test principle using intrinsic factor specific for vitamin B12. The sample is first incubated with the vitamin B12 pretreatment 1 and pretreatment 2 during which bound vitamin B12 is released. The pretreated sample is then incubated with the ruthenium labeled intrinsic factor and a vitamin B12-binding protein complex is formed, the amount of which is dependent upon the analyte concentration in the sample. After addition of streptavidin-coated microparticles and vitamin B12 labeled with biotin, the still-vacant sites of the ruthenium labeled intrinsic factor become occupied, with formation of a ruthenium labeled intrinsic factor-vitamin B12 biotin complex. The entire complex becomes bound to the solid phase via interaction of biotin and streptavidin. The reaction mixture is

aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier. Results are determined via a calibration curve which is instrument-specifically generated by 2-point calibration and a master curve provided via the reagent barcode.

Specimen handling and preparation

Serum specimens collected using standard sampling tube (tubes containing separating gel) and plasma specimens collected with Na-heparin or K3-EDTA as an anticoagulant from fasting patients were tested and found acceptable. A routine test requires 150 µL for the sample cup. Specimens collected in the field should be kept cold and protected from light. After processing, specimens should be frozen and shipped on dry ice by overnight mail. Once received, they should be stored at $<-20^{o}_{C}$ until analyzed. For long term storage, specimens should always be frozen at -70°C. Serum B12 is fairly stable at -20°C to -70°C and it can withstand 3 freeze/thaw cycles. Refrigerated samples may be used if they are kept cold and brought promptly (within 2 hours) from the site of blood collection.

Specimen acceptance and rejection criteria

- Specimen with incomplete information
- Clotted, gross hemolysis, lipemic and icterus sample.
- Miss labeling and unlabelled sample
- Wrong tube drawn
- Insufficient sample
- Two or more specimens labelled with the same ID.

Safety Precautions

Use Universal Precautions (treat as potentially infectious) while handling material. Wear PPE while handling. Avoid contact with skin and eyes. If splashing occurs, rinse affected area immediately. Do not empty contents into drains. Discard material into biohazard receptacles

Calibration

Every Elecsys Vitamin B12 reagent set has a barcoded label containing the specific information for calibration of the particular reagent lot. The predefined master curve is adapted to the analyzer by the use of Elecsys Vitamin B12 CalSet II. Calibration must be performed once per reagent lot using fresh reagent (i.e. not more than 24 hours since the reagent kit was registered on the analyzer). Renewed calibration is recommended as follows:

- every 28 days when using the same reagent lot
- every 7 days if using the same reagent kit.
- as required:

Quality Control

Roche controls (Elecsys PreciControl Varia (PCV1 and PCV2) are used for quality control. In addition, other suitable control material can be used. Controls for the various concentration ranges should be run individually at least once every 24 hours when the test is in use, once per reagent kit, and following each calibration. The control intervals and limits should be adapted to each laboratory's individual requirements. Values obtained should fall within the defined limits. Each laboratory should establish corrective measures to be taken if values fall outside the defined limits.

Procedure Refer to *Cobas e 411 manual*

Calculations

The analyzer automatically calculates the analyte concentration of each sample (either in pmol/L or pg/ml).

Result Interpretation

- Values below detection limit are reported < 36.9 pmol/L or < 50.0 pg/mL.
- values above the Analytical range are reported > 1476 pmol/L or > 2000 pg/mL
- Samples with vitamin B12 concentrations above the measuring range can be manually diluted 1:2 with Diluent Universal. The concentration of the diluted sample must be > 738 pmol/L or > 1000 pg/mL. After manual dilution, multiply the results by the dilution factor 2.

Critical Value:- Not applicable

Limitations

- In patients receiving therapy with high biotin doses (i.e. >5 mg/day), no sample should be taken until at least 8 hours after the last biotin administration.
- For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.
- Assay needs to be performed within 2 hours of the samples being placed on board the instrument to minimize the effect of evaporation.

Expected value

Because differences may exist with respect to population and dietary status, it is recommended that normal ranges be determined by each laboratory over a suitable period of time and in a statistically significant number of assays before clinical significance is attached to the results of these tests . Clinical reference ranges reported in the Roche Vitamin B12 package insert are:

N	Median		Range (2.5th-97.5th percentile) (pg/mL)	
	Pmol/L	pg/ml	Pmol/L	pg/ml
135	425	314	145-569	197-771

Instrument linearity

Parameter	Linearity Range	Units
Vitamin B12	50 - 2000	50 pg/mL.

Declaration

I the undersigned, declare that this is my original work and has not been presented for a degree in this work or any other university and all source of materials used for this thesis have been acknowledged.

Name:- Genet Kefeany

Signature_____

Place:-Addis Ababa, Ethiopia

This thesis has been submitted with our approval as University advisors.

Name_____Signature_____

Name_____Signature_____

Name_____Signature_____

Place_____ Date of submission_____