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ORIGINAL RESEARCH

Assessment of Serum Vitamin B_{12} and Folate Levels and Macrocytosis in Patients with Type 2 Diabetes Mellitus on Metformin Attending Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia: A Cross-Sectional Study

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Background: Metformin is the first-line drug in the treatment of type 2 diabetes mellitus. Monitoring vitamin B_{12} deficiency associated with long-term and high-dose therapy is not a common practice in many clinical settings in Ethiopia.

Objective: The study aimed to measure levels of serum vitamin B_{12} and folate and to assess the macrocytic status of type 2 diabetes mellitus patients on metformin.

Methods: A cross-sectional study was conducted on 80 type 2 diabetes mellitus patients who had been on metformin for 5 months or more at the diabetic clinic of Tikur Anbessa Specialized Teaching Hospital. Serum vitamin B_{12} and folate levels were quantified by chemiluminescent immunoassays. Mean corpuscular volume was determined by complete blood count. Differences in vitamin B_{12} and folate levels and mean corpuscular volume between different groups were assessed using Kruskal–Wallis *H* and Mann–Whitney *U* tests.

Results: Vitamin B_{12} and folate deficiency were documented in 5% and 23.8% of participants, respectively, and 6.2% of patients were macrocytic. Levels of vitamin B_{12} and folate in patients who had been on metformin >1,500 mg/day ≥4 years were significantly lower those who had been on metformin 1,000–1,500 mg/day and <1,000 mg/day <4 years, respectively.

Conclusion: Low serum vitamin B_{12} and folate levels and macrocytosis were found to be associated with prolonged metformin treatment.

Keywords: macrocytosis, metformin, type 2 diabetes mellitus, vitamin B_{12}, folate

Background

Metformin has been used widely in the treatment of type 2 diabetes for decades.[1,2] The exact mechanism of metformin action has only been partially explored and remains controversial. However, it has been found that the glucose-lowering effect of metformin is mainly due to the inhibition of hepatic glucose output.

Various guidelines advocate the use of metformin as the first-line glucose-lowering agent concurrently with lifestyle-modification approaches if there are no contraindications like renal and hepatic dysfunction.[3,4,4] For example, the American Diabetes Association and American Association of Clinical Endocrinologists recommend the use of metformin as first-line treatment for type 2 diabetes mellitus and prediabetes to prevent progression of the disease. It has been

reported that metformin is a pharmacological cause of vitamin B deficiencies in patients with type 2 diabetes mellitus which leads to long-term deleterious neurological and hematological effects.[5–7]

Vitamin B_{12} is a water-soluble vitamin that plays a fundamental role in DNA synthesis, optimal hemopoiesis, and neurological function. In the human body, vitamin B_{12} is converted enzymatically into its two coenzyme forms, methylcobalamin and adenosylcobalamin.[8–10] 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 to make DNA, the genetic material in all cells. The clinical picture of vitamin B_{12} deficiency is hence predominantly of features of hematological and neurocognitive dysfunction.[8]

In the mitochondria, for example, propionyl-CoA is enzymatically carboxylated to methylmalonyl-CoA, which is then reversibly isomerized to succinyl-CoA by the B_{12}-dependent enzyme methylmalonyl-CoA mutase. As such, deficiency in vitamin B_{12} blocks the production of succinyl-CoA and leads to elevated methylmalonic acid, which induces neuron destabilization and consequential neurological deficits.[11,12] In the cytoplasm, the methylation of homocysteine to produce methionine uses 5-methyltetrahydrofolate as the methyl donor in a reaction catalyzed by methionine synthase that requires methylcobalamin.[10,12] Therefore, vitamin B_{12} deficiency leads to a condition known as folate trap (functional folate deficiency), where free folate is trapped in the form of methyltetrahydrofolate. Therefore, deficiency in any form of vitamin B_{12} and folate ultimately affect methionine metabolism to the point where homocysteine can no longer remethylate back to methionine, causing hyperhomocysteinemia.[13,14]

Many studies have reported that higher doses and prolonged duration of metformin treatment induce vitamin B_{12} deficiency and consequential complications in type 2 diabetes mellitus patients.[5,7,15] This effect is most often seen after the patient has received long-term treatment (ie, ≥6 months) and high doses (ie, >1 g/day) of metformin.[7,16]

Different proposed mechanisms explain metformin-induced vitamin B_{12} deficiency among patients with type 2 diabetes mellitus: alterations in small-bowel motility activating bacterial overgrowth, competitive inhibition or inactivation of vitamin B_{12} absorption, alterations in intrinsic factor (IF) levels, and interaction with the cubulin endocytic receptor.[6] Metformin has also been shown to prevent calcium-dependent absorption of the vitamin B_{12}–IF complex at the terminal ileum.[17]

Even though metformin is a cornerstone in the treatment of type 2 diabetes, many reports and the package insert of metformin advise patients using high doses for a long time to have routine serum vitamin B_{12} measurements done every 2–3 years besides the hematological assessments recommended to be done on annual basis.[18] However, screening type 2 diabetes mellitus patients using high doses for a long time to monitor metformin's side effects like vitamin B_{12} deficiency is not a common clinical practice in many clinical settings in Ethiopia. Therefore, this study aimed to measure levels of serum vitamin B_{12} and folate and assess the macrocytic status of type 2 diabetes mellitus patients on metformin.

Methods

Between October and December 2019, a total of 80 patients with type 2 diabetes mellitus who fulfilled the American Diabetes Association criteria (reported of the expert committee on the diagnosis and classification of diabetes mellitus, 1997), aged 35 to 79 years and who had been on metformin for 5 months and above, were recruited for this cross-sectional study using a convenient sampling method. All patients were from the Diabetic Clinic of Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia.

Exclusion Criteria

Patients with thyroid disease, liver disease, pernicious anemia, active cancer, end-stage renal disease, chronic pancreatitis, *Helicobacter pylori* infection, or HIV, having undergone gastrectomy or ileal resection, documented diagnosed reason for vitamin B_{12} malabsorption (alcoholism, atrophic gastritis, celiac disease, Crohn's disease, gastric banding, or bypass), those on oral/intramuscular vitamin B_{12} supplements, H_2-receptor blockers, proton-pump inhibitors, methotrexate, or chloramphenicol, and vegetarians, pregnant women, alcoholics, and smokers were excluded.

Ethics Approval and Informed Consent

The study adhered to Declaration of Helsinki principles. The study protocol was approved by Addis Ababa University Biochemistry Department Ethics and Research Committee (DRERC). Ethics approval (SOM/BCHM/02/2012) was obtained from the departmental research and ethics review committee of the Department of Medical

Biochemistry and College of Health Sciences Institutional Review Board (DRERC). Informed consent was obtained from participants before sample collection. Confidentiality and privacy were assured.

Data and Blood Collection

Sociodemographic, clinical, and therapeutic data related to type 2 diabetes mellitus were collected from participants' medical history charts and interviews using an Amharic-language structured questionnaire administered by experienced nurses. After subjects had been fasted overnight, 6 mL venous blood was collected from each participant and dispensed into blood collecting tubes with serum separator and EDTA (4 mL for serum separation and 2 mL for complete blood counts). The 4 mL blood samples were allowed to retract and were then centrifuged at 3,000 rpm for 10 minutes to obtain serum samples that were kept at −20°C until analysis for vitamin B_{12} and folate levels. Complete blood countswere done immediately after collection. Laboratory testing was done at the National References Laboratory for Clinical Chemistry, Ethiopian Public Health Institute.

Determination of Serum Vitamin B_{12}, Folate, and MCV

Biochemical tests for serum vitamin B_{12} and folate were analyzed and determined with a Cobas 6000 immunoassay analyzer. A UniCelDxH 800 analyzer (Beckman Coulter) was used for hematology testing to determine mean corpuscular volume (MCV). Vitamin B_{12} deficiency was defined as a serum concentration <197 pg/mL and folate deficiency as serum concentration <4.6 ng/mL. Macrocytosis was defined as MCV <96 fL.

Statistical Analysis

After checking the distribution of vitamin B_{12}, folate, MCV, and other variables for normality using histograms, Mann–Whitney *U* and Kruskal–Wallis *H* tests for abnormally distributed data, were used to determine differences in medians (vitamin B_{12}, folate, and MCV). Independent *t*-tests and one-way ANOVA with Welch tests for normally distributed data were used to determine differences in means of age and BMI. Data with normal distribution are presented as means ± SD, while data with abnormal distribution are presented as medians (IQRs). Spearman's correlation coefficient was used to measure relationships among variables. $P<0.05$ was considered statistically significant. SPSS 25 was used to perform all analyses.

Results

Mean participant age was 56.35±10.60 years, of whom 64 (80%) were above the aged >56 years, 57.5% female, and 93.8% resided in an urban setting. Mean daily dose of metformin was 1,200±644.35 mg, with 4 (1–20) yearsthe median duration of metformin intake. There were 43.8% and 28.7% of patients using insulin and glibenclamide, respectively, in addition to metformin. Vitamin B_{12}- and folate-deficient patients numbered four (5%) and 19 (23.8%), respectively, while the other five (6.2%) patients had macrocytosis (Table 1).

In sum, 43 and 16 participants were taking metformin doses <1,000 mg and 1,000–1,500 mg, respectively, but none had developed vitamin B_{12} deficiency. However, of the 21 who used metformin >1,500 mg, vitamin B_{12} deficiency was observed in four. This accounted for 5% of 80 participants (Figure 1).

As depicted in Figure 2, although there were cases of folate deficiency in patients taking metformin <1,000 mg,

Table 1 Demographic and Clinical Characteristics of Participants (n=80)

Female, n (%)	46 (57.5)
Age (years), mean ± SD	56.35±10.60
Urban-dwelling, n (%)	75 (93.8)
Duration of type 2 diabetes mellitus (years), median (IQR)	5 (1–21)
Duration of metformin treatment (years), median (IQR)	4 (1–20)
Daily dose of metformin (mg), mean ± SD	1,200 ±644.35
Use of insulin, n (%)	35 (43.8%)
Use of glibenclamide, n (%)	23 (28.7%)
Use of antihypertensives, n (%)	36 (45%)
Use of statins, n (%)	50 (62.5%)
Vitamin B_{12} levels, n (%)	
<197 pg/mL	4 (5%)
≥197 pg/mL	76 (95%)
Folate levels, n (%)	
<4.6 ng/mL	19 (23.8%)
≥4.6 ng/mL	61 (76.2%)
MCV, n (%)	
80–96 fL	75 (93.8%)
>96 fL	5 (6.2%)

Abbreviation: MCV, mean corpuscular volume.

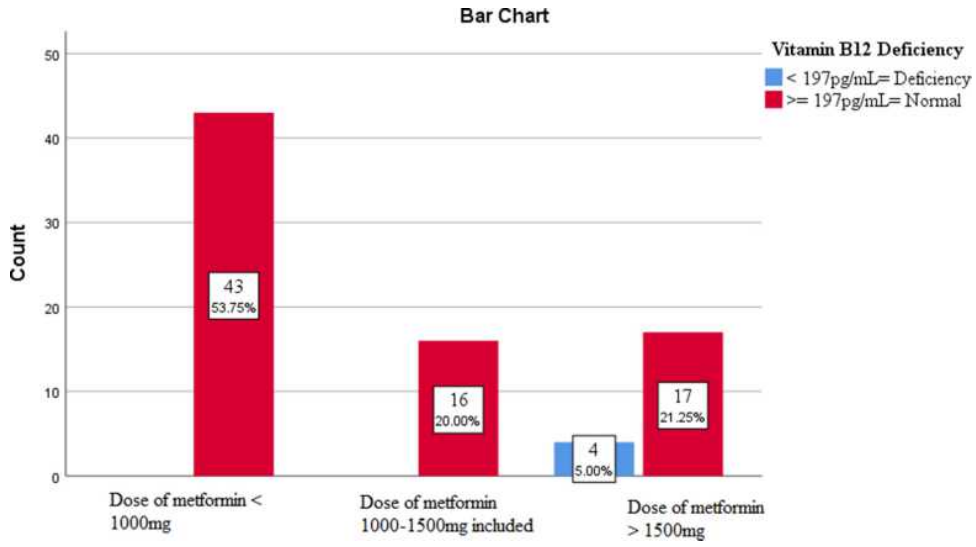

Figure 1 Serum vitamin B_{12} status of selected type 2 diabetes mellitus patients based on daily dose of metformin.

and 1,000–1,500 mg per day, the highest percentage of folate deficiency was observed in patients on metformin >1,500 mg per day. As the dose increased, the percentage of patients with serum folate levels <4.6 ng/mL increased as well (Figure 2).

Based on metformin intake, patients were divided into two groups: <4 years' intake and ≥4 years. Four patients who were found to be vitamin B_{12}-deficient had been taking metformin ≥4 years. However, no patients who had been on metformin <4 years experienced vitamin B_{12} deficiency. Similarly, though there were cases of folate deficiency in patients who had taken metformin <4 years, higher (34.3%) folate deficiency was observed in patients who had been taking metformin ≥4 years (Table 2).

Note: Data expressed as n (%)

As shown in Table 3, patients were divided into 3 groups based on daily metformin dose. The median level of vitamin B_{12} in patients on >1,500 mg/day was significantly lower (P<0.001) than patients on of 1,000–1,500 mg/day and <1,000 mg/day. Those on <1,000 mg/day had the highest median B_{12}. Similarly, median serum folate of patients on >1,500 mg/day was significantly lower than patients on

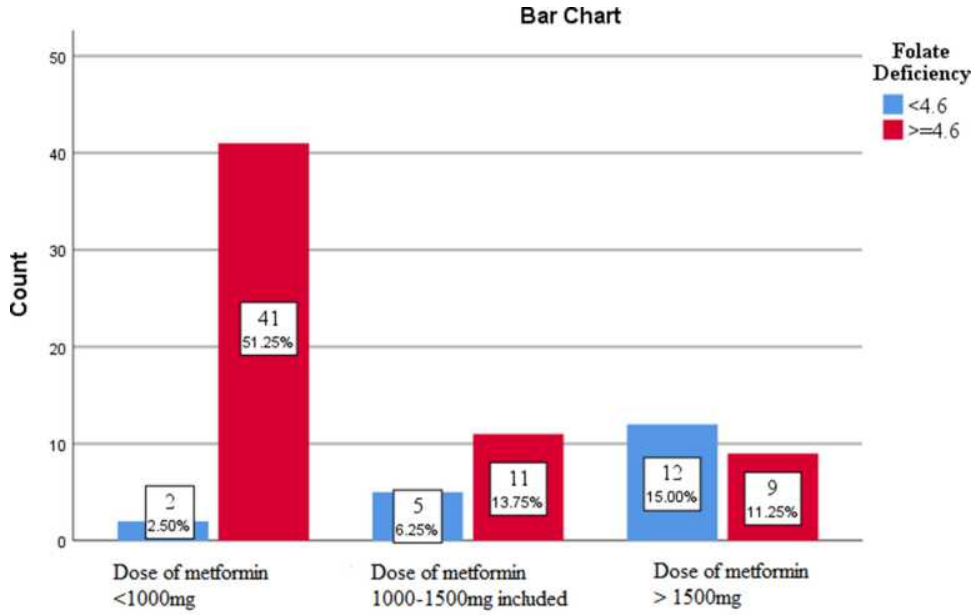

Figure 2 Serum folate status of selected type 2 diabetes mellitus patients based on daily dose of metformin.

Table 2 Serum Vitamin B_{12} and Folate Status of Selected Type 2 Diabetes Mellitus Patients Based on Duration of Metformin Intake

	Vitamin B_{12} levels		Folate levels	
	<197 pg/mL	≥197 pg/mL	<4.6 ng/mL	≥4.6 ng/mL
Duration of metformin intake				
<4 years	0	45 (100)	7 (15.6)	38 (84.40
≥4 years	4 (11.4)	31 (88.6)	12 (34.3)	23 (65.7)

1,000–1,500 mg/day and <1,000 mg/day (P=0.002). Differences in median MCV with the different daily doses was not statistically significant (P=0.518). Similarly, there was no statistically significant difference in mean BMI or age of patients on different doses (Table 3).

Based on duration of metformin intake, patients were again divided into two groups; <4 years and ≥4 years. Patients who had used metformin ≥4 years had significantly lower median vitamin B_{12} than patients who had used it <4 years (P<0.001). Furthermore, median serum folate of patients who had used metformin <4 years was significantly higher than patients who had used it ≥4 years (P=0 0.01). However, neither MCV (P=0.207) nor BMI (P=0.207) was associated with duration of intake (Table 4).

Spearman's correlation coefficients between variables showed negative correlations between doses (r=−0.324***, P=0.003; r=−0.523***, P=0) and duration (r=−0.313***, P=0.005; r=−0.218***, P=0.052) of use with serum vitamin B_{12} and folate levels, respectively. MCV values were positively associated with dose and (r=0.036***, P=0.752) and negatively associated with duration of treatment (r=−0.020***, P=0.864).

Discussion

This study was conducted to determine serum vitamin B_{12} and folate levels and assess macrocytosis in type 2 diabetes mellitus patients on metformin.

Controlled clinical trials of metformin have reported that 7% of patients havesubnormal levels of previously normal serum vitamin B_{12} without clinical manifestations. As well as the package insert for metformin, research has recommended that patients susceptible to low vitamin B_{12} should have routine serum vitamin B_{12} blood tests done every 2–3 years in addition to hematological parameters that are recommended on an annual basis.[18] 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} results in better patient health.[5,7,15,16]

This study indicated an incidence of vitamin B_{12} levels below the lower reference limit of 5%, and is thus in concordance with other reports.[19–21] This could be due to interference with vitamin B_{12} absorption from the B_{12}–IF complex as a consequence of prolonged metformin use[18] or due to nutritional deficiency.[22]

The 23.8% incidence of folate deficiency in patients on higher doses and longer metformin use is in line with other studies.[15,23] However, comparing the percentage of

Table 3 Serum Levels of Vitamin B_{12} and Folate Other Variables Based on daily dose of metformin

	<1,000 mg (n=43)	1,000 mg–1,500 mg (n=16)	>1,500 mg (n=21)	*P*
Age(years)	55.97±10.86	59.5±11	54.71±9.7	0.476
BMI (kg/m^2)	28.0012±5.02907	28.2719±5.66339	26.8614±5.72798	0.374
MCV (fL)	88.2 (80.7–122.1	88.8(82.9–104.7)	90.1 (80.1–107.5)	0.518
Folate (ng/mL)	7.54 (4.44–20)	5.56 (4.20–8.55)	4.55 (2.49–20)	0.002*
Vitamin B_{12} (pg/mL)	475.5 (237.6–930)	297.6 (206–375.6)	240.4 (135.4–389.3	<0.001*

Note: *Data shown as means ± SD or median (range). *P<0.05.

Table 4 Serum Levels of Vitamin B_{12} and Folate and Other Variables Based on Duration of Metformin Intake

	<4 Years (n=45)	≥4 Years (n=35)	*P*
Age (years)	56.17±11.17	56.57±9.96	0.873
BMI (kg/m^2)	28.46±5.44	27.2±5.13	0.315
MCV (fL)	88 (80.1–97.4)	89.1 (80.9–122)	0.207
Folate (ng/mL)	7.54 (3.88–20)	5.95 (2.49–11.85)	0.01*
Vitamin B_{12} (pg/mL)	457.3 (221.7–930)	263.4 (135.4–548.9)	<0.001*

Note: *Data shown as means ± SD or medians (range). *P<0.05.

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 several factors should be borne in mind. For example, different techniques are used in determining serum vitamin B_{12} and folate levels, such as high-performance liquid chromatography vs immunoassay and different cutoff values used for deficiency determination. Furthermore, we did not include type 2 diabetes mellitus patients without metformin intake as a control group, which is among factors that have to be considered when comparing our results and those in the literature.

Many studies have investigated the effect of dose and duration of metformin use on vitamin B_{12} levels and found that lower of vitamin B_{12} levels are highly prevalent in patients on higher doses and longer duration of metformin treatment.[15,19,23–26] In this study, the smallest value (240 pg/mL) for vitamin B_{12} was recorded in patients with type 2 diabetes mellitus with daily intake >1,500 mg. This is entirely consistent with previous studies, where lower levels of vitamin B_{12} were observed in patients on higher doses than control groups.[19,25,27] However, this study is inconsistent with Nervo et al,[22] where vitamin B_{12} had no association with the daily dose, but did for duration of use.

In terms of the metformin-treatment duration, as it, lower levels of vitamin B_{12} in patients on prolonged treatment have been reported by a number of studies.[19,24,26] This is entirely consistent with this study, with 457.3 pg/mL vitamin B_{12} level observed in patients on metformin <4 years and a significant drop to 263.4 pg/mL in those on metformin ≥4 years. However, Chen et al[11] did not find any significant association between metformin duration and vitamin B_{12} deficiency.

Many studies have 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. For example, in Ko et al, subjects on metformin >10 years and on >2 g showed about fourfold the risk of vitamin B_{12} deficiency of those with duration <4 years and on 1 g.[25] Similarly, each 1 g daily intake caused a near-threefold increase (OR 2.88, $p<0.001$) in risk of developing vitamin B_{12} deficiency. Additionally, metformin treatment ≥3 years more than doubles (OR 2.39, $p=0.001$) the risk of developing vitamin B_{12} deficiency according to Ting et al.[31] This could explain the lower levels of vitamin B_{12} in our patients on metformin >4 years and intake >1,500 mg/day than the other group of patients in our study cohort.

The exact pathogenic mechanisms through which high doses of metformin and long duration of treatment cause vitamin B_{12} deficiency have not been fully elucidated. However, different proposed mechanisms 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 IF levels, and interaction with the cubulin endocytic receptor, among others.[6,10] 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.[17]

According to several cohort and case–control studies, there is a negative causal relationship between dose and duration of metformin treatment and level of vitamin B_{12}.[15,23,24,26] This study also revealed a negative cross-sectional relationship between these variables. However, due to its design, it was not quite unequivocal about the direction of causality or relationship, as patients would have had serum vitamin B_{12} lower than normal even before they had started metformin treatment. Therefore, further carefully designed research is required to be done in Ethiopia to ensure the consistency of our findings with those reported in previous clinical studies.

The folate levels in patients on prolonged and higher dos of metformin in this study are partly consistent with the results reported in other studies.[14,23] It has been reported that type 2 diabetes mellitus patients with vitamin B_{12} deficiency had folate levels decreased from normal.[15,23,25] However, our observations could not confirm that low folate levels were due to vitamin B_{12} deficiency, and how metformin affects folate status has not yet been elucidated. Deficiency in any form of vitamin B_{12} can be due to different factors, including disease, drug use, and low-vitamin diet, which can lead to a condition known as folate trap (functional folate deficiency), where free folate is trapped in the form of methyltetrahydrofolate.[30] This could be a possible reason for type 2 diabetes mellitus patients with vitamin B_{12} deficiency in our study having serum folate levels below normal. We assessed folate levels in terms of dose and duration of metformin treatment. However, studies on levels of serum folate in relation to dose and duration of metformin treatment are limited and rare. As such, it was difficult to correlate the results of our study, the first of this kind in Ethiopia, with those of previous articles.

The percentage of patients with macrocytosis was in agreement with Ko et al,[20] but not with Robert et al[27] who did not find any increase in MCV. Studies investigating the association of metformin-related vitamin B_{12} deficiency with MCV are rare and varied. However, considering the physiological importance of vitamin B_{12} and folate in the development of red blood cells, probable reasons for the observed higher MCV values in patients on doses of metformin for prolonged duration could be low serum vitamin B_{12} and folate levels. Though there were few with macrocytosis, this finding should not be ignored, as both vitamin B_{12} and folate are required substrates for the maturation of blood cells, and deficiencies in them could lead to a classic form of anemia known as megaloblastic anemia.[32]

In the present study, age did not show any specific pattern as daily dose and was and was insignificantly higher in patients on metformin >4 years. Other studies have shown conflicting results, with some associating age with vitamin B_{12} deficiency[21] while others finding no relationship.[17,25] In our study, mean BMI was insignificantly lower in patients on metformin >4 years. In Akinlade et al, patients on metformin for a prolonged duration had mean BMI insignificantly higher than controls.[2] On the other hand, in terms of daily dose, BMI did not show a specific pattern of consistency and was not comparable to findings in previous studies. Therefore, a strong unequivocal relationship connecting age, BMI, and metformin-related vitamin B_{12} deficiency could not be established.

Strengths and Limitations

This first Ethiopian study of this kind has several positive aspects. Firstly, as we assessed the folate levels and macrocytosis in addition to serum vitamin B_{12} levels, it could serve as a starting point for further investigations. Secondly, it could be used by physicians as a reference to consider the usefulness of monitoring vitamin B_{12} levels in patients on metformin. This study also has important limitations. Given its cross-sectional design, we were unable to assess time as a factor or hence establish a causal relationship. Age-matched type 2 diabetes mellitus patients not using metformin were not included as a control group. The cohort was also relatively small (80) and there was no age difference among participants. Therefore, to draw clinically significant conclusions, it is recommended that further investigations proceed with larger cohorts, and include biochemical tests for methylmalonic acid and homocysteine levels, considering that these have been identified as better indicators of functional vitamin B_{12} deficiency than actual serum vitamin B_{12} level itself.[33,34]

Conclusion

The findings of this study show that low serum vitamin B_{12} and folate levels were associated with higher doses and a long duration of metformin treatment. Macrocytosis was also observed in patients on prolonged and high-dose metformin use, but with no statistical significance. Therefore, yearly routine monitoring of serum levels of vitamin $B1_{12}$, folate and hematological parameters like MCV and blood film in type 2 diabetes mellitus patients on higher doses and longer duration of metformin treatment are required to monitor the effect of metformin on levels of vitamin B_{12} and folate and macrocytosis.

Abbreviations

BMI, body-mass index; DRERC, Departmental Research and Ethics Review Committee; EPHI, Ethiopian Public Health Institute; IF, intrinsic factor; MCV, mean corpuscular volume.

Data Sharing Statement

The data sets used during the current study are available from the corresponding author on reasonable request.

Author Contributions

All authors contributed to data analysis, drafting, or revising the article, have agreed on the journal to which the article will be submitted, gave final approval to the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no conflicts of interest for this work.

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