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SERUM VITAMIN B_{12} AND FOLATE LEVELS AND MACROCYTOSIS IN PATIENTS WITH TYPE 2 DIABETES MELLITUS ON METFORMIN ATTENDING TIKUR ANBASSA SPECIALIZED HOSPITAL

BY

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SERUM VITAMIN B_{12} AND FOLATE LEVELS AND MACROCYTOSIS IN PATIENTS WITH TYPE 2 DIABETES MELLITUS ON METFORMINATTENDING TIKUR ANBASSA SPECIALIZED HOSPITAL

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This is to clarify that Master's Thesis prepared by Wondwossen Waletitled As, "Serum vitamin B_{12} and folate levels and, macrocytosis in patients with type 2 diabetes mellitus on metformin attending Tikur Anbassa Specialized Hospital" is submitted in partial fulfillment of therequirement for the Degree of Master of Sciences in Medical Biochemistry complies with the regulations of the University and meets the accepted standards with respect to the originality and quality.

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ABBREVIATIONS AND ACRONYMS

AdoCbl: AdenosylCobalamin

AMP: Adenosine MonoPhosphate

AMPK: AMP- activated Protein Kinase

BHMT: Betaine-Homocysteine MethylTransferase

DCCT: Diabetes Control and Complications Trial

DM: Diabetes mellitus

DNP: Diabetic Peripheral Neuropathy

ECL: Electroluminescence

ECLIA: ElectroChemiLuminescence Immuno Assay

EDTA: Ethylene Di Amine Tetra Acetic Acid

ENAO: Ethiopian National Accreditation Office

EPHI: Ethiopian Public Health Institute

FBP: Folate Binding Protein

IDF: International Diabetic Federation

IQC: Internal Quality Control

ISO: International Organization for Standardization

NADH: Nicotinamide Adenine Dinucleotide

NGSP: National Glycohemoglobin Standardization Program

SOP: Standard Operating Procedure

TASH: Tikur Anbesa Specialized Hospital

ABSTRACT

Introduction:-Metformin is an orally administered drug used for lowering blood glucose concentrations in patients with type 2 diabetes mellitus. However, its long term and high dose therapy is associated with vitamin B_{12} malabsorption. Furthermore, screening and monitoring of vitamin B_{12} level of patients with metformin is not a common practice.

Objective of the study:- Aim of this study was to measure serum vitamin B_{12} and folate levels and, to assess macrocytic status of type 2 diabetes mellitus patients who were on metformin treatment.

Methodology:- Hospital based cross-sectional study was used as an appropriate study design for this particular study. Serum vitamin B_{12} and folate levels and, MCV were determined using COBAS 6000 immunoassay analyzer and UniCel DxH 800 analyzer (Beckman Coulter, USA) respectively in 80 type 2 diabetes mellitus patients who had been on metformin for 5 months or more. Differences in vitamin B_{12} and folate levels and, MCV between different groups were assessed based on daily dose and duration of metformin intake using Mann- Whitney U and Kruskal- Wallis H tests, and $P < 0.05$ was considered as statistically significant.

Result:-Serum vitamin B_{12} and folate values below the lower reference limits were documented in 5% and 23.8% of type 2 diabetes mellitus patients respectively, and 6.2% of patients had MCV values above the higher reference limit. The median level of vitamin B_{12} in patients who were on metformin at a dose of >1500 mg/ day for ≥ 4 years was significantly lower (*p< .001*) compared to patients who were on metformin at a dose of 1000-1500 mg/day and < 1000mg/day for < 4 years respectively. Insignificantly higher value of MCV was recorded among high dose and long duration of metformin takers. The median level of folate in patients who were on metformin at a dose of >1500 mg/ day for ≥ 4 years was significantly lower compared to patients who were on metformin at a dose of 1000-1500 mg/day and < 1000mg/day for < 4 years respectively (*p= 0.002* for dose; and *p=0.010* for duration).

Conclusion:- Type 2 diabetics with longer duration and higher dose of metformin intake were found to have lowered serum vitamin B_{12} and folate levels. Thus, monitoring vitamin B_{12} status of type 2 diabetics on higher and prolonged duration of metformin treatment would have a beneficial impact in minimizing complications that could be worsened due to metformin.

Keywords: Anemia, Diabetic peripheral neuropathy, Folate, Homocysteine, Vitamin B_{12}.

1. INTRODUCTION

1.1. BACKGROUND

Diabetes mellitus is defined as a metabolic disorder resulting from a defect in insulin secretion, insulin action, or both. Insulin deficiency in turn leads to chronic hyperglycemia with disturbances of carbohydrate, fat, and protein metabolism. This chronic hyperglycemia is most frequently associated with long-term failure, dysfunction, and damage of different organs, especially the kidneys, eyes, nerves, heart, and blood vessels (Lebovitz, 2001; American diabetic association, 2019).

There are four main types of diabetes: type 1 diabetes, type 2 diabetes, gestational diabetes and specific types of diabetes due to other causes, e.g. diabetes due to drugs or chemicals, diseases of the exocrine pancreas (American diabetes association, 2019): type 1 diabetes, which occurs most frequently in children and adolescents, is caused by an autoimmune reaction where the body's immune system attacks the insulin-producing beta cells in the islets of the pancreas gland. As a result, the body produces zero to very little insulin with a relative or absolute deficiency of insulin. The causes of this destructive process are not fully understood but a combination of genetic susceptibility and environmental triggers such as viral infection, toxins or some dietary factors have been implicated (You and Henneberg, 2016).

The second type is type 2 diabetes. In this type, hyperglycemia is the result of an inadequate production of insulin and inability of the body to respond fully to insulin, defined as insulin resistance (Evans *et al*., 2000; Holman *et al*., 2015). Gestational diabetes mellitus is a type of diabetes diagnosed in the second or third trimester of pregnancy(American diabetes association, 2019).

Based on plasma glucose, diabetes may be diagnosed according to three main criteria. (1) The fasting plasma glucose (FPG) value or (2) the 2-h plasma glucose (2-h PG) value during a 75-g oral glucose tolerance test (OGTT), or (3) A1C criteria. However, in patients with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose > 200mg/dL (11.1mmol/L) can be used as a diagnosis criterion (American diabetes association, 2019).

Table 1: Diagnosis of diabetes according to American diabetes association, 2019.

FPG (fasting plasma glucose)>126 mg/dL (7.0 mmol/L). Fasting is defined as no caloric intake for at least 8 h.* OR
2-h PG (plasma glucose) ≥200 mg/dL (11.1 mmol/L) during oral glucose tolerance test OGTT. The test should be performed as described by the WHO, using a glucose load containing the equivalent of 75-g anhydrous glucose dissolved in water.* OR
A1C (hemoglobin A1C)>6.5% (48 mmol/mol). The test should be performed in a laboratory using a method that is NGSP certified and standardized to the DCCT assay.* OR
In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose >200 mg/dL (11.1 mmol/L).

*In the absence of unequivocal hyperglycemia, diagnosis requires two abnormal test results from the same sample or in two separate test samples.

Type 2 diabetes mellitus is the most familiar type of diabetes. Its prevalence has reached epidemic proportions worldwide and promotes the risk for cardiovascular diseases and early mortality. Prevention and management of type 2 diabetes has become a major public health challenge around the world (Viollet *et al*., 2012).

Patients with type 2 diabetes are suffering from complications that affect organs including kidney, eye, nerve and vessels leading to serious complications that worsen their quality of life if left untreated. Furthermore, these patients are subjected to various side effects of the drugs they take. For example, metformin is the most widely prescribed drug to treat hyperglycemia in individuals with type 2 diabetes and is recommended, in conjunction with lifestyle modification (diet, weight control and physical activity), as a first line oral therapy in the recent guidelines of the American Diabetes Association and European Association of the Study of Diabetes (Nathan *et al*., 2009). Even if metformin is being used as first line therapy for patients with type 2

diabetes, it is now associated with vitamin B_{12} deficiency if it is taken for long period of time. It is believed that metformin causes vitamin B_{12} deficiency due to malabsorption at gastro intestine (Tomkin *et al*., 1971).

Due to different lower reference values used by different studies across countries, it has been found difficult to define vitamin B_{12} deficiency. For example, it is defined as serum concentration of <200 pg/dL, borderline deficiency as 200 – 300 pg/dL and >300 pg/dL as normal (Akinlade *et al.*, 2015). Others defined it as serum level ≤300 pg/dL (Ko *et al.*, 2014). In general the prevalence increases with age (Allen, 2008; Chatthanawaree, 2011). Vitamin B_{12} deficiency impacts red blood cell synthesis, resulting in megaloblastic anemia due to abnormal DNA synthesis. In addition, it impairs neurological function, in particular demyelination of nerves in part due to abnormal methylation, leading to peripheral neuropathy, dementia, poor cognitive performance, and depression. Other effects of vitamin B_{12} deficiency or depletion are increased risk of neural tube defects in fetus, osteoporosis in adult, cerebrovascular and cardiovascular diseases (Allen, 2012). Vitamin B_{12} deficiency is considered to be one of the causes that worsen diabetic peripheral neuropathy and anemia complications among type 2 diabetic who have been taking metformin for long period of time (Jianbo, 2011). Therefore, early diagnosis is essential, because of the latent nature of this disorder and the risk of permanent neurological damage (Allen , 2012; Chatthanawaree, 2011). However, despite the confirmed association between metformin and vitamin B_{12} deficiency, data those quantify the real size of the problem particularly in Ethiopia are quite limited. Thus, this cross-sectional study tried to investigate vitamin B_{12}, folate and MCV status of type 2 diabetics.

1.2. LITERATURE REVIEW

1.2.1. METFORMIN: PHARMACOKINETICS AND PHARMACODYNAMICS

Metformin (dimethylbiguanide) appears as a current first-line pharmacological treatment for type 2 diabetes in almost all guidelines and recommendations worldwide. It is an orally administered drug used for lowering blood glucose concentrations in patients with type 2 diabetes; particularly in those overweight and obese as well as those with normal renal function. It has been found that the glucose lowering effect of metformin is mainly due to the inhibition of hepatic glucose output, and therefore, the liver is presumably the main site of metformin action (Song, 2016).

Pharmacologically, metformin is a biguanide class of antidiabetes drugs. It has a superior safety profile and is well tolerated when compared to the other biguanides that were introduced for diabetes therapy in late 1950s. The other two biguanides, phenformin and buformin, were withdrawn from the market in the early 1970s due to a potential risk of lactic acidosis and increased cardiac mortality. The incidence of lactic acidosis with metformin at therapeutic doses is rare (less than three cases per 100,000 patient-years) and is not greater than with non-metformin therapies. In addition to its use in type 2 diabetes, there is interest in the use of metformin for the treatment of polycystic ovary disease, diabetic nephropathy, and gestational diabetes (Viollet *et al*., 2012).

The optimal oral metformin dose for many diabetic patients is ~2 g/day. After a single oral dose, metformin is rapidly distributed to many tissues following partial absorption by the small intestine, but the luminal concentration in the gastrointestinal tract remains high. The peak plasma concentration occurs in 3 hr (increasing from 1.0 to 1.6 μg/ml [about 6 to 10 μM] after a 0.5 g dose and to ~3 μg/ml [about 18 μM] after a 1.5 g dose) with a mean plasma half-life of about 20 hrs. (Tucker *et al*., 1981).

Metformin has been used widely in the treatment of type 2 diabetes for over 50 years and has been found to be safe and efficacious both as monotherapy and in combination with other oral antidiabetic agents and insulin. It provides the major clinical advantage of not inducing hypoglycemia or weight gain and ameliorates hyperglycemia with remarkable cardiovascular safety (Foretz *et al.*, 2014). However, the mechanism of metformin action is only partially

explored and remains controversial. In mammals, oral bioavailability of metformin is ~50% and is absorbed through the upper small intestine (duodenum and jejunum) (Graham *et al.*, 2011) and then is delivered to the liver, circulates unbound essentially, and finally is eliminated by the kidneys.

Note that metformin is not metabolized and so is unchanged throughout the journey in the body. The concentration of metformin in the liver is three- to five fold higher than that in the portal vein (40–70 μmol/L) after single therapeutic dose (20 mg/kg/day in humans or 250 mg/kg/day in mice) (Foretz *et al.*, 2014;He and Wondisford, 2015), and metformin in general circulation is 10–40 μmol/L (He and Wondisford, 2015).

Hepatic mechanisms of metformin that have been suggested include the activation of AMPK through liver kinase B1 and decreased energy charge (Zhou *et al.*, 2001; Kawaguchi *et al.*, 2002; Shaw *et al.*, 2005), the increase of the AMP/ATP ratio by restricting NADH-coenzyme Q oxidoreductase (complex I) in the mitochondrial electron transport chain (El-Mir *et al.*, 2000), the inhibition of glucagon-induced cAMP production by blocking adenylyl cyclase (Miller *et al.*, 2013), and, more recently, the reduction of lactate and glycerol metabolism to glucose through a redox change by inhibiting mitochondrial glycerophosphate dehydrogenase (Madiraju *et al.*, 2014).

Various guidelines advocate the use of metformin as the first line glucose lowering agent concurrently with life style modification approaches if there are no contraindications like renal and hepatic dysfunction (Day, 2012; American Diabetes Association, 2019). For example, the American Diabetes Association and the American Association of Clinical Endocrinologists recommend the use of metformin as first-line treatment for both type 2 diabetes mellitus and prediabetes to prevent progression of the disease (American Diabetes Association, 2019). However, the drug has so many adverse effects when taken for long period of time. One of the serious adverse effects of metformin is vitamin B_{12} malabsorption which leads to long-term deleterious neurological and hematologic effects (Bell , 2010; Wile and Toth, 2010).

It has been found that approximately 30% of patients taking metformin do not properly absorb vitamin B_{12} (Tomkin *et al.*, 1971). This effect is most often seen after the patient has received

long-term treatment (i.e ≥6 months) and high doses (i.e >1 g/day) of metformin (Ting *et al.*, 2006; Wile and Toth, 2010).

There are different proposed mechanisms that explain metformin induced vitamin B_{12} deficiency among patients with type 2 diabetes mellitus. Alterations in small bowel motility which activates bacterial overgrowth and consequential vitamin B_{12} deficiency, competitive inhibition or inactivation of vitamin B_{12} absorption, alterations in intrinsic factor (IF) levels and interaction with the cubulin endocytic receptor are among the others (Andre`s *et al.*, 2002). Metformin has also been shown to prevent the calcium dependent absorption of the vitamin B_{12}-IF complex at the terminal ileum. This inhibitory effect is reversed with calcium supplementation (Bauman *et al.*, 2000).

1.2.2. VITAMIN B_{12}: METABOLISM, PHYSIOLOGIC ROLE AND ITS DEFICIENCY

Vitamin B_{12} ('B12') is critical for humans and most forms of life. It was after treatments of deadly pernicious anemia with (B_{12}-containing) raw liver extracts in the beginning of the 20th century that the lifesaving effect of vitamin B_{12}had been revealed. Because the structure and functions of the therapeutically active anti- pernicious factor (vitamin B_{12}) were still unexplored at those times, these treatments mark the starting point of vitamin B_{12} research (Zelder, 2015).

The amount of vitamin B_{12} our body needs each day depends on our age. Average daily recommended amounts for different ages are listed below in micrograms (mcg) (US Department of Health and Human Services, 2019).

Table 2: Average daily recommended amounts of vitamin B_{12} based on life stage.

Life Stage	Recommended Amount ,micrograms (mcg)
Birth to 6 months	0.4 mcg
Infants 7–12 months	0.5 mcg
Children 1–3 years	0.9 mcg
Children 4–8 years	1.2 mcg
Children 9–13 years	1.8 mcg
Teens 14–18 years	2.4 mcg
Adults	2.4 mcg
Pregnant teens and women	2.6 mcg
Breastfeeding teens and women	2.8 mcg

*US Department of Health and Human Services, National Institute of Health Office of Dietary Supplements, 2019

Structurally, vitamin B_{12} or cobalamin consist of a central cobalt ion that attached to different R-groups (Zelder, 2015).It is a water soluble vitamin that plays a very fundamental role in DNA synthesis, optimal haemopoesis and neurological function. The clinical picture of vitamin B_{12} deficiency hence, is predominantly of features of hematological and neuro-cognitive dysfunction (Kibirige and Mwebaze, 2013; Farland *et al*., 2015).

The principal source of vitamin B_{12} is animal proteins and it is also found in some packed foods and pharmacologically it is available as vitamin B_{12} injections, nasal spray and as oral supplements.

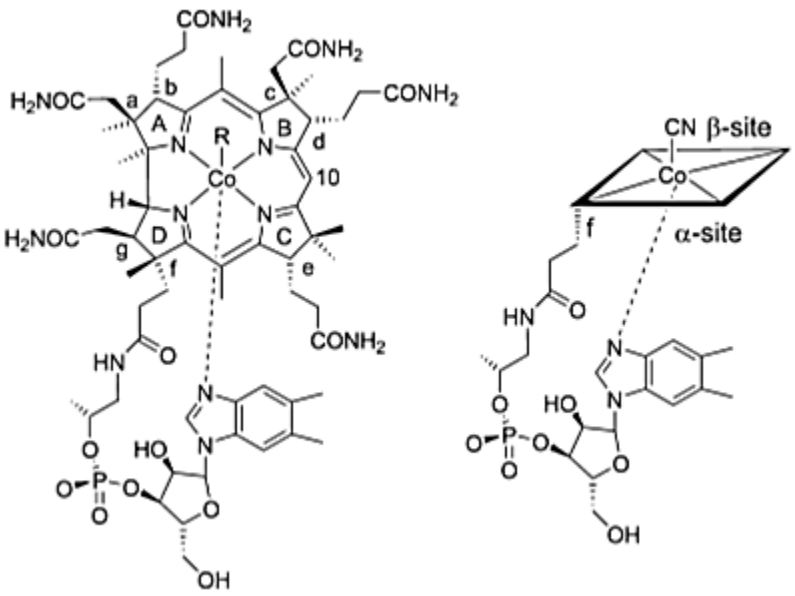

Figure 1: Chemical structure of vitamin B_{12}. Where; R = CN: cyanoCbl, CNCbl or B12; R = OH: hydroxoCbl, HOCbl; R = CH 3: methylCbl, MeCbl; R = 50-deoxy-50-adenosyl: adenosylCbl, AdoCbl (Zelder, 2015).

The major step in the metabolism of dietary vitamin B_{12} involves its release from animal sources, a process mediated by the action of pepsin and gastric acid. After the release, dietary vitamin B_{12} then binds to the R-protein secreted by the salivary glands. The R-protein (Haptocorrin) is then hydrolyzed to release vitamin B_{12} which later binds with the intrinsic factor (IF) secreted by the gastric parietal cells. The hydrolysis of R-protein takes place in the duodenum and it requires the presence of alkaline medium and pancreatic proteases. Vitamin B_{12} which is released from the R-protein is now free to bind with IF and make vitamin B_{12}-IF complex.

The vitamin B_{12} –IF complex which is extremely resistant to proteolytic degradation then attaches at its specific receptors on the mucosa of the terminal ileum, a site where its absorption occurs. This phase of vitamin B_{12} absorption is calcium mediated.

The intracellular vitamin B_{12} released following IF degradation is now free to bind to another protein carrier, transcobalamin –II (TC-II) and is later released into the circulation. This vitamin B_{12} – TC-II complex, also referred to as holo TC-II is then actively taken up by different body

parts like the liver, bone marrow and other vital body cells. The liver serves as the main storage site of up to 90% of the body's total vitamin B_{12}. Within each cell of the body, the transcobalamin II–vitamin B_{12} complex is taken up by a mode of endocytosis and free cobalamin is released and then converted enzymatically into its 2 coenzyme forms, methylcobalamin and adenosylcobalamin (Oh and Brown, 2003; Andrès *et al*., 2004)**.**

In the mitochondria, cobalamin is converted to something known as adenosylcobalamin (AdoCbl), a coenzyme involved in the conversion of methylmalonyl-CoA (MM-CoA) to succinyl-CoA. In the cytoplasm, cobalamin functions in its active form, methylcobalamin, as a coenzyme in the conversion of homocysteine to methionine by the enzyme methionine synthase (Andrès *et al*., 2004).

Thus, through its active forms vitamin B_{12} exerts its physiological effects through mediating important enzymatic pathways that help keep the body's nerve and blood cells healthy, and helps make DNA, the genetic material in all cells. Vitamin B_{12} also helps prevent a type of anemia called megaloblastic anemia (Kibirige and Mwebaze, 2013).

A disruption in any steps of vitamin B_{12} metabolism described above will result into clinical or biochemical vitamin B_{12} deficiency. This includes insufficient dietary intake especially among alcoholics and vegetarians and malabsorption due to several conditions like chronic atrophic gastritis mainly in the elderly, pernicious anemia, HIV/AIDS, celiac disease, chronic kidney disease, liver disease, chronic pancreatitis(Affenberger *et al,*. 2007; Koplay *et al.,* 2011; Patil *et al.,* 2016).

Vitamin B_{12} can interact with some medicines that can lower its level in the body by interfering with the body's absorption or use of vitamin B_{12}. Examples of drugs that have the potential to lower vitamin B_{12} in the body includes metformin, a drug used to treat type 2 diabetes mellitus, Chloramphenicol, an antibiotics used to treat certain infections, omeprazol, a proton pump inhibitor which is used to treat peptic ulcer disease and histamine H2 receptor antagonists, such as ranitidine and cimetidine that are used to treat peptic ulcer disease (Kibirige and Mwebaze, 2013; Farland *et al*., 2015).

1.2.3. FOLATES: ITS BIOCHEMISTRY, FUNCTIONS, AND DEFICIENCIES

Folates are a family of B_9 vitamins which comprise a family of chemically related compounds based on the folic acid structure. Folates in tissues act as donors and acceptors of one-carbon units in metabolic reactions known as one-carbon metabolism. These one-carbon units can be at the oxidation level of different forms like methanol (5-methyl-tetrahydrofolate), formaldehyde (5,10-methylene-tetrahydrofolate), or formate (5- or 10-formyl-tetrahydrofolate or 5,10-methenyl-tetrahydrofolate). Practically all tissue folates are polyglutamate forms. Since the polyglutamate forms are much more effective substrates for folate-dependent enzymes than are monoglutamates, which are the transport forms of the vitamin, the conversion of folates to polyglutamate forms is needed for their biological activity. Conversion of folates to polyglutamates of chain length greater than three or more is also required for effective retention of folate by tissues (Shane, 2008).

Folic acid, which is not an active form of the coenzyme, does not occur in nature and is rarely found in unfortified foods. It is the most common form of folate used in supplements and in fortified food products because it is highly bioavailable, chemically stable, and is readily reduced to tetrahydrofolate, the active coenzyme form of folate.

Folates present in food typically occur in a reduced, polyglutamyl form. Before absorption, they are cleaved to their monoglutamyl forms by a brush border glutamylhydrolase, sometimes called intestinal folate conjugase (Devlin *et al.*, 2000). Folates are absorbed in the proximal small intestine by a saturable, pH-sensitive transporter that transports oxidized and reduced folates (Qiu *et al.*, 2006). The highest amount of dietary folate, and folic acid in the diet, is metabolized to 5-methyl-tetrahydrofolate during its passage across the intestinal mucosa.

Folate coenzymes are involved in three major interrelated metabolic cycles in the cytosol of cells. These cycles are required for the synthesis of thymidylate and purines, precursors for DNA and RNA synthesis, and for the synthesis of methionine from homocysteine and the interconversion of serine and glycine. 5,10-Methylene-tetrahydrofolate plays a central role in these cycles, as it can be used directly for thymidylate synthesis, or it can be reduced to 5-methyltetrahydrofolate in the methionine synthesis cycle, or can be oxidized to 10-formyl-tetrahydrofolate to be used in purine synthesis. Although these synthetic cycles are located in the

cytosol, mammalian cells also contain a large mitochondrial folate pool, which is also involved in the provision of one-carbon precursors for cytosolic one-carbon metabolism (Shane, 2008).

For example, the methylation of homocysteine to produce methionine uses 5-methyl-tetrahydrofolate as the methyl donor in a reaction catalyzed by methionine synthase, one of only two vitamin B_{12}-dependent enzymes in mammals (Shane and Stokstad, 1985). 5-Methyl-tetrahydrofolate is generated from 5,10-methylene-tetrahydrofolate in a reaction catalyzed by the flavoprotein methylenetetrahydrofolate reductase (MTHFR). Methionine can be metabolized to S-adenosylmethionine, which acts as the methyl donor in many reactions, including the methylation of DNA, histones and other proteins, neurotransmitters, and phospholipids, and the synthesis of creatine. These methylation reactions play important roles in development, gene expression, and genomic stability. S-Adenosyl-homocysteine, the product of methylation reactions, is a potent inhibitor of many methyltransferases and is catabolized by hydrolysis to adenosine and homocysteine (Shane, 2008).

Homocysteine in liver and kidney can be converted back to methionine via the folate-independent betaine-homocysteine methyltransferase (BHMT), which catalyzes the transfer of one of the methyl groups of betaine to homocysteine to generate methionine and dimethylglycine. Betaine arises from choline oxidation in liver mitochondria. BHMT is present in high concentrations in liver, and limited studies in humans indicate that up to 30% of homocysteine remethylation may occur through this reaction (Mudd and Poole , 1975). In the liver and kidneys, homocysteine can also be metabolized to cysteine via the transsulfuration pathway, which involves two PLP-dependent enzymes, cystathionine β-synthase and cystathionase. In most other tissues, homocysteine is exported to the circulation or is reconverted back to methionine via the folate dependent methionine synthase reaction. Methylation reactions account for a large proportion of the methyl group intake in humans, and the methionine synthase reaction allows salvage of its backbone after its use for methylation. The folate-dependent methionine cycle is very sensitive to inadequate folate status. When folate status is poor, the decreased ability to remethylate cellular homocysteine results in an increased plasma homocysteine level, and the plasma total homocysteine level is an indirect indicator of folate insufficiency(Refsum *et al.*, 1998; Jacques *et al.*, 1999).

Folate deficiency may occur at all ages, particularly in persons ingesting a poor diet or suffering from intestinal malabsorption or who have excessive alcohol intake or have excessive demands as in hemolytic anemia, psoriasis or other medical conditions with increased cell proliferation or use of certain drugs. Individuals with reduced folate status have elevated levels of homocysteine. Thus, individuals with low or borderline levels of folate and elevated levels of homocysteine can be defined as having 'metabolically significant' folate deficiency. Individuals with combined folate and vitamin B_{12} deficiency may be defined as 'metabolically significant' combined deficiency if they have low or borderline levels of folate and vitamin B_{12} and elevated homocysteine (Clarke *et al*., 2003; Allen , 2008).

1.2.4. VITAMIN B_{12} AND FOLATE RELATED MACROCYTIC ANEMIA

The presence of abnormally large RBCs in the peripheral blood characterizes a state of anemia known as macrocytic anemia. This abnormality is usually recognized by the automated blood cell counter and confirmed on review of the peripheral blood smear. There are different etiologies that cause macrocytic anemia. Vitamin B_{12} and folate deficiencies are among the others.Macrocytic anemia can usually be divided into two categories, megaloblastic and nonmegaloblastic, based on the examination of the bone marrow. Megaloblastic anemia is due to vitamin B_{12} and folate deficiency while liver disease and chronic alcoholism cause nonmegaloblastic anemia. This classification is important and frequently aids in determining the etiology of the anemia. Furthermore, a careful review of the peripheral blood smear noting the morphology of the RBCs, as well as the other cellular elements and features on the smear, can provide important clues as to the etiology of the anemia (Aslinia *et al*., 2006).

1.2.5. RELATIONSHIP BETWEEN METFORMIN INDUCED VITAMIN B_{12} DEFICIENCY AND ONE CARBON METABOLIC PATHWAYS

One carbon metabolism describes reactions including the addition, transfer or removal of 1-C units in cellular metabolic pathways. The central methylation pathway, the methylation cycle occurs in the cytoplasm of every cell where the formation of S-adenosyl methionine from adenosine triphosphate and methionine is catalyzed by methionine adenosyl transferase. In turn, S-adenosyl methionine is converted to S-adenosyl homocysteine and then to homocysteine. Methionine is reproduced when a methyl group from 5-CH3-tetrahydofolate is transferred to

homocysteine. This last step of the cycle requires the presence of vitamin B_{12} as a cofactor for methionine synthase. When concentrations of available vitamin B_{12} are inadequate, folate becomes trapped as 5-methyltretrahydrofolate, and the regeneration of methionine is inhibited, and the concentrations of homocysteine and its metabolites increased (Scott , 1999). Methionine deficiency is a commonly used animal model to demonstrate intrauterine growth retardation and fatty liver disease (Kalhan , 2009).

Metabolism of homocysteine occurs through intersecting enzymatic pathways: (1) remethylation, which requires vitamins B_{12} and B_9, (2) transsulphuration, which requires vitamin B_6 and (3) the catalysis by betaine-homocysteine methyltransferase of the transfer of one of the amino groups of betaine to homocysteine to form methionine. The importance of labile methyl groups and homocysteine remethylation in liver function and diabetes has been demonstrated in humans and animals (Mato *et al.*, 2008).

The remethylation of homocysteine to methionine intersects with the folate cycle where methylenetetrahydrofolate reductase catalyzes the reduction of 5,10 methyl tetrahydrofolate to 5 methyl tetrahydrofolate, which is a co-substrate for the methylation of homocysteine to methionine. Folate is the methyl acceptor and donor in this cycle where methionine is regenerated. The folate cycle is essential for purine and pyrimidine nucleotide synthesis, which are essential for the formation and stability of DNA, RNA and nucleoside triphosphates such as adenosine triphosphate (Sinclair *et al.*, 2007; Lillycrop , 2011).

The conversion of S-adenosyl methionine to S-adenosyl homocysteine is a critical step in the central methylation cycle. S-adenosyl methionine serves as a methyl donor and methyl groups may be added to many methyl acceptor substrates including DNA. Methylation of DNA nucleotides is an important epigenetic mechanism for control of gene expression. This control of gene expression is particularly important during critical periods of growth and development and may help explain why nutritional imbalances are associated with fetal phenotypes, which increase the risk for subsequent diseases (Jimenez-Chillaron *et al.*, 2012).

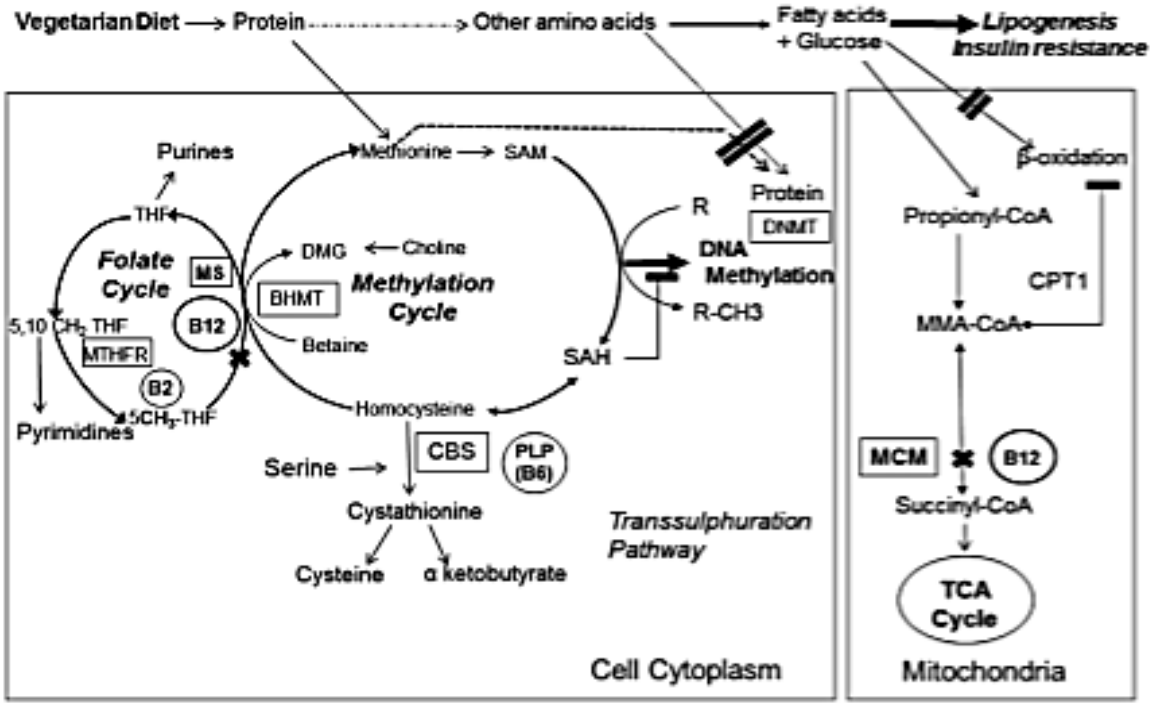

Figure 2: One carbon metabolic pathways, vegetarian diet and effects of B12 insufficiency: BHMT, Betaine-homocysteine S-methyltransferase; CPT1, Carnitine palmitoyltransferase; CBS, Cystathionine-bsynthase; DNMT, DNA methyltransferase; GNMT, Glycine N-methyltransferase; MCM, Methylmalonyl-CoA mutase; MMA-CoA, MethylmalonylCoA; MTR, Methionine synthase; MTHFR, ethylenetetrahydrofolate reductase; MS, Methionine Synthase; R Methyl acceptors, including adenosine and cytosine; R-CH3 Methylated acceptor; SAH, S-adenosyl homocysteine; SAM, S-adenosyl methionine; THF, Tetrahydrofolate (Rush *et al*., 2014).

In liver, kidney, small intestine and pancreas, homocysteine may enter the trans-sulphuration pathway and be converted to cystathionine by the addition of serine. The active form of vitamin B_6, pyridoxal-5-phosphate, is a cofactor for this step (Shane, 2008).

In the mitochondria, beta-oxidation of uneven number carbon unit fatty acids results in the formation of propionyl-CoA, a three carbon unit. Propionyl-CoA is enzymatically carboxylated to methylmalonyl CoA which is then reversibly isomerized to succinyl-CoA by the B_{12} dependent enzyme, methyl malonyl-CoA mutase. Deficiency of vitamin B_{12} blocks the

production of succinyl-CoA, and leads to elevated methyl malonic acid (MMA) and methyl malonyl CoA (MMACoA). Increased concentrations of malonyl CoA inhibit the activity of carnitine palmitoyltransferase (CPT1), the enzyme that controls the rate of long chain fatty acyl-CoA transfer into the mitochondria. The outcome is inhibition of beta-oxidation. Thus, there is accumulation of fatty acids in the cytosol of the nucleus and increased inclusion of fatty acids into glycerolipids (Rush *et al.*, 2014). Thus, vitamin B_{12} influences folate-dependant reactions and mitochondrial energy and lipid metabolic pathways, and any form of vitamin B_{12} deficiency could potentially lead to the following abnormalities.

1.2.5.1. HYPOMETHYLATION AND ALTERED GENE EXPRESSION

Methylation of DNA nucleotides is an important epigenetic mechanism for control of gene expression as it has been discussed above. Thus, vitamin B_{12} deficiency may lead to alterations in the supply of one carbon units and could influence DNA methylation and therefore gene expression by determining which genes are switched on and off and when (Sinclair *et al.*, 2007). Evidence from sheep models (Sinclair *et al.*, 2007) and growing evidence in humans (Dominguez-Salas *et al.*, 2012) suggests that environmental insults, particularly of availability of folate and vitamin B_{12} in utero, lead to differences in DNA methylation in the offspring and the patterns of DNA methylation that are established in utero could induce stable changes in gene expression lasting through the life of the individual. Thus, epigenetic alterations can have profound and life-long effects on structure and function (phenotype). For example, the cell cycle may be switched from proliferation to differentiation with adverse consequences for total cell number and function (Fowden *et al.*, 2006). In addition, there is evidence that genetic polymorphism of the methylenetetrahydrofolate reductase modulates genomic DNA methylation (Friso and Choi, 2005). Therefore, it is tempting to speculate that the balance of nutrient intake might ultimately affect patterns of epigenetic modifications such as DNA methylation in a population- and individual-specific manner may lead to abnormalities (Barbosa *et al.*, 2008).

1.2.5.2. INSULIN RESISTANCE

Deficiency of vitamin B_{12}, as discussed above, blocks the production of succinyl-CoA, and leads to elevated methyl malonic acid (MMA) and MMACoA. Increased concentrations of malonyl CoA inhibit the activity of carnitine palmitoyltransferase (CPT1), the enzyme that controls the

rate of long chain fatty acyl-CoA transfer into the mitochondria. This inhibits beta-oxidation in the mitochondria and promotes accumulation of fatty acids in the cytosol leading increased lipogenesis, obesity and insulin resistance (Kelley *et al.*, 2002).

1.2.5.3. COGNITIVE AND NEUROLOGICAL DEFICITS

Neural tube defects and other neurological problems may be partially attributed to deficits in myelination and also to increased inflammation. Myelin is 80% lipid and the mechanism for defects in myelination may be related to accumulation of MMA and myelin destabilization (Black, 2008) which in turn is due to vitamin B_{12} deficiency (Rush *et al.*, 2014).

1.2.5.4. VITAMIN B_{12} DEFICIENCY, FOLATE TRAP AND HYPERHOMOCYSTEINEMIA

Vitamin B_{12} deficiency due to different factors including disease, drugs and low vitamin diets lead to a condition known as folate trap(functional folate deficiency) where free folate is trapped in the form of methyl tetrahydrofolate. Thus, deficiency of any forms of vitamin B_{12} and folate ultimately affect methionine metabolism where homocysteine can no longer remethylated back to methionine causing hyperhomocysteinemia (Faeh *et al*., 2006).

1.2.5.5. VITAMIN B_{12} DEFICIENCY ASSOCIATED PERIPHERAL NEUROPATHY

Vitamin B_{12}deficiency-associated peripheral neuropathy can also remain subclinical and, possibly, interact with that of type 2 diabetes mellitus. Such neuropathy is thus likely to be misdiagnosed as diabetic neuropathy. The long-term use of metformin, mediated by vitamin B_{12} deficiency, may contribute to increase the substantial burden of peripheral neuropathy in type 2 diabetes mellitus patients (Briani *et al.*, 2013). There are a number of conflicting findings whether metformin-associated vitamin B_{12} deficiency may contribute to the clinical burden of diabetic peripheral neuropathy. For example, Wile and Toth suggest long term metformin use exacerbate peripheral neuropathy in patients with type 2 diabetes (Wile and Toth, 2010)and the other study suggests there is no association between metformin induced vitamin B_{12} deficiency and peripheral neuropathy (Ahmed *et al.*, 2016).

1.2.6. METFORMIN INDUCED VITAMIN B12 DEFICIENCY AND ASSOCIATED RISK FACTORSAMONG PATIENTS WITH TYPE 2 DIABETES MELLITUS

Vitamin B_{12} absorption and its levels start to decrease just after 4-6 months following metformin use (Wulffelé *et al.*, 2003). However, the clinical manifestations of vitamin B_{12} deficiency emerges after 5–10 years owing to the large body stores in the liver mainly that are not quickly depleted (Andre`s *et al.*, 2002).

Different cross- sectional, case report and randomized controlled trials studies stated the association of metformin use and vitamin B_{12} deficiency. For example, a randomized controlled trial by Defronzo *et al.,* showed that metformin decreased the serum vitamin B_{12}levels by 22% and 29% compared to placebo and glyburide respectively (DeFronzo *et al*., 1995).

A report from the National Health and Nutrition Examination Survey (NHANES) from 1999–2006 in USA, biochemical B_{12} deficiency was present in 5.8% of those diabetic patients on metformin compared to 2.4% of those type 2 diabetics not on metformin (P = 0.0026) and 3.3% of those without the disease (P = 0.0002). Among those with diabetes, metformin use was associated with biochemical B_{12} deficiency (adjusted odds ratio 2.92; 95% CI 1.26–6.78) (Reinstatler *et al*., 2012).

According to a randomized control trial study conducted in the outpatient clinics of three non-academic hospitals in the Netherlands, it has been found that long term (4.5 years) metformin treatment was correlated with a decrease in vitamin B_{12} concentration of 19% ($P<0.001$) and in folate concentration of 5% ($P=0.033$), and an increase in homocysteine concentration of 5% ($P=0.091$) as compared with placebo after 4 years (De Jager, 2010).

Not only long term metformin treatment, but also short term metformin therapy has also been associated with vitamin B_{12} deficiencies according to studies. For example, i the same country (Netherlands), a placebo-controlled, randomized trial was conducted to investigate the effect of short term (16 weeks) metformin treatment on serum concentrations of homocysteine, folate and vitamin B_{12} in type 2 diabetes mellitus and the study found, amongst those who completed 16 weeks of treatment, metformin use, as compared with placebo, was associated with an increase

in homocysteine of 4% (P = 0.039) and with decreases in folate of 7% (P = 0.024] and vitamin B_{12} of 14% (P < 0.0001] (Wulffelé *et al.*, 2003).

Other interventional study by Iftikhar *et al.,* in Pakistan revealed that after 12 months of metformin therapy, serum vitamin B_{12} levels were low in 35 patients (31%) on metformin as compared to only 9 patients (8.6%) among controls,(p value 0.002). Mean vitamin B_{12} levels were significantly low in metformin group 311 pg/mL (±194.4), p value 0.03 (Iftikhar *et al*., 2014).

Furthermore, a cross- sectional study by Zalaket *et al.,* from Lebanon, 22.5% of type 2 diabetes mellitus patients among 200 had metformin related vitamin B_{12} deficiency (Zalaket *et al*., 2018).

A study to determine the serum level of vitamin B_{12} in Nigerian patients with type 2 diabetes mellitus on metformin was carried out and according to the result; vitamin B_{12} deficiency and borderline deficiency were recorded in 8.6% and 26.0% out of 81 patients respectively (Akinlade *et al*., 2015). A similar cross-sectional study at diabetes clinics of two public hospitals in South Africa was done and the prevalence of metformin related vitamin B_{12} deficiency, among 121 participants, was found to be 28.1% (Ahmed *et al*., 2016). In 2017, unpublished data in Ethiopia reported a 21% of metformin related vitamin B_{12} deficiency in patients with type 2 diabetes mellitus (Genet, 2017).

According to studies, the risk of developing metformin associated vitamin B_{12} deficiency increase with age, metformin dose and duration of use (De Jager *et al*., 2010). For example, a cross-sectional study by Ting *et al.,* reported that each 1-g/d of metformin dose increment conferred an odds ratio of 2.88 (95% confidence interval, 2.15-3.87) for developing vitamin B_{12} deficiency (P<.001). Among those using metformin for 3 years or more, the adjusted odds ratio was 2.39 (95% confidence interval, 1.46-3.91) (P = .001) compared with those receiving metformin for less than 3 years (Ting *et al.*, 2006).

Many cross-sectional studies have revealed that dose of metformin had inverse correlation with vitamin B_{12} levels in that, the serum vitamin B_{12} levels decreased as the dose of metformin increased in a linear way. For example, based on case control study by Iftikhar *et al.,* dose of metformin had inverse correlation with vitamin B_{12} levels and the difference was statistically

significant with p-value < 0.001 (Iftikhar *et al*., 2014). Another study in Lebanon has also revealed the levels of vitamin B 12 was inversely correlated and it was found to be highly significant (p = 0) (Zalaket *et al.,* 2018).

According to a study by Akinlade and his colleagues, it was observed that the median level of vitamin B_{12} was significantly lower (Vitamin B_{12} (pg/dL) = 306.98 (244.22–389.36)) in patients who were on metformin at a dose of >1000 mg/ day compared with patients who were on metformin at a dose of <1000 mg/day (Vitamin B_{12} (pg/dL) = 417.29 (295.94–505.49)) with p-value of 0.004 (Akinlade *et al.,* 2015).

Based on studies the duration of metformin intake was also a significant factor as the vitamin B_{12} levels decreased with the increasing duration of metformin use. It has been found that subjects using metformin for about three months, the average vitamin B_{12} level was 232.11 pmol/L and that decreased to 177.83 pmol/L for those taking metformin for more than 12 months (p = .004) (Zalaket *et al.,* 2018).

In one study all the patients were divided into 2 groups based on duration of metformin use: <10 years and >10 years. The median vitamin B_{12} level was significantly lower (Vitamin B_{12}(pg/dL) = 299.63 (261.12–373.05)) in participants who have used metformin for>10 years compared with patients who have used metformin for <10 years (Vitamin B_{12} (pg/dL) = 429.48 (304.17–510.42)) (Akinlade *et al.*, 2015).

Age is also the other associated factor related to vitamin B_{12} deficiency among type two diabetic patients on metformin. However, regarding the association between age and vitamin B_{12} deficiencies, there are different studies with different results. For example, according to the study by Aroda *et al.,* age was not related to vitamin B_{12} deficiency or vitamin B_{12} levels. Because it was observed that, in the metformin group, vitamin B_{12} deficiency increased over time in all age categories (Aroda *et al.,* 2016).

According to the study by Ahmed *et al.,* however, vitamin B_{12} deficient participants were significantly older than those with normal vitamin levels (62.3 vs. 57 years, P = 0.012) (Ahmed *et al*., 2016).

1.3. STATEMENT OF THE PROBLEM

The high glucose level in the serum of type 2 diabetes mellitus patients can lead to serious diseases affecting the heart and blood vessels, kidneys, eyes, and nerves. Cardiovascular complications, peripheral neuropathy, nephropathy, and retinopathy are among the major complications most frequently observed among diabetic patients (Atlas, 2015). Patients with chronic medical conditions like diabetes mellitus take several medications, e.g. metformin, with various dose and duration of treatment. If the side effects of these drugs are left unmonitored they add a further complication to these patients. Metformin is a cornerstone of type 2 diabetes mellitus management. Despite its clinical benefits however, it has been reported that metformin is becoming a pharmacological cause of vitamin B_{12} deficiencies in patients with type 2 diabetes mellitus (Gilligan, 2002; Liu *et al.*, 2006). The prevalence of vitamin B_{12} deficiency in type 2 diabetes mellitus patients on metformin has been reported to be between 5.8% and 41% (Reinstatler *et al*, 2012; Owhin *et al.*, 2019).

A long line of studies reported that higher dose and prolonged duration of metformin treatment induce vitamin B_{12} deficiency and a consequential peripheral neuropathy, anemia and cardiovascular complications. For example, although the accurate mechanism is not well understood, it is proposed that the high levels of homocysteine, which results from vitamin B_{12} malabsorption due to long term metformin therapy, is responsible for the worsening of cardiovascular complications among type 2 diabetic patients (Wulffelé *et al.*, 2003; Sadeghian *et al*., 2006; De Jager *et al*., 2010). Anemia and diabetic peripheral neuropathy (DNP) are also other complications most frequently observed in diabetic patients. Folate, the active form of vitamin B_9, due to deficiency of vitamin B_{12}, get trapped in the form of methy-tetrahydrofolate resulting a condition known as functional folate deficiency that ultimately leads to anemia. Diabetic peripheral neuropathy (DPN), which occurs in up to 50% of diabetic patients and causes sensory, motor, and/or autonomic dysfunction, is also a serious complication among diabetic patients (Wile and Toth, 2010; Jianbo *et al.*, 2011). Thus, it is now believed that vitamin B_{12} deficiency due to high dose and long term metformin therapy is the root causes of increased levels of homocystein, decreased levels of folate that may contribute to the worsening of the aforementioned complications (Aroda *et al*., 2016). Despite these scientific facts, availability of

published data on vitamin B_{12}, folate and MCV in relation to the dose and duration of metformin treatment is limited in Ethiopia.

1.4. SIGNIFICANCE OF THE STUDY

Screening type 2 diabetes mellitus patients with higher dose and long duration of metformin treatment to monitor its side effects especially vitamin B_{12} deficiency is not a common clinical practice in many clinical settings in Ethiopia. Therefore, to minimize complications and maximize quality of life of people with diabetes mellitus through monitoring metabolites (vitamin B_{12} and folate), the findings of this study could serve as a reference for clinicians to consider metformin related vitamin B_{12} deficiency and related folate deficiency and macrocytosis. Furthermore, considering the limited availability of data on this area, the final result of this study could serve as baseline information for further investigations on this area.

2. OBJECTIVE

2.1. GENERAL OBJECTIVE

- ✓ To measure serum vitamin B_{12} and folate levels and, to assess macrocytic status of type 2 diabetes mellitus patients who were on metformin treatment attending TASH, Addis Ababa, Ethiopia.

2.2. SPECIFIC OBJECTIVE

- ✓ To determine serum vitamin B_{12} and folate levels of type 2 diabetes mellitus patients on metformin
- ✓ To compare the difference in serum vitamin B_{12} and folate levels between high and low dose metformin intake
- ✓ To compare the difference in serum vitamin B_{12} and folate levels between long and short duration of metformin intake
- ✓ To investigate whether there was difference in mean corpuscular volume between high and low dose metformin intake
- ✓ To examine whether there was difference in mean corpuscular volume between long and short duration of metformin intake

3. MATERIALS AND METHODS

3.1. STUDY AREA

The study was conducted at Tikur Anbassa Specialized Teaching Hospital, Addis Ababa, Ethiopia that provide teaching and medical services for people of Addis Ababa and people who are referred from other parts of the country. TASH, under the administration of Addis Ababa University, is located in Lideta Sub City of Addis Ababa. This referral hospital has about 700 beds and is the main teaching hospital for both clinical and preclinical training of most disciplines.

3.2. STUDY DESIGN AND PERIOD

Hospital based cross-sectional study was conducted from Oct, 2019 to Dec, 2019.

3.3. SOURCE POPULATION

The source population was all type 2 diabetes mellitus patients who visited Tikur-Anbessa Specialized Hospital, Addis Ababa, Ethiopia.

3.4. STUDY POPULATION

The study populations were type 2 diabetics attending the diabetic clinic of TASH and who have been taking metformin during the study period.

3.5. ELIGIBILITY CRITERIA

3.5.1. INCLUSION CRITERIA

Adult type 2 diabetes mellitus patients of both sexes (≥18 years of age) who have been on metformin for 5 months and more were included in the study.

3.5.2. EXCLUSION CRITERIA

Patients with any of the following **documented** conditions:

- ✓ Thyroid disease, liver disease, pernicious anemia, active cancer, end-stage renal disease, pregnant

- ✓ Patients known to have diseases causing vitamin B_{12} malabsorption (alcoholism, atrophic gastritis, celiac disease, Crohn's disease, gastric banding or bypass, partial or complete gastrectomy, Helicobacter pylori infection, human immunodeficiency virus, ileal resection, or chronic pancreatitis).
- ✓ Patients with oral or intramuscular vitamin B_{12} supplement, patients on histamine 2 receptor blocker, Methotrexate, antibiotic (chloramphenicol), and vegetarians. Patients who had documented vitamin B_{12} deficiency and macrocytic anemia prior to the initiation of metformin, and if they had a habit of smoking and drinking alcohol daily. Patients who didn't give informed consent were excluded from the study.

3.6. SAMPLING TECHNIQUE

To recruit study subjects convenient sampling technique was used and type 2 diabetes mellitus patients who came to diabetic center during the study period and those who met the inclusion criteria were selected for this study.

3.7. SAMPLE SIZE DETERMINATION

The required sample size was determined using single population formula for estimating single population proportion. The prevalence of vitamin B_{12} deficiency in type 2 diabetes mellitus patients on metformin has been reported to be between 5.8% and 41%(Reinstatler *et al,* 2012; Owhin *et al.,* 2019). However, we used 5.8% prevalence for our study since it was a National Health and Nutrition Examination Survey. The formula for calculating the sample size (n) would be: $n = \frac{\left(\frac{Za}{2}\right)^2 P(1-p)}{d^2}$, considering the following assumptions:

P= .058

Level of significance = 0.05, and Non-response rate= 10%

Where:

n= sample size

Z(a/2) = Z-score at 95% confidence interval is 1.96;

P= 5.8% = 0.5 (positive prevalence); 1-P=Q= 0.942 (negative prevalence)

d= marginal error=0.05 (5%)

Therefore n becomes: $n = \frac{(1.96)^2 * 0.058(1-0.058)}{0.05^2}$

$$n = \frac{(3.8416)x0.058x(0.942)}{0.0025} \cong 84$$

Due to practical constrains like finance (the cost per sample for vitamin B_{12} and folate were very expensive), a total of 80 study subjects were included in the study.

3.8. STUDY VARIABLES

3.8.1. INDEPENDENT VARIABLES

- ✓ Socio-demographic characteristics (marital status, educational level, sex, residence)
- ✓ Anthropometric parameters (height, weight, BMI)
- ✓ Behavioral factors (smoking, alcohol consumption)
- ✓ Dose of metformin
- ✓ Duration of metformin therapy

3.8.2. DEPENDENT VARIABLES

- ✓ Serum vitamin B_{12} concentration
- ✓ Serum folate concentration
- ✓ Mean corpuscular volume

3.9. OPERATIONAL (WORKING) DEFINITIONS

- ✓ Vitamin B_{12} deficiency is defined as serum vitamin B_{12}concentration of <197 pg/dL (Using the EPHI reference point).
- ✓ Serum folate levels < 4.6ng/mL is considered to be folate deficiency. (Using the EPHI reference point).
- ✓ Macrocytosis is defined as the mean corpuscular volume >96fL (Using the EPHI reference point).

- ✓ Vegans are people who do not eat any animal flesh, dairy milk, dairy cheese, eggs or any other products derived from animal.
- ✓ Literate: a person who can read and write or someone who is educated in a specific area of knowledge.
- ✓ Illiterate: a person who cannot read and write or someone who is not educated in a specific area of knowledge.

3.10. DATA AND BLOOD SAMPLE COLLECTION PROCEDURE

Socio-demographics, clinical and therapeutic data related to type 2 diabetes mellitus were collected from the participants medical history charts (***i-*** care) and interview using Amharic version structured questionnaire by experienced nurse professionals. After an overnight fast, 6 mL of venous blood was collected from each participant and was dispensed into blood collecting tubes; Serum separator and EDTA. (4mL for serum separation, and 2mL for complete blood count). The 4mL blood samples were allowed to retract and then centrifuged at 3000 rpm for 10 minutes to obtain serum samples which were kept at -20^0C until analyzed for vitamin B_{12} and folate levels. CBC was done immediately after collection. Analysis was done at National References Laboratory for Clinical Chemistry, Ethiopian Public Health Institute (EPHI).

3.11. DATA QUALITY CONTROL AND MANAGEMENT

The biochemical tests were analyzed on calibrated COBAS 6000 instrument after internal quality control (IQC) was done. The tests that strictly followed laboratory's SOP were done by well trained and experienced professionals. The laboratory was received accreditation certificate with ISO 15189 standard from Ethiopian National Accreditation Office (ENAO) on May 2017. The hematology test was done using a quantitative, automated UniCel DxH 800 analyzer (Beckman Coulter, USA) after internal quality control was done each day. Data coding, entering, verifying, and cleaning were performed by the investigator with a maximum care.

3.11.1. TEST PRINCIPLES OF THE LABORATORY ANALYTES

MEASUREMENT OF SERUM VITAMIN B_{12}

This study used the electrochemiluminescence immunoassay "ECLIA" which is intended for use on Elecsys and cobas immunoassay analyzers. This method applies various test principles (such as competitive principle, sandwich and bridging) for the measurement. Competitive principle is the most common one in measuring vitamin B_{12} concentration and it is applied to low molecular weight molecules. The Elecsys Vitamin B_{12} II assay applies a competitive test principle using intrinsic factor specific for vitamin B_{12}. In this principle vitamin B_{12} in the sample competes with the added vitamin B_{12} labeled with biotin for the binding sites on the ruthenium‑labeled intrinsic factor complex.

Test principle: Competition principle: Total period of assay: 27 minutes.

▪ 1stincubation: by incubating the sample (15 µL) with the vitamin B_{12} pretreatment 1 and pretreatment 2, bound vitamin B_{12} is released.

▪ 2nd incubation: by incubating the pretreated sample with the ruthenium labeled intrinsic factor, a vitamin B_{12}-binding protein complex is formed, the amount of which is dependent upon the analyte concentration in the sample.

▪ 3rd incubation: after addition of streptavidin-coated microparticles and vitamin B_{12} labeled with biotin, the still-vacant sites of the ruthenium labeled intrinsic factor become occupied, with formation of a ruthenium labeled intrinsic factor vitamin B_{12} biotin complex. The whole complex becomes bound to the solid phase via interaction of biotin and streptavidin.

▪ the reaction mixture is then aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then separated with ProCell/ProCell M. Application of a voltage to the electrode then cause chemiluminescent emission which is measured by a photomultiplier.

▪ the final results are then determined via a calibration curve which is apparatus-specifically generated by two‑point calibration and a master curve delivered via the reagent barcode or e‑barcode.

The analyzer automatically calculates the analyte concentration of each sample (either in pmol/L or pg/mL) (Mathew *et al.*, 2005, Marchese *et al.*, 2009).

Conversion factors: pmol/L x 1.36 = pg/mL

pg/mL x 0.738 = pmol/L

MEASUREMENT OF SERUM FOLATE

This study used electrochemiluminescence binding assay method using Elecsy folate III assay which applies a competitive test principle using natural folate binding protein (FBP) specific for folate. In this technique folate in the sample competes with the added folate (labeled with biotin) for the binding sites on FBP.

Competition principle: Total period of assay: 27 minutes.

▪ 1st incubation: by incubating 25 µL of sample with the folate pretreatment reagents 1 and 2, bound folate is released from endogenous folate binding proteins.

▪ 2nd incubation: by incubating the pretreated sample with the ruthenium labeled folate binding protein, a folate complex is formed, the amount of which is dependent upon the analyte concentration in the sample.

▪ 3rd incubation: after addition of streptavidin‑coated microparticles and folate labeled with biotin, the unbound sites of the ruthenium labeled folate binding protein become occupied, with formation of a ruthenium labeled folate binding protein‑folate biotin complex. The whole complex becomes bound to the solid phase via interaction of biotin and streptavidin.

▪ the reaction mixture is then aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then separated with ProCell/ProCell M. Application of a voltage to the electrode then causes chemiluminescent emission which is measured by a photomultiplier.

▪ the final results are then determined via a calibration curve which is apparatus-specifically generated by 2‑point calibration and a master curve provided via the reagent barcode. The analyzer automatically calculates the analyte concentration of each sample (either in nmol/L or ng/mL). **Conversion factors**: nmol/L x 0.44 = ng/mL

ng/mL x 2.27 = nmol/L

MEASUREMENT OF MEAN CORPUSCULAR VOLUME

A complete blood count (CBC) is performed in duplicate on all study participants aged 1 year and older. The CBC is performed on the Coulter® DxH 800 analyzer using the study participant's EDTA blood tubes. It is a blood panel test that measures the cells that make our blood: red blood cells, white blood cells, and platelets. MCV is derived from RBC histogram using UniCel DxH 800 analyzer (Beckman Coulter, USA). The average volume of individual erythrocytes is derived from the RBC Histogram where the system multiples the number of RBC in each channel by the size of the RBC in that channel. The products of each channel between 36 and 360 femtoliters (fL) are then added. This sum is divided by the total number of RBC between 36 and 360 fL. The analyzer then multiplies by a calibration factor (Hedley *et al*., 2011; Tan *et al.,* 2011).

3.12. DATA PROCESSING AND ANALYSIS

Data were entered into SPSS of version 25 for cleaning, editing, and analysis. Descriptive analysis such as mean, median, standard deviation, range, frequency and percentage were used to present socio-demographic and magnitude of serum biochemical vitamin B_{12} and folate levels. Mann-Whitney U and Kruskal- Wallis H tests were used to determine differences in medians of the variables as appropriate. Independent T and one way ANOVA with welch tests were used to determine differences in means of the variables (age and BI). Spearman correlation coefficient (r) to measure relationship between variables was used. Data with normal distribution were presented as mean ± standard deviation while data with non-normal distribution were presented as median (range). P-values less than 0.05 were considered to be statistically significant. Serum vitamin B12 and folate levels were determined using Electroluminescence (ECL) method. The

reference values used were 197–866pg/mL for vitamin $B_{12,}$ 4.6– 18.7ng/mL for folate, and 80-96fL for MCV according to EPHI.

3.13. ETHICAL CONSIDERATION

Participants were enrolled into this study after explaining the purpose and aims of the study, and obtaining a written informed consent from each of them. Also, an ethical approval (SOM/BCHM/2011) was obtained from departmental research and ethics review committee (DRERC) of the Department of Medical Biochemistry and College of Health Sciences Institutional Review Board.

RESULT

4.1. Socio-demographic and therapeutic characteristics of selected type 2 diabetes mellitus patients who were on metformin

A total of 80 patients with type 2 diabetes mellitus, aged 35 to79 years and who had been on metformin for at least one year, were recruited into this cross-sectional study using a convenient sampling method. The mean ±SD of participant's age was 56.35±10.60, of which 64(80%) were above the age of 56, (57.5%) were female, more than half were married and 97.5% were literate. In addition, the mean ±SD participant's daily dose of metformin was 1200±644.35 mg with 4 years (1-20) as the median duration of metformin intake. In addition, there were respectively 43.8% and 28.7% of patients who were using insulin and glibinclamide in addition to metformin (Table-3).

Table 3: Socio-demographic and therapeutic characteristics of selected type 2 diabetes mellitus patients who were on metformin, Tikur Anbesa Specialized Hospital (TASH), Addis Ababa, Ethiopia, 2019.

Variables	Frequency	Percent
Gender		
Female	46	57.5%
Male	34	42.5%
Age		
≤56 years of age	16	20%
> 56 years of age	64	80%
Marital Status		
Unmarried	7	8.8%
Married	56	70%
Divorced	8	10%
Widowed	9	11.2%
Education		
Illiterate	2	2.5%
Literate	78	97.5%
Residential Area		
Urban	75	93.8%
Rural	5	6.2%
Use of Statins		
Yes	50	62.5%
No	30	37.5%
Use Antihypertensive		
Yes	36	45%
No	44	55%
Use of Glibinclamide		
Yes	23	28.7%
No	57	71.3%
Use of Insulin		
Yes	35	43.8%
No	45	56.2%

4.2. Serum vitamin B_{12}, folate and macrocytic statuses of selected type 2 diabetes mellitus patients

Among all participants, according to the reference values used for vitamin B_{12} (197–866pg/mL), folate (4.6– 18.7ng/mL) and MCV (80- 96fL); the number of patients with vitamin B_{12} values below the lower reference limit and deficient were 4(5%), the number of patients with folate values below the lower reference limit and deficient were 19 (23.8%), and the number of patients with MCV values above the higher reference limit and macrocytic were 5 (6.2%).

Table 4: Serum vitamin B_{12}, folate and macrocytic statuses of selected type 2 diabetes mellitus patients, TASH, Addis Ababa, Ethiopia, 2019.

Variables	Frequency	Percent
Serum Vitamin B_{12} levels		
< 197pg/mL	4	5%
≥ 197pg/mL	76	95%
Serum Folate levels		
< 4.6ng/mL	19	23.8%
≥ 4.6ng/mL	61	76.2%
MCV values		
80- 96fL	75	93.8%
>96fL	5	6.2%

4.3. Serum vitamin $B_{12}$and folate statuses of selected type 2 diabetes mellitus patients based on daily dose of metformin

A total of 43 and 16 participants respectively had a daily dose of metformin below 1000mg, and between 1000mg and 1500mg but none of them developed vitamin B_{12} deficiency. However, vitaminB_{12} deficiency was observed only in 4 out of 21 patients who have used metformin above the dose of 1500mg and it accounted for 5 % out of 80 participants (figure 3).

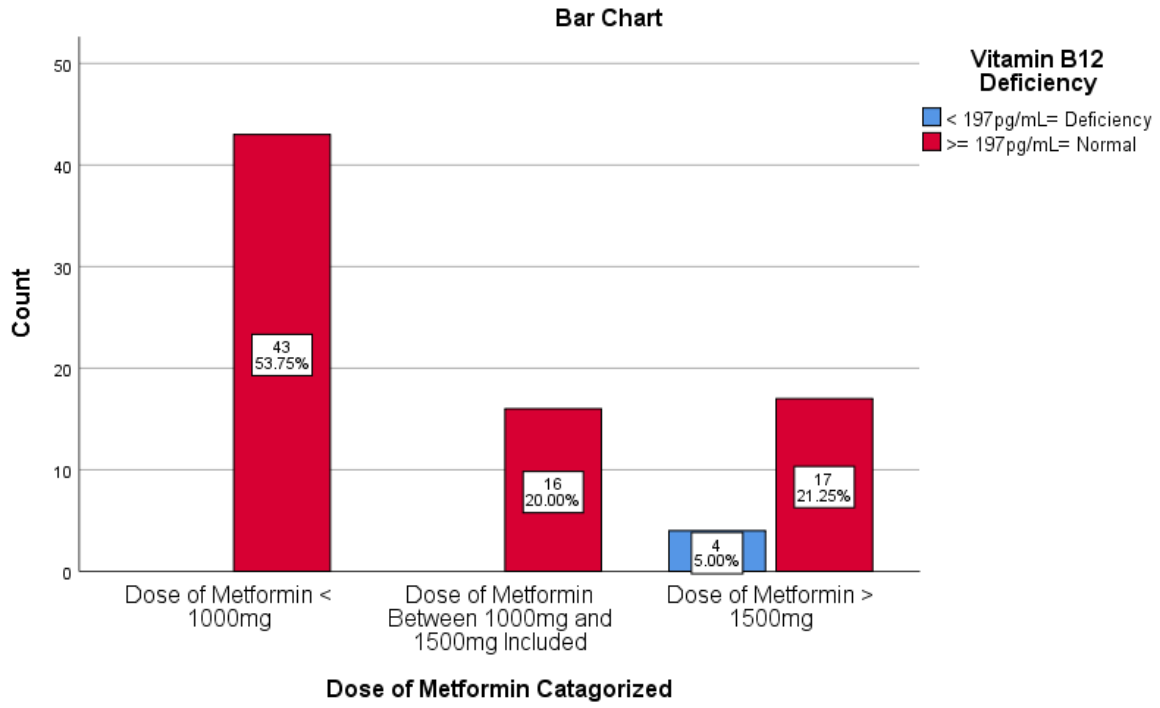

Figure 3: Serum vitamin B_{12} status of selected type 2 diabetes mellitus patients basedon daily dose of metformin catagorized, TASH, Addis Ababa, Ethiopia, 2019.

As it is depicted in the figure below, from 19 patients who had serum folate level below 4.6ng/mL, 12 (63.1%) have taken metformin above the dose of 1500mg, 5(26.3%)between 1000mg and 1500mg and, only 2 (10.6%) were found to have taken metformin below the dose of 1000mg. From 61 patients who had serum folate level above 4.6ng/mL, the vast majority 41(67.2%) of patients were found to have the daily dose of metformin below the dose of 1000mg, 11(18%) between 1000mg and 1500mg and, 9(14.8%) had daily dose of metformin

above the dose of 1500mg. As the dose increases, the percentage of patients with serum folate level above 4.6ng/mL decreases (figure 4).

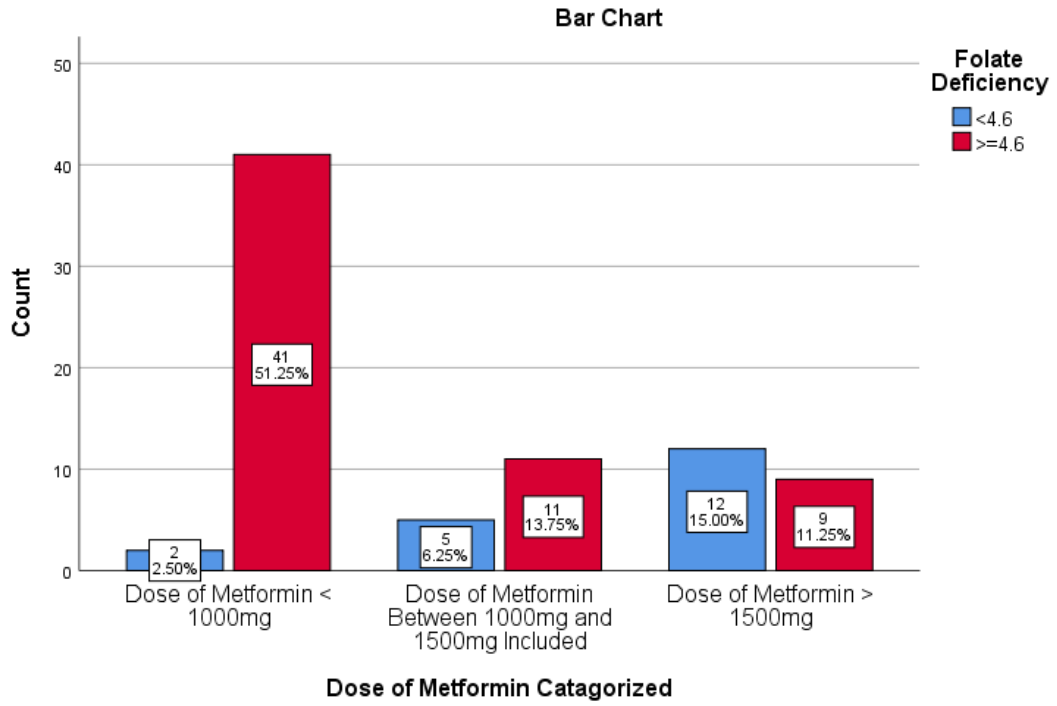

Figure 4: Serum folate status of selected type 2 diabetes mellitus patients based on daily dose of metformin catagorized, TASH, Addis Ababa, Ethiopia, 2019.

4.4. Serum vitamin B_{12} and folate statuses of selected type 2 diabetes mellitus patients based on duration of metformin intake

By taking 4 years as a median year of duration of metformin intake, patients were divided into two groups, those with the duration below 4 years and those with 4 and above years of metformin intake respectively. All patients who had serum vitamin B_{12} below 197pg/mL were found to have taken metformin for more than 4 years. Among patients who have taken metformin for less than 4 years none had serum vitamin B_{12} below 197pg/mL (Table-5).

Table 5: Serum vitamin B_{12} status of selected type 2 diabetes mellitus patients based on duration of metformin intake, TASH, Addis Ababa, Ethiopia, 2019.

Variables	**Vitamin B_{12} levels**		
	< 197pg/Ml	≥ 197pg/mL	Total
Duration of Metformin Intake			
< 4 years			
Count	0	45	45
%	0	100%	100%
≥ 4 years			
Count	4	31	35
%	11.4%	88.6%	100%
Total			
Count	4	76	80
%	5%	95%	100%

From 45 patients who took metformin for less than 4 years, 38(84.4%) had serum folate level above 4.6ng/mL. Among 35participants who took metformin for 4 years and above, 12(34.3%) had serum folate below 4.6ng/mL. It was found that from a total of 19 patients who were initially considered to be serum folate deficient, (63.16%) patients were on metformin for 4 years and above, and only 36.84% patients had duration of metformin intake less than 4 years (Table-6).

Table 6: Serum folate status of selected type 2 diabetes mellitus patients based on duration of metformin intake, TASH, Addis Ababa, Ethiopia, 2019.

Variables	**Folate levels**		
	< 4.6ng/mL	≥ 4.6ng/mL	Total
Duration of Metformin Intake			
< 4 years			
Count	7	38	45
%	15.6%	84.4%	100%
≥ 4 years			
Count	12	23	35
%	34.3%	65.7%	100%
Total			
Count	19	61	80
%	23.8%	76.2%	100%

4.5. A comparison of median serum levels of vitamin B_{12}, folate and MCV in selected type 2 diabetes mellitus patients on different daily dose of metformin

As shown in Table 7, patients were divided into 3 groups based on the daily dose of metformin. It was observed that the median level of vitamin B_{12} in patients who were on metformin at a dose of **>1500** mg/ day was significantly lower ($p<$ ***0.001***) compared to patients who were on metformin at a dose of **1000-1500** mg/day and <**1000**mg/day respectively. Patients who were on metformin at a dose <**1000**mg/day had the highest median level of vitamin B_{12} compared to the other groups of patients. Similarly, the median level of serum folate of patients who were on metformin at a dose >**1500**mg/day was significantly lower compared to patients who were on metformin at a dose of **1000**mg-**1500**mg/day and at dose of <**1000**mg/day respectively ($p=$***0.002***). The median level of MCV of patients was increased from **88.2**fL (for patients on metformin at a dose of **< 1000mg**) to **90.1**fL (for patients on metformin at a dose of **> 1500**mg). Thus, it was insignificantly (***p= 0.518***) higher in patients on the daily dose of metformin above **1500**mg. On the contrary, the mean BMI and age of patients were random values and were not significant.

Table7: Comparison of median serum levels of vitamin B_{12}, folate, and some characteristics of selected type 2 diabetes mellitus patients based on daily dose of metformin, TASH, Addis Ababa, Ethiopia, 2019.

Variables	Daily dose of metformin < 1000mg (n=43)	Daily dose of metformin between 1000mg and 1500mg Included (n= 16)	Daily dose of metformin > 1500mg (n=21)	*P-value*
Age(years)	55.97±10.86	59.50±11.00	54.71±9.70	*0.476*
BMI(kg/m^2)	28.0012±5.02907	28.2719±5.66339	26.8614±5.72798	*0.374*
MCV(fL)	88.2000(80.70-122.10	88.8000(82.90-104.70)	90.10(80.10-107.50)	*0.518*
Folate(ng/mL)	7.54(4.44-20.00)	5.56(4.20-8.55)	4.55(2.49-20.00)	***0.002****
Vitamin B_{12} (pg/mL)	475.50(237.60-930.00)	297.60(206.00-375.60)	240.40(135.40-389.30	***< 0.001****

*Kruskal- Wallis H test. The significance level is 0.05.

4.6. A comparison of median serum levels of vitamin B_{12}, folate and MCV in selected type 2 diabetes mellitus patients on different duration of metformin intake

Based on the duration of metformin intake, patients were again divided in to 2 groups; < 4 years and ≥ 4 years. Patients who have used metformin for more than 4 years and above had significantly lower median level of vitamin B_{12} compared to patients who have used metformin for less than 4 years (*p*= ***< 0.001***). Furthermore, the median level of serum folate of patients who have used metformin for less than 4 years was significantly higher compared to patients who have used metformin for more than 4 years and above (*p*=***0 .010***). The median level of MCV of patients was increased from **88**fL (for patients on metformin for **< 4years**) to **89.1**fL (for patients on metformin for**≥ 4 years**). Thus, it was insignificantly (***p= 0.207***) higher in patients on metformin for ≥ 4 years. On the other hand, the mean BMI was insignificantly lower in patients who have used metformin for ≥ 4 years and, age of patients had insignificant higher values among patients with metformin for ≥ 4 years.

Table 8: Comparison of median serum levels of vitamin B_{12}, folate and some characteristics of selected type 2 diabetes mellitus patients based on duration of metformin intake, TASH, Addis Ababa, Ethiopia, 2019.

Variables	Duration of metformin intake< 4Years(n=45)	Duration of metformin intake ≥ 4 Years(n=35)	*P- value*
Age(Years)	56.17±11.17	56.57±9.96	0.873
BMI(kg/m^2)	28.46±5.44	27.20±5.13	0.315
MCV(fL)	88.00(80.10-97.40)	89.10(80.90-122.00)	0.207
Folate(ng/mL)	7.54(3.88-20.00)	5.95(2.49-11.85)	**0.010***
Vitamin B_{12}(pg/mL)	457.30(221.70-930.00)	263.40(135.40-548.90)	**< 0.001***

*Mann-whitney U test. The significance level is 0.05.

4.7. The relationship between dose and duration of metformin with serum vitamin B_{12} levels

Spearman correlation coefficient (r) to measure relationship between variables was used. It was observed that the relationship of dose and duration of metformin use with serum vitamin B_{12} levels was, respectively, r = **-0.324******,** p = **0.003** and r = **-0.313******,** p = **0.005.** Thus, according to the results, it was found that the dose and duration of metformin had negative relationship with serum vitamin B_{12} levels.

5. DISCUSSION

This study was conducted in Tikur Anbassa specialized teaching hospital with aim of measuring serum vitamin B_{12} and folate levels and, assessing macrocytosis in type 2 diabetes mellitus patients who were taking metformin.

Controlled clinical trials of metformin reported that 7% of patients had subnormal level of previously normal serum vitamin B_{12} without clinical manifestations. Many reports and the package insert of metformin recommend that patients susceptible to low vitamin B_{12} should have routine serum vitamin B_{12} measurements done every 2 to 3 years beside hematological parameters which are recommended to be done on annual basis (Squibb, 2008). Furthermore, to prevent neurological deterioration rather than treat it once it has begun, it has been suggested that annual monitoring of serum vitamin B_{12} measurements had a better impact on patient's health (Ting *et al*., 2006; Wile and Toth, 2010; Kibirige and Mwebaze, 2013). It is also recommended to assess vitamin B_{12} serum concentration prior to starting metformin therapy and then yearly thereafter (Kibirige and Mwebaze, 2013).

The result of this study indicates percentages of patients with vitamin B_{12} levels below the lower reference limit was 5% and is in concordance with the report of Nervo *et al*., 2011; Reinstatler *et al.,* 2012; Akinlade *et al*., 2015; Ahmed *et al.,* 2016; Owhin *et al.,* 2019. This observation could possibly be due to interference with vitamin B_{12} absorption from the B_{12}-intrinsic factor complex as a consequence of prolonged metformin use (Squibb, 2008) or due to nutritional deficiency. The prevalence of vitamin B_{12} deficiency related to metformin use, as reported, varies according to the study population. National Health and Nutrition Examination Survey by Reinstatler and his friends showed that vitamin B_{12} deficiency was present in 5.8% of those with diabetes using metformin compared with 2.4% of those not using metformin (Reinstatler *et al.,* 2012). Similarly, the prevalence of vitamin B_{12} deficiency in patients with type 2 diabetes using metformin was 6.9% from a comparable study reported from Brazil (Nervo *et al*., 2011). Furthermore, cross-sectional studies by Akinlade *et al*., and Ahmed *et al.,* in Nigeria and South Africa respectively reported a 8.6% and 28.1% prevalence of vitamin B_{12} deficiency (Akinlade *et al*., 2015; Ahmed *et al.,* 2016). In 2019, Owhin *et al.,* reported a 41% prevalence (Owhin *et al.,* 2019).

In this study, folate level below the lower reference limit was observed in 23.8% of patients with highest and higher percentages of folate deficiency being observed in patient with highest and longer duration of metformin treatment respectively. This finding is in line with other studies by De Jager and his friemds where folate deficiency was 5% and by Wuffele *et al.* where folate deficiency was 7% (Wulffele *et al.*, 2003; De Jager *et al.*, 2010). However, comparing the percentage of vitamin B_{12} and folate deficiencies of this study with the prevalence of vitamin B_{12} and folate deficiencies in other studies is not straightforward, and should take into consideration several factors. For example, different techniques used in determining serum vitamin B_{12} and folate levels (HPLV vs immunoassay), different cut-off values used for vitamin B_{12} and folate deficiencies determination and, the fact that we didn't include type 2 diabetes mellitus patients without metformin as a control groups are among the factors that should take into consideration.

Many studies investigated the effect of categorical dose and duration of metformin use on vitamin B_{12} level and reported that lower level of vitamin B_{12} is highly prevalent in patients with higher dose and long duration of metformin treatment (Wulffele *et al*., 2003; De Jager *et al*., 2010; Iftikhar *et al.*, 2014; Ko *et al.*, 2014; Akinlade *et al*., 2015; Zalaket *et al.*, 2018). For example, a 417.29pg/dL of vitamin B_{12} level, which was recorded in patients who were on metformin at a dose of <1000 mg/ day, was significantly dropped to 306.98pg/dL in patients on metformin at a dose of ≥ 1000mg (Akinlade *et al*., 2015). In this study a 240pg/mL of vitamin B_{12} level being the lowest was recorded in patients with type 2 diabetes mellitus who had been taking metformin at a dose of > 1500mg compared to the other groups and this finding is entirely consistence with the previous studies by Ko *et al*., 2014; Akinlade *et al.*, 2015; Alharbi *et al*., 2018 where lower level of vitamin B_{12} was observed in patients who took metformin at higher dose compared to their counterparts. However, Nervo *et at.*, reported that vitamin B_{12} has no association with the daily dose of metformin except for duration of metformin use (Nervo *et al*., 2011).

In terms of duration of metformin treatment, as it has been reported by a number of studies, lower level of vitamin B_{12} was recorded in patients with prolonged metformin treatment. For example, a 414 pg/mL of vitamin B_{12} level, which was recorded in patients who took metformin for less than 1 year, was significantly dropped to 188 pg/mL in patients who took it for more

than 1 year (Iftikhar *et al.,* 2014). Furthermore, a 429.48pg/dL and 232.11pmol/L of vitamin B_{12} levels, which were recorded in patients who took metformin for <10 years and 3 months, were significantly dropped to 299.63pg/dL and 177.33pmol/L in patients who took it for ≥10 years and 12 months respectively (Akinlade *et al.,* 2015; Zalaket *et al.,* 2018). This is entirely consistence with the finding of this study where a 457.30pg/mL of vitamin B_{12} level which was observed in patients who took metformin for <4 years was significantly dropped to 263.40pg/mL in those type 2 diabetes mellitus patients who had been on metformin for 4 years or more. However, quite contrary to our study and all other supporting studies, Chen *et al* did not find any significant association between metformin duration and vitamin B_{12} deficiency (Chen *et al.,* 2012).

A long line of studies had already concluded that dose and duration of metformin treatment are the most important risk factors of vitamin B_{12} deficiency in type 2 diabetes mellitus patients on metformin. For example, according to a report by Ko *et al*, subjects with metformin use ≥10 years and daily dosage ≥2,000 mg showed about a 4-fold higher risk of vitamin B_{12} deficiency compared to those with metformin use of <4 years and daily dosage of ≤1,000 mg (Ko *et al*., 2014). Similarly, each 1g daily intake of metformin caused a ratio of 2.88 increases in the risk of developing vitamin B_{12} deficiency. Additionally, metformin treatment for 3 years or more, caused a ratio of 2.39 increases in the risk of developing vitamin B_{12} deficiency as reported by Ting et al, (Ting *et al*., 2006). This potentially could explain our observed lower level of vitamin B_{12} in patients with metformin use for ≥4 years and taking it at >1500mg/day compared to the other group of patients.

The exact pathogenic mechanisms through which high dose of metformin as well as long duration of metformin treatment cause vitamin B_{12} deficiency have not been fully elucidated. However, there are different proposed mechanisms that explain metformin induced vitamin B_{12} deficiency among patients with type 2 diabetes mellitus. Alterations in small bowel motility which stimulates bacterial overgrowth and consequential vitamin B_{12} deficiency, competitive inhibition or inactivation of vitamin B_{12} absorption, alterations in intrinsic factor (IF) levels and interaction with the cubulin endocytic receptor are among the others (Andre`s *et al.,* 2002; Wulffele *et al.,* 2003; Ting *et al.,* 2006). Metformin has also been shown to inhibit the calcium

dependent absorption of the vitamin B_{12}-IF complex at the terminal ileum. This inhibitory effect is reversed with calcium supplementation (Bauman *et al.*, 2000).

According to a number of cohort and case control studies, there is a negative causal relationship between dose and duration of metformin treatment and the level of vitamin B_{12}. This study also revealed a negative cross-section relationship between these variables. However, due to the design of the study, this study was not quite sure about the direction of causality or relationship as patients would have had their serum vitamin B_{12} decreased than normal even before they had started metformin treatment. Thus, due to difference in the design of the study used, the consistency of our finding to the previous studies would not be confirmatory and require further investigation to be done in Ethiopia.

In this study, the recorded low serum folate levels in patients with prolonged and higher dose of metformin treatment in this study are somehow consistent with some other studies. Furthermore, patients who were vitamin B_{12} deficient had their serum folate levels decreased than normal. It has been reported that those type 2 diabetes mellitus patients with vitamin B_{12} deficiency had their serum folate levels decreased than normal (Wulffele *et al*., 2003; De Jager *et al*., 2010; Ko *et al*., 2014). However, this study could not confirm that low folate levels were due to vitamin B_{12} deficiency and how metformin affect folate status is not crystal clear. However, deficiency of any forms of vitamin B_{12} due to different factors including disease, drugs and low vitamin diets may lead to a condition known as folate trap(functional folate deficiency) where free folate is trapped in the form of methyl tetrahydrofolate (Faeh *et al*., 2006). Thus, this could be the possible reasons that type 2 diabetes mellitus patients with vitamin B_{12} deficiency have their serum folate levels below normal in our study. This study assessed folate levels in terms of categorical dose and duration of metformin treatment. However, studies on the levels of serum folate in relation to categorical dose and duration of metformin treatment are quite limited and rare. Thus, it has been found difficult to make communication between findings of this study to the previous ones. Thus, it has been found to be tempting to conclude that the finding is first of its kind at least in Ethiopia.

In our study, the observed macrocytosis in five patients (6.2%) from the total study subjects is in agreement with the study by Ko and his friends (0.5%) (Ko *et al*., 2014) and in disagreement

with the study by Raizada *et al* where they didn't find any increase in MCV (Raizada *et al.*, 2017). Studies that investigated the association of metformin related vitamin B_{12} deficiency with MCV are rare. Moreover, the relationship between MCV and metformin related vitamin B_{12} deficiency varied among studies. For example, Ko *et al.*, reported that there was no association of serum vitamin B_{12} levels with MCV. On the other hand, according to the report by Alharbi *et al*., mean MCV in the metformin group was 89.1 fl compared to 90.2 in the non-metformin group ($p = 0.08$) (Alharbi *et al*., 2018).

Considering the physiological importance of vitamin B_{12} and folate in the development RBC, the probable reasons for the observed higher MCV value in patients who had taken higher dose of metformin for prolonged duration could be low serum vitamin B_{12} and folate levels or otherwise due to some other reasons. Even though there was only small number of patients with macrocytosis, it is tempting to say that the number would have increased if there had been enough number of participants. Thus, this finding should not be ignored as both vitamin B_{12} and folate are the required substrates for the maturation of blood cells and their deficiency could lead to a classic form of anemia known as megaloblastic anemia (Aslinia *et al.,* 2006).

The data available on the association between age, BMI and metformin related vitamin B_{12} deficiency is sparse and controversial. For example, Aroda *et al.*, reported age was not related to vitamin B_{12} deficiency or vitamin $B1_2$ levels. Because it was observed that, in the metformin group, vitamin B_{12} deficiency increased over time in all age categories (Aroda *et al.,* 2016). On the contrary, a univariate analysis by Ahmed *et al*., revealed that vitamin B_{12} deficient participants were significantly older than those with normal vitamin B_{12} levels (62.3 vs. 57 years, $p = 0.012$) (Ahmed *et al.,* 2016). In our study however, age of participants didn't show a specific pattern of consistency as the daily dose of metformin increases and was not significant regarding to the dose of metformin, and insignificantly higher in patients who took metformin for more than 4 years. The mean BMI was insignificantly lower in patients who have used metformin for $\geq$ 4 years. This result is found to be against the report by Akinlade *et al*., where the mean BMI was insignificantly higher in patients who took metformin for $\geq$ 10 years (Vs < 10 years) (Akinlade *et al*., 2015). On the other hand, based on the daily dose of metformin, the BMI of participants were not of specific pattern of consistency and was not comparable to findings in

previous studies. Quite the contrary, there was no association of serum vitamin B_{12} levels with sex, age and BMI according to the report by Ko *et al.*, (Ko *et al.*, 2014). Thus, according to the results of this study, the relationship between age, BMI and metformin related vitamin B_{12} is not crystal clear.

6. STRENGTH AND LIMITATION OF THE STUDY

The main strength of this study was the fact that we tried to assess a medically important yet neglected area in the monitoring of diabetes mellitus patients on metformin. We submitted the laboratory results to the endocrine unit of internal medicine department so that patients could see their vitamin statuses. This study did not answer several questions due to several limitations, however; still we believe this study may have the strength to attract the medical community's attention to this common problem.

This study has important limitations. First, it is a cross-sectional study design and it cannot assess time as a factor, and therefore the results were associations and not causal relationships. Second, age-matched type 2 diabetes mellitus patients who were not using metformin, as a control group, were not included in this study and the sample size is small. Therefore, the observed statistically significant p-values alone cannot be used to make a clinically big picture conclusion. Thus, we cannot be certain that our results can be generalizable.

In a number of cases, normal or high serum vitamin B-12 levels can sometimes be seen in a B_{12} deficient state, and can therefore be misguiding. Additionally, serum total vitamin B_{12} levels may not accurately reflect vitamin B_{12} status of the body and therefore biochemical deficiency often does not result in clinical deficiency (Oberley *et al.*,2013). High levels of Methylmalonic Acid (MMA) and Homocysteine (HC) have been identified as better indicators of functional vitamin B_{12} deficiency than the actual serum vitamin B_{12} level itself (Bailey *et al.*, 2011; Obeid *et al.*, 2013). Thus, the fact that we couldn't assess patient's homocysteine and methylmalonic acid levels is considered to be the other important limitation of this study. Finally, due to financial constraints, this study could not do additional blood test to figure out if vitamin B_{12} and folate deficiencies were causing the observed macrocytosis.

7. CONCLUSION

Our finding is not novel as it has been reported before by a number of studies. However, this study is intended to arouse the clinician's interests to do more on this area. Thus, based on the findings of this study, it could be concluded that those type 2 diabetes mellitus patients with longer duration and higher dose of metformin intake had significantly lowered serum vitamin B_{12} and folate levels. The higher dose and longer duration of metformin intake had negative correlations with serum level of vitamin B_{12} even though it was non-causal and macrocytosis was insignificantly observed in patients with prolonged and higher dose of metformin use.

8. RECOMMENDATIONS

To make a clinically significant conclusion, we would like to recommend any interested bodies to undertake similar kind of study with larger sample size and better study design including biochemical tests for methymalonic acid and homocysteine levels. Although there is no national guideline that recommends routine screening for vitamin B_{12} deficiency among patients with type 2 diabetes on metformin, we would like to suggest the need for routine vitamin B_{12} monitoring in those types 2 diabetes mellitus patients who are on higher daily dose and prolonged duration of metformin treatment.

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10. ANNEXES

Annex I: Information sheet (English version)

Addis Ababa University, School of Medicine, Department of Medical Biochemistry

Research title: Serum vitamin B_{12} and folate levels and, macrocytosis in patients with type 2 diabetes mellitus on metformin attending Tikur Anbassa Specialized Hospital, Addia Ababa, Ethiopia.

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Introduction

You are invited to take part in this research study. Before you decide, it is important for you to understand why the research is done and what it will involve. Please take a time to read or listen the following information carefully and discuss it with others if you wish.

My Name is ----------; I am working in ----------. I am working as data collector in a study conducted by the research team member of Addis Ababa University College of Health Sciences. I would like to inform you that I would have a short interview concerning this study. Before we go to our discussion, I will ask you to listen carefully to what I am going to tell you about the purpose and general condition of the study and tell me whether you agree or disagree to participate in this study.(for those who can't read or write)

Purpose of the study

The aim of this study is to check the serum levels of vitamin B_{12}, folate and, associated macrocytosis in type 2diabetes mellitus patients who are taking metformin. This will be importantfor better treatment strategies and improve prognosis of patients with various compilations that could happen due to the drug they are taking.

Procedure

If you agree to take part in this study, you will be given the consent form to sign, and interviewed by health professional to assess whether you qualify to participate in the study or not. If you are fit for the study, the data collector will ask some questions which are important for our study like socio-demographic. Physical measurements like weight and height will be taken. 6mL of blood sample will be also collected for laboratory examination of vitamin B_{12}, folate and CBC.

Potential risks and Discomforts

There will be minor discomfort during blood sample collection. During sample collection, appropriate precaution will be taken and all samples will be collected by trained health professionals. If anything happened, appropriate medical care will be provided to you.

Potential Benefits

You will not receive any payment during participation in this study as compensation. But,you will have the chance to know your general health status from the project without any direct incentive because the cost will be covered by the project. In addition, the result of the study will be beneficial for the early management of diabetic complications. Hence, you are indirectly benefiting other patients and the society in this respect.

Confidentiality

We respect your privacy and confidentiality. Any information that identifies you will not be shared with anyone else outside the study team. The information we will collect from you as part of the study will be kept in a locked file cabinet, or be protected by a password on the computer

only accessible to personnel involved in the study. There is no sensitive issue that you will be asked related with your social desirability but any information that is obtained in connection with this study and that can be identified with you will remain confidential.

Sharing the result

At the end of the study, the study findings will be published and they will be disseminated to other scientists through publication, and also to your doctor.

The right to refuse

The participation is completely voluntary and you have the right not to participate in this study. You may withdraw at any time and place without consequences of any kind. You may also refuse to give any sample. You can ask any questions regarding to this study and you have a right to get a laboratory diagnosis result for free.

Annex II: Informed Consent (English Version)

Department of medical Biochemistry, School of graduate studies, College of Health Sciences, Addis Ababa University, Consent form for the participation of the study participants in the research project.

Code number _____

Participant's Name _____

By name below, I confirm that I have read and understood this informed consent. I have been informed about the study that aims to measure vitamin B_{12}, folate and associated macrocytosis in type 2 diabetes mellitus patients on metformin. The objective and the application of the study were explained to me and I have been told that the results obtained will help me and the community. I understand that this is a research study and that my participation is voluntary. I understand that I may change my mind about participating at any time, without my medical care or legal rights being affected. I have had the opportunity to ask questions and my questions have been answered. I have been given adequate explanation and understand the purpose, procedures, risks and benefits of the research study. By signing this form, I give my permission for the researchers to have access to my blood sample for the study.

Name of participant	date	signature
Name of investigator	date	signature

Annex III: Questionnaire

Dear respondents the aim of this questionnaire is to collect the necessary data to measure serum vitamin B_{12}, folate and associated macrocytosis in type 2 diabetes mellitus patients on metformin. It is a part of the study for the masters of degree in medical biochemistry at Addis Ababa university school of graduate studies. This study is purely academic and all your responses will be used for accomplishing the requirements of this study. Therefore, you are kindly requested to give correct information accordingly. Thank you for your time and kindness.

SignatureDate

Participant: _____ _____

Interviewer: _____

Please answer every question in the questionnaire by marking "X" in the space or filling the necessary information.

I. Personal information

1. Patient code _____
2. Residential area□ urban □ rural
3. Age (in years) _____
4. Gender: Male ☐ Female ☐
5. Marital status: married ☐ single ☐ widow ☐ divorced ☐
6. Pregnancy: A.YESB. NO
7. Educational status: Can you read, write or took formal education?
   A. Yes B. No

II. Behavioral characteristics

1. Do you drink alcohols such as beer, wine, '**Tela**', '**Areke**' daily?

   A. Yes B. No

2. Do you **smoke tobacco** products daily?

   A. Yes B. No

3. Are you vegan? A. yes B. no

III. Anthropometric measurements

1. Weight _____ kg
2. Height _____cm
3. Body Mass Index_____kg/m^2

IV. Therapeutic information

1. Duration of **Type 2 DM** ----------
2. How many tablets of metformin have you taken per day? dose----------mg/day
3. How long have you taken metformin? duration----------years
4. How many tablets of glibinclamide have you taken per day? dose---------mg/day
5. How long have you taken glibinclamide? duration----------years
6. How many IU of insulin have you taken per day? dose----------IU/day
7. How long have you taken insulin duration?----------years
8. Others

---------- mg/day

----------mg/day

----------mg/day

V. Laboratory information:

1. Serum vitamin B_{12} ---------- pg/dL
2. Serum folate---------- ng/dL
3. MCV----------fL

VI. Check list whether or not patients had the following documented medical conditions and medications in their medical chart. Thus, patients with anyone of the following medical conditions or medications were excluded.

(For data collectors)

Medical condition	YES	NO
Liver disease		
HIV/AIDS,chronic pancreatitis, atrophic gas		
Thyroid disease		
Active cancer		
End-stage renal disease		
Macrocytic anemia		
Vitamin B 12 deficiency		
Gastrectomy		
Pernicious anemia		

Medication	YES	NO
Methotrexate		
Omeprazole		
Ranitidine		
Multi-vitamin sup.		
IM- vitamin B12		
Chloramphenicol		

Annex ሀ፡ **የጥናቱ ተሳታፊዎች የመረጃ ቅፅ**

አዲስ አበባ ዩኒቨርሲቲ የጤና ሳይንስ ኮሌጅየሕክምና ፋካልቲባዮኬሚስተሪ ት/ክፍል

የጥናቱ ርዕስ: Serum vitamin B_{12} and folate levels and, macrocytosis in patients with type 2 diabetes mellitus on metformin

የጥናቱባለቤት፡ወንድወሰን ዋለ

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አማካሪዎች

ሶሎሞን ገነት(ፒ.ኤች.ዲ)

ናናሴኬራን(ፒ.ኤች.ዲ)

ጌታሁን ታረቀኝ (ዶ/ር)

መግቢያ፡ -እባክዎ በዚህ ጥናት ላይ ለመሳተፍ ከመወሰኖ በፊት ጥናቱ ለምን እደሚካሄድና ምንምን ዓይነት ነገሮች እንደሚያስፈልጉት ለማወቅ ጥቂት ግዜ ይወስዱና የሚከተለውን ስለጥናቱ በተመለከተ መረጃ ይመልከቱ አስፈላጊ ከሆነም ከሌሎች ሰዎች ጋር ይወያዩ፡፡ማንኛውም ግልፅ ያልሆነ ነገር ካለ ወይም ተጨማሪ መረጃ ከፈለጉ የጥናቱ ባለቤትን መጠየ ቅይችላሉ፡፡

እኔ አቶ/ወ/ሮ---------- የተባልኩ የጤና ባለሙያ በጥቁር አንበሳ ሆስፒታል የምሰራ ስሆን ጥናቱን በተመለከተ የተወሰኑ ጥያቄዎችን እጠይቆታለሁ፡፡ በተጨማሪም የጥናቱን አላማ አብራራልዎታለሁ፡፡ እርሶም በዚህ ጥናት ላይ ለመሳተፍ ፍላጎኛ መሆኖን እና አለመሆኖን ይነግሩኛል፡፡

የጥናቱአላማ፡-የዝህ ጥናት አላማ **ሜትፎረሚን** ብሚወስዱ የስኳር በሽታ አይነት 2 ታማሚዎች ላይየቫይታሚን ቢ 12 እና ፎሌት መጠን እንዲሁም ተጉዋዳኝ macrocytosis ማወቅ ሲሆን ይህም በመዳኒቱ ምክንያት ሊከሰቱ የሚችሉ የጎንዮሽ ጉዳቶችን አስቀድሞ ለመከላከል ይጠቅማል፡፡

የጥናቱሂደት፡-በዚህ ጥናት ላይ ለመሳተፍ ፍቃደኛ ከሆኑ ከእርሶ የሚጠበቁት የሚከተሉት ናቸው፡፡

1. ምርምሩን ለመስራት የሚያስፈልገው የደም መጠን ስድስት ሲሲ ጥናቱን ለመስራት ብቻ እሚውል፡፡

2. የጥናቱ ባለቤት ጥናቱን በተመለከተ አንዳንድ ጥያቄዎችን ሊጠይቅ ይችላል፡፡

ጉዳት፡- ከዚህ ጥናት ጋር በተያያዘ በጤናም ሆነ በሚያገኙት ተገቢህ ክምና ምንም አይነትጉዳት ስለማያስከትል አይስጉ፡ ፡

በዚህ ጥናት መሳተፍ ሊገኙ የሚችሉ ጥቅሞች:- ይህ ጥናት የማስተርስ ዲግሪ መመረቂያ እንደመሆኑ መጠን በዚህ ጥናት በመካፈልዎ በገንዘብ የሚያገኙት ጥቅም ባይኖርም ከጥናቱ በሚገኘው ውጤት ግን ተጠቃሚ ነዎት፡፡የእርሶዎ ተሳትፎ የእርስዎንና የወገንዎን የስኮር በሽታ እንዲሁም መዳኒቱ ሊያስከትላቸው የሚችለውን ተዛማጅ በሽታዎች ለማወቅና ለማከታተል ከፍተኛ ጥቅም ይኖረዋል፡፡

ሚስጥራዊነት፡-ማንኛውም ከዚህ ጥናት ጋር የተያያዘ የግል መረጃ ሚስጥራዊነት የተጠበቀ ነው፡፡ስለዚህ የጥናቱ መረጃ ይፋ የሚሆነው ለእርሶ ብቻ ነው፡፡ስለሚወሰደው ማንኛውም መረጃዎች ሆነ የጥናት ውጤት ለማሰራጨት በስም ሳይሆን በሚስጥር (ኮድ) የሚመዘገብ ይሆናል፡፡

የተሳትፎ መብት፡-በዚህ ጥናት መሳተፍ ሙሉ በሙሉ በርሶ ፍቃድ የተመሰረተ መሆኑን ልናሳስብ እንወዳለን፡፡በመሆኑም በማንኛውም ግዜ ምንም ዓይነት ምክንያት ሳይሰጡ ከጥናቱ ራስን የማግለል መብት የተጠበቀ ነው፡፡ የሰጡት ደም ናሙና ለዚህ ጥናት እንደሚውል ማድረግ በእርሶ ሙሉ ፈቃድ ብቻ ሲሆን በጥናቱ ላይ ለመሳተፍ መወሰን ወይም አለመወሰን መድሐኒት ወይም ሌላ የጤና አገልግሎት የማግኘት መብት አሁንም ሆነ ለወደፊቱ ምንም አይነት ተፅእኖ አያሳድርብዎትም፡፡

Annex ለ: የተሳታፊ ፊርማ ስምምነት ማረጋገጫ

የሚስጥር ቁጥር ስም----------

እኔስሜከላይየተጠቀሰውተሳታፊ "**የቫይታሚን ቢ 12 እና ፎሌት መጠን እና ፤ ተ□ዳኝ macrocytosis ሜትፎረሚን በሚወስዱ የስኳር በሽታ አይነት 2 ታማሚዎች ላይ**" በሚለው ጥናት ላይ በቂ ገለጻ ተደርጎልኛል፡፡ለጥናቱም የደም ናሙና እንደሚያስፈልግ ተገልጸልኛል፡፡የጥናቱንም አላማዎችም ተረድቻለሁ፡፡

በመጠይቁ ላይ የገለጽኳቸው መረጃዎች በሙሉ በሚስጥር የተጠበቁ እንደሚሆኑ ተነግሮኛል፡፡በጥናቱ ላይ ያለመሳተፍና ማንኛውንም መረጃ ያለመስጠት እንዲሁም በማንኛውም ጊዜ ከጥናቱ ራሴን የማግለል መብቱ የተጠበቀ እንደሆነ ተገልጸልኛል፡፡ስለዚህ ለዚህ ጥናት መረጃና የስምምነት ቃሌን የሰጠሁት በአጠቃላይ ሁኔታውን በመረዳትና በፍጹም ፍቃደኝነት ነው፡፡በተጨማሪም ጥያቄ ለመጠየቅ ተፈቅዶልኝ ለማወቅ የፈለኩትን ያህል ማብራሪያ አግኝቻለሁ፡፡የዚህ ጥናት ተሳታፊ በመሆኔ የማገኘው ጥቅም የሁሉንም ምርመራ ውጤት በነጻ ማግኘት እንደሆነ ተረድቻለሁ፡፡በአጠቃላይ እኔ ከላይ በመተማመኛ ቅፅ የተጠቀሱትን ሁሉ በሚገባና በተረጋጋ መንፈስ አንብቤዋለሁ፡፡ስለዚህ በዚህ ጥናት ለመሳተፍ ፈቃደኛ መሆኔን በፊርማዬ አረጋግጣለሁ፡፡

ፊርማ _____ **ስም** _____

የስምምነቱን ቅጹ ማንበብ ለማይችሉ ተሳታፊዎች መረጃ በሚሰበስበው/በምትሰበስበው አማካሪ ነርስ ይነበባል፡ ፡

Annex ሐ: መጠይቅ

ውድ ተሳታፊያችን የዚህ መጠየቅ አላማ ለጥናቱ የሚያስፍልጉ መረጃዎችን ማሰባሰብ ሲሆን ለጥናቱ መሳካት ትክክለኛ መረጃ በመስጠት ይተባበሩን፡፡

የሚከተሉትን ጥያቄዎች ከተዘረዘሩት አማራጮች በመክበብ ወይንም የተቀመጡትን ክፍት ቦታዎች በመሙላት መልሳችሁን አስቀምጡ

Code ----------

ሀ. የማህበረሰብ እና ስነ ህዝብ ባህሪያትን በተመለከተ

1. ዕድሜ ---------
2. ያታA. ወ B. ሴ
3. ቁመት --------ሜ ሴቲ-------
4. ክብደት--------- Kg
5. የጋብቻ ሁኔታ፡A.ያገባ B.ያላገባ C. የፈታ D.የሞተበት
6. እርግዝና ሁኔታ(ለ ሴት)----------
7. የት/ት ሁኔታ፡ ማንበብ ወይም መፃፍ ወይም መደበኛ ት/ት ተከታትለዋል?
   A. አዎ/ተካታትያለሁ B. አልችልም/አልተከታተልኩም
8. የመኖሪያ ቦታ፡ A. ከተማ B. ገጠር

ለ. የግል ባህሪን በተመለከተ

9. ሲጋራ ያጨሳሉ?
   A. አዎ B. አላጨስም
10. አልኮሆል ለምሳሌ ቢራ ፡ ጠላ፡ ወይን ፡አረቂ የመሳሰሉ መጠጦችን በየቀኑ ይጠጣሉ?

    A. አልጠጣም B. ጠጣለሁ

11. ስጋ እና የእነሰሳት ተዋፅዖ ለምሳሌ ወተት፣ አይብ፣ ቅቤ፣ እንቁላል አትመገቡም ?

    A. አዎ B. አይደለም

ሐ. የስኳር በሽታ አይነት 2 መድሃኒትን በተመለከተ

12. የስኳር በሽታ ከጀመርዎት ምን ያህል አመት ሆነ?----------አመት
13. በቀን ምን ያህል metformin ይወስዳሉ?

A. 500mg C. 1500mg

B. 1000mg D. 2000mg

E. 2500mg

ከዛ በላይ ከሆነይግልፁልን ----------

14. ለምን ያህልጊዜ metformin ወሰዱ(ከ 5 ወርያላነሰ)፡ ----------አመት

15. በቀን ምን ያህል Glibinclamide ይወስዳሉ?

A. 5mg C. 15mg

B. 10mg D. 20mg

16. ለምን ያህልጊዜGlibinclamide ወሰዱ? ----------አመት

17. በቀን ምን ያህል Insulin ይወስዳሉ? dose----------IU/day

ለምን ያህል ጊዜ Insulin ወሰዱ? duration----------years

18. ሌሎች

----------mg/day

----------mg/day

----------mg/day

----------mg/day

መ. ላቦራቶሪ

Serum vitamin B_{12}----------pg/mL

Serum folate----------ng/mL

MCV----------fL

ሠ.በተሳታፊው የጤና የግል ማህደር ዉስጥ ከታች የተዘረዘሩ በሽታዎች ወይንም መድሃኒቶች ተመዝግበው ከተገኙ በቀጥታ ከዚህ ጥናት ውጪ ይደረጋል፡፡ (ሚጃውን በሚበስበው ጠፍ ባለማቆው ሚጋገጥ)

Medical condition	YES	NO
Liver disease		
HIV/AIDS, chronic pancreatitis, atrophic gas		
Thyroid disease		
Active cancer		
End-stage renal disease,		
Macrocytic anemia,		
Vitamin B 12 deficiency		
Gastrectomy,		
Pernicious anemia		

Medication	YES	NO
Methotrexate		
Omeprazole		
Ranitidine		
Multi-vitamin sup.		
IM- vitamin B12		
Chloramphenicol		