https://doi.org/10.31925/farmacia.2021.5.7 **ORIGINAL ARTICLE**

THE FREQUENCY OF VITAMIN B_{12} DEFICIENCY IN PATIENTS WITH TYPE 2 DIABETES MELLITUS TREATED WITH METFORMIN FOR AT LEAST FIVE YEARS

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Abstract

This study was carried out in order to determine the frequency of vitamin B_{12} deficiency in Romanian patients with type 2 diabetes mellitus (T2DM), treated with metformin for at least five years. The study group included 39 T2DM patients and 33 control subjects. A complete blood count was performed and serum vitamin B_{12} concentration was determined. The T2DM patients on metformin had statistically significant lower levels of B_{12} vitamin, adjusted for age and gender, and of haemoglobin compared to control subjects. Older patients, as well as male patients, had statistically significant lower B_{12} vitamin levels. No statistically significant association was found between the vitamin B_{12} level and the duration of metformin treatment. These results suggest the importance of assessing vitamin B_{12} status in T2DM patients treated with metformin on long term.

Rezumat

Scopul studiului a fost evaluarea frecvenței deficitului vitaminei B_{12} la pacienți cu diabet zaharat de tip 2 (DZ2) din România, tratați cu metformin de cel puțin cinci ani (39 de pacienți comparați cu 33 de subiecți sănătoși). A fost efectuată o hemoleucogramă completă și s-a determinat concentrația serică a vitaminei B_{12}. Pacienții tratați cu metformin au avut niveluri semnificativ statistic mai scăzute ale vitaminei B_{12}, ajustate în funcție de vârstă și sex, și ale hemoglobinei în comparație cu subiecții din grupul martor. Nivelurile vitaminei B_{12} au fost semnificativ statistic mai mici la pacienții mai în vârstă, precum și la pacienții de sex masculin. Nu s-a găsit o asociere semnificativă statistic între nivelul vitaminei B_{12} și durata tratamentului cu metformin. Rezultatele sugerează importanța evaluării statusului vitaminei B_{12} la pacienţii cu DZ2 trataţi cu metformin pe termen lung.

Keywords: anaemia, metformin, type 2 diabetes mellitus, vitamin B_{12} deficiency

Introduction

Metformin is the most commonly prescribed drug in the treatment of type 2 diabetes mellitus (T2DM) [31]. The beneficial effects of the treatment with metformin are multiple. It improves the markers of metabolic syndrome, the sensitivity of peripheral tissues to insulin, and reduces the risk of cardio-vascular mortality [10, 14, 22, 35]. Some studies have shown that metformin exerts anti-aging effects [22]. Patients on metformin monotherapy, compared to those on sulphonylurea monotherapy, have a lower risk of mortality [27]. An association between metformin use and the prevention or reduced risk of cancer was observed also [17].

However randomized control trials and cross-sectional studies have reported that metformin users had significantly lower plasma levels of vitamin B_{12} than non-metformin users [8]. In a previous study that evaluated the vitamin B_{12} status in a non-vegetarian sample of the urban adult population in Romania was identified a large number of subjects (50.67%) with marginal depletion of vitamin B_{12} [9]. There are no studies assessing long term metformin treatment-induced vitamin B_{12} deficiency in Romanian patients

with T2DM. Taking into account these observations and the consequences of the untreated vitamin B_{12} deficiency, this study aimed to evaluate the frequency of vitamin B_{12} deficiency in Romanian T2DM patients treated with metformin for at least five years.

Materials and Methods

We performed a cross-sectional analytical study that compared T2DM patients with healthy controls. The study was carried out over two months, from November to December 2018, and was performed in line with the Declaration of Helsinki principles. „Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania Ethics Committee approved the study and all participants signed a written informed consent before being included in the study.

The T2DM group included 39 patients treated with metformin for at least five years. In some patients, the treatment with metformin was associated with other antidiabetics (Amaryl®, Bydureon®, Diaprel®, Januvia®, Forsiga®, Qtern®).

We selected patients from three regional medical centres in Cluj-Napoca, Romania. The control group consisted of non-diabetic voluntary subjects, from the same region, who were similar regarding gender and age with the study group (Figure 1).

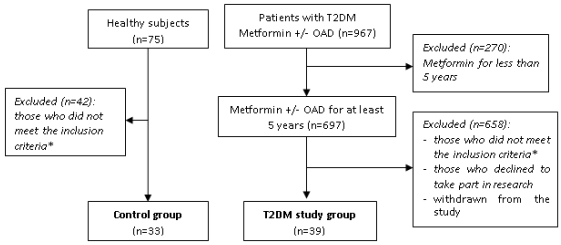

Figure 1.

Flow diagram of the study OAD: oral antidiabetic drugs; T2DM: type 2 diabetes mellitus

*We excluded vegetarians, subjects who have a habit of holding a religiously-motivated fast, and those declaring using vitamin B_{12} supplements or vitamin B_{12} - fortified food before the entrance into the study. Furthermore, we excluded those who presented the following risk factors for B_{12} deficiency: hypothyroidism, renal dysfunction, pancreatic insufficiency, history of partial or total gastrectomy, ileum resection, malabsorption syndromes, medication such as proton pump inhibitors, histamine H_2 - receptor antagonists.

All subjects declared no previous assessments for B_{12} deficiency. All this clinical information was obtained from each participant using a self-administered structured questionnaire.

Venous blood samples were collected after an over-night fast in vacutainer tubes with clot activator for serum vitamin B_{12} assay and in vacutainer tubes with K3-EDTA for complete blood count test. The sample analysis was performed on the day of collection. The serum vitamin B_{12} assays were performed using a chemiluminescence immunoassay on an ADVIA Centaur XPT Immunoassay System (Siemens Healthcare Diagnostics, Tarrytown, NY, USA). The laboratory uses the reference range recommended by Siemens Diagnostics: 211 - 911 pg/mL (information provided in the vitamin B_{12} package inserts). A complete blood count test was performed on an ADVIA 2120i haematology analyser (Siemens Healthcare Diagnostics, Erlangen, Germany). The assays were performed by "Bioclinica" Laboratory in Cluj-Napoca, Romania.

Vitamin B_{12} deficiency was defined at serum levels under 200 pg/mL and a concentration between 200 and 350 pg/mL was defined as borderline level [33]. Anaemia was defined as haemoglobin < 13 g/dL or haematocrit < 38.9% (males) and haemoglobin < 12 g/dL or haematocrit < 34.8% (females) [37].

Statistical analysis

Categorical data were expressed as counts and percentages. Associations between categorical variables were checked with Fisher exact test. For subgroup comparisons, Bonferroni correction was applied. Continuous data following the normal distribution were compared with the t-test for independent samples and presented as means and standard deviations. Continuous data that didn't follow the normal distribution were compared with the Wilcoxon rank-sum test and were presented as medians and quartiles. The normality of the data was checked with Shapiro Wilk test and QQ plots. B_{12} vitamin levels were predicted using simple and multiple linear regressions for the diabetic group having age, gender, and disease duration as explanatory variables; and for all the subjects, having age, gender, and group (diabetic/control) as explanatory variables. All the models were checked for the normality of residuals, heteroscedasticity and linearity assumptions. The multivariate models were checked for multi-collinearity assumptions. For all the built models, these assumptions held, except for heteroskedasticity, where robust confidence intervals were computed. For all models, the coefficients along with 95% confidence intervals were shown. For all statistical tests, a level of significance of 0.05 was used, and the two-sided p-value was computed. All analyses were made using the R environment for statistical computing and graphics, version 3.6.1.

Results and Discussion

The age of the subjects ranged from 42 to 79 years. The characteristics of patients and controls are shown in Table I.

The groups were similar regarding thir demographic characteristics. Among the 39 T2DM patients 87.17% used a total daily dose of metformin of 2000 mg.

The values for serum vitamin B_{12} concentration were significantly lower in the T2DM group compared to the control group. Furthermore, vitamin B_{12} deficiency and borderline B_{12} levels were significantly more

frequent in the T2DM group (Table I). Fifty-seven percent of the patients with vitamin B_{12} deficiency were part of the group with a 5-year duration of the disease and metformin treatment.

Table I

General characteristics of the studied groups

Characteristics	T2DM metformin treated patients (n = 39)	Control group (n = 33)	p-value
Age (years), mean (SD)	63.92 (6.81)	60.91 (8.42)	0.098
Female/male, n (%)	17 (43.59)/22 (56.41)	19 (57.58)/14 (42.42)	0.237
Metformin treatment duration (years), n (%)			
5	12 (30.76)		
6 - 10	13 (33.33)		
11 - 20	13 (33.33)		
> 20	1 (2.56)		
Metformin total daily dose in mg, n (%)			
< 2000	4 (10.25)		
2000	34 (87.17)		
> 2000	1 (2.56)		
Vitamin B_{12} (pg/mL), median (IQR)	281 (225 - 329.5)	389 (325 - 473)	< 0.001
Vitamin B_{12} deficiency: < 200 pg/mL, n (%)	7 (17.94)	0 (0)	0.026
Borderline B_{12} level: 200 - 350 pg/mL, n (%)	27 (69.23)	12 (36.36)	0.018
Haemoglobin (g/dL), median (IQR)	13.3 (12.6 - 14.4)	13.9 (13.4 - 14.9)	0.022
Low haemoglobin, n (%)	10 (25.64)	1 (3.03)	0.008
Haematocrit %, median (IQR)	39.5 (37.75 - 43.1)	42.45 (40.67 - 44.62)	0.007
Low Haematocrit, n (%)	10 (25.64)	1 (3.03)	0.008
RDW %, median (IQR)	14 (13.45 - 14.6)	13.55 (13.17 - 14.03)	0.018
High RDW %, n (%)	13 (33.33)	6 (18.18)	0.146
MCV (fL), median (IQR)	87.6 (84.2 - 91.4)	89.6 (86.1 - 92.3)	0.244

T2DM – type 2 diabetes mellitus; RDW – red blood cell distribution width; MCV – mean corpuscular volume; SD – standard deviation; IQR – interquartile range

Statistically, significantly lower values were observed for the haemoglobin concentration and haematocrit in the T2DM group but the mean corpuscular volume did not differ significantly between the groups. Red cell distribution width was significantly higher in T2DM patients than in control subjects (Table I). Of all patients with T2DM 25.64% were anaemic. Among patients with anaemia, 70% had borderline level of vitamin B_{12}, and 20% had vitamin B_{12} deficiency. However, we did not find a statistically significant correlation between the level of vitamin B_{12} and the concentration of haemoglobin ($p = 0.152$; 95% CI: 0.07 - 0.28), or between haemoglobin concentration and the age of patients ($p = 0.066$; 95% CI: 0.38 - 0.08). Moreover, the lack of association between haemoglobin and vitamin B_{12}, persisted in multivariate analyses adjusted for age, gender, diabetes status. T2DM patients had significantly lower haemoglobin values (by 0.9 g/dL (95% CI: 0.8 - 2.0), $p = 0.009$), compared to controls, adjusted for age and gender.

Next, we wanted to verify if the association between lower values of B_{12} vitamins holds after adjustment in a multivariate model. T2DM patients had statistically significantly lower values of B_{12} vitamin, adjusted for age and gender. The multivariate model was statistically significant ($p < 0.001$) and had a determination coefficient of 0.36. Older subjects, as well as male subjects, had significantly lower B_{12} vitamin levels (Table II).

Table II

Multiple linear regression predicting vitamin B_{12} (pg/mL) levels, using the group (T2DM patients *vs*. Control group), adjusted for age and gender, as well as univariate analyses

	B unadjusted	(95% CI)	p	B adjusted	(95% CI)	p
Age (years)	-5.29	(-8.57 - -2.01)	0.002	-4.58	(-7.42 - -1.74)	0.002
Gender (male *vs*. female)	-51.61	(-103.8 - 0.58)	0.053	-50.44	(-93.38 - -7.5)	0.022
Group (T2DM patients *vs*. Control group)	-125.46	(-170.19 - -80.73)	< 0.001	-104.6	(-147.74 - -61.46)	< 0.001

Within the T2DM patients' group, no statistically significant association was found between the vitamin B_{12} level and the duration of metformin treatment, with or without adjustment for age and gender (Table III). The multivariate model was statistically significant, $p < 0.001$, and had a determination coefficient of 0.08.

This is the first study to report the impact of long term treatment with metformin on the vitamin B_{12} status, in Romanian T2DM patients. This observational study showed that subjects with T2DM treated with metformin had lower values of vitamin B_{12}, even after adjustment for other variables. The present study

highlights not only a high frequency of vitamin B_{12} deficiency but also a borderline level of vitamin in a large number (69.23%) of T2DM patients compared to the control group. The association between low vitamin B_{12} levels and metformin use has been highlighted by many other previous studies [8, 28].

Table III

Multiple linear regression predicting vitamin B_{12} (pg/mL) levels for the T2DM patients, adjusted for age and gender, and metformin treatment duration (identical to disease duration) as well as univariate analyses

	B unadjusted	**(95% CI)**	**p**	**B adjusted**	**(95% CI)**	**p**
Age (years)	-5.29	(-8.58 - -2.01)	0.002	-4.55	(-7.68 - -1.42)	0.007
Gender (male *vs*. female)	-51.61	(-102.9 - -0.32)	0.053	-28.09	(-77.76 - 21.57)	0.275
Metformin treatment duration (years)	-2.08	(-6.73 - 2.56)	0.385	-2.29	(-6.69 - 2.11)	0.314

Our study found 17.94% frequency of vitamin B_{12} deficiency in T2DM patients. Different prevalence rates of vitamin B_{12} deficiency (5.8 - 30%) in T2DM patients have been reported in the literature. These wide variations may be due to differences in the definition of vitamin B_{12} deficiency as well as in the study design [8].

In the current study, the duration of treatment with metformin was between 5 to 25 years and 20 patients in the study group were over 65 years of age. By multiple linear regression analysis, we found that older patients had statistically significantly lower serum vitamin B_{12} levels. A significant negative relationship between age and vitamin B_{12} level in T2DM patients using metformin for 1 - 10 years was reported by Koduri *et al*. [18].

The vitamin B_{12} status depends on both the amount ingested and the proper functioning of the gastrointestinal tract. Vitamin B_{12} in foods is protein-bound and in the stomach is dissociated from food proteins under the action of pepsin and hydrochloric acid secreted by gastric epithelial cells. The absorption of vitamin occurs into the terminal ileum and is mediated by the intrinsic factor, a glycoprotein produced by the parietal cells of the stomach [7].

The elderly is a group at risk of becoming vitamin B_{12} deficient, and this is due to a marked increase of the incidence of pernicious anaemia with age and to a progressive reduction with age in the ability of parietal cells to secrete hydrochloric acid. In the elderly, hypochlorhydria causes reduced pepsin formation and lower pepsin activity, and thus leads to food vitamin B_{12} malabsorption [2, 6]. In a population-based study, Loikas *et al*. observed that in aged persons vitamin B_{12} deficiency was more common in men than in women and the probability for low vitamin B_{12} was twofold greater for men compared to woman [21]. In a study conducted on healthy Swiss seniors, Risch *et al*. reported significant differences in vitamin B_{12} concentration between females and males only in the age group of 70 - 79 years [29]. These observations support our finding concerning the significantly lower levels of vitamin B_{12} in male patients.

In patients with T2DM treated with metformin, the decrease in vitamin B_{12} uptake is also due to the medication. Metformin, administered orally is absorbed in a proportion of approximately 50% through the upper small intestine. The unabsorbed amount accumulates in the gut mucosa of the distal intestine where the absorption is very low and is eliminated with faeces [5, 12, 34]. In the terminal ileum, active absorption of vitamin B_{12} also occurs. The intrinsic factor (IF)-B_{12} complex is absorbed by calcium-dependent endocytosis via a receptor complex between cubilin and amnionless, termed cubam [25]. Active absorption of vitamin B_{12}, IF-mediated, is efficient, but is limited (1.5 - 2 μg). This way the excess of vitamin B_{12} that is not internalized by cells and is secreted with bile (1 - 10 μg/day) is also absorbed. This enterohepatic circuit contributes to the efficient absorption of vitamin B_{12} [15, 30].

One of the most suggested mechanisms by which metformin treatment induces lower plasma levels of vitamin B_{12} in T2DM patients is the inhibition of the calcium dependent absorption of vitamin B_{12}–IF complex at the terminal ileum. Metformin, at physiological pH exists in its cationic form, and in the terminal ileum it competes with calcium for the mucosal cell membrane thus interfering with vitamin B_{12} absorption [12, 25]. Bauman *et al*. observed, in patients with T2DM treated with metformin, that the decrease in the serum vitamin B_{12} level is associated with a decrease in the concentration of holotranscobalamin (holoTCII) which represents the bioavailable form of vitamin B_{12}. This mechanism is supported by the fact that oral calcium administration reverses this effect [3]. Not only the absorption of dietary vitamin B_{12} but also the enterohepatic cycle of the vitamin is inhibited by this mechanism. On the other hand, Greibe *et al*. observed in an experimental study in rats that metformin increases the liver accumulation of vitamin B_{12} associated with decreased vitamin levels in plasma and kidney [13].

In the present study, we also observed the lack of association between the vitamin B_{12} level and the duration of therapy with metformin. This result is consistent with those reported in other studies [4, 19]. In contrast, some authors have shown that the reduction of serum vitamin B_{12} concentration is associated with duration of metformin therapy and is induced in a dose-dependent manner [16, 18, 20, 38]. Our observation concerning the high frequency of anaemia in T2DM metformin treated patients is in

agreement with the results of other studies. Yakubu *et al*. found, based on the haemoglobin level, a 45% prevalence of anaemia among the T2DM patients included in the study and a 32% frequency of vitamin B_{12} deficiency [38]. Zalaket e*t al*. reported a clear association between the level of vitamin B_{12} and macrocytosis as well as anaemia. The presence of anaemia was associated both with borderline and low levels of vitamin B_{12} [39]. The study conducted by De Groot-Kampuis *et al*. reported that anaemia was present in 23.3% of patients treated with metformin and the frequency of anaemia was higher in patients with vitamin B_{12} deficiency [11]. In agreement with our findings, De Groot-Kampuis *et al*. and Abdel-Moneim *et al*. did not observe statistically significant differences concerning the mean MCV in T2DM patients using metformin compared with the control group [1, 11]. In addition, in accordance with the current study, significantly higher RDW values in T2DM patients than in control subjects were also reported by Abdel-Moneim *et al*. and Nada [1, 24]. The complex interaction between the oxidative stress and inflammation in T2DM patients contribute to the alteration of erythrocyte homeostasis and cause an increase in RDW values [1, 23, 26]. Vitamin B_{12} possesses antioxidant properties, and subclinical vitamin B_{12} deficiency may contribute to oxidative stress through various mechanisms [36]. Analysis of the results of large population studies showed an increase in the concentrations of total plasma homocysteine and methylmalonic acid, metabolic markers of vitamin B_{12} deficiency, in subjects with levels of vitamin B_{12} in the low-normal range (< 300 pmol/L) [32].

A limitation of our study is that we determined only the serum concentration of B_{12} vitamin without evaluating the serum level of methylmalonic acid and homocysteine, indicators of cellular vitamin B_{12} deficiency. The small number of subjects who met the criteria for inclusion in the study is also a limiting factor. The use of a control group of short-term metformin-treated patients would have allowed a better assessment of the impact of long term treatment with metformin on vitamin B_{12} status. As with any observational study the associations found might be affected by residual confounders, even with all the care we took by a long list of exclusion criteria and the multiple regression used to take into account several confounders. Further studies with larger sample size, over for long periods, are required to evaluate the consequences of B_{12} deficiency in metformin-treated patient.

Conclusions

Metformin is an invaluable antidiabetic drug, and each patient is unique in terms of vitamin B_{12} status at the beginning of therapy, age, diet, metformin dose, duration of therapy. The increased frequency of vitamin B_{12} borderline levels and vitamin B_{12} deficiency as well as of anaemia observed in T2DM patients treated on long term with metformin, shown the importance of monitoring the status of vitamin B_{12} both prior to the onset and during metformin therapy, to prevent the detrimental effects induced by vitamin B_{12} deficiency.

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Conflict of interest

The authors declare no conflict of interest.

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