Relationship Between Metformin Use and Vitamin B12, Homocysteine and Methylmalonic Acid Levels in Patients with Type 2 Diabetes

Relação entre o Uso de Metformina e os Níveis de Vitamina B12, Homocisteína e Ácido Metilmalónico em Doentes com Diabetes Tipo 2

I. Inácio[1], T. Azevedo[1], A. M. Balsa[2], M. Alves[1], R. Dantas[1], S. Ferreira[1], P. Rosinha[1], J. Guimarães[1,3,4]

1 – Endocrinology Department, Centro Hospitalar do Baixo Vouga, Aveiro, Portugal.

2 – Endocrinology Department, Centro Hospitalar de Trás-os-Montes e Alto Douro, Vila Real, Portugal.

3 – Faculty of Health Sciences of the University of Beira Interior, Covilhã, Portugal.

4 – Department of Medical Sciences of the University of Aveiro, Aveiro, Portugal.

Abstract

Background: Long-term use of metformin may be associated with vitamin B12 (B12) deficiency. Homocysteine (Hcy) and methylmalonic acid (MMA) are more sensitive biomarkers, the latter being more specific. This study aimed to estimate B12 deficiency in Portuguese patients with type 2 diabetes (T2D) taking metformin according to the B12, Hcy and MMA levels and evaluate the correlation with diabetes duration, metformin exposure and doses, anemia and neuropathy.
Methods: Cross-sectional study of Portuguese patients with T2D taking metformin (MET) versus a control group not taking metformin (nMET). A comprehensive analytical profile included B12, Hcy and MMA was measured. Neuropathy was assessed through the Michigan Neuropathy Screening Instrument (MNSI).
Results: A total of 56 patients were studied (64.3% males) with median T2D duration of 12.0 (9.0 - 17.8) years. MET group was composed of 40 and nMET by 16 patients. MET patients had significantly lesser B12 levels [329.5 (246.5-455.0) *vs.* 485.0 (335.0-580.0), p = 0.035]. The prevalence of B12 deficiency (B12 ≤ 211 pg/mL) was 12.5% in MET group and 6.3% in nMET (p = 0.662). Median MMA levels were greater in patients with larger doses (≥2000 mg) of metformin (18.2 *vs.* 6.1 g/L, p = 0.033), and were positively correlated with T2D duration (r = 0.439, p = 0.010) and duration of metformin treatment (r = 0.454, p = 0.026). No statistically significant correlations were found with hemoglobin or MNSI scores.
Conclusions: Portuguese patients taking metformin had lower B12 levels. Despite the small sample size of our study population, our findings suggest the need for routine screening of B12 deficiency in patients with T2D taking metformin at higher daily doses (≥ 2000 mg) and eventually with the additional use of more sensitive biomarker MMA.

Keywords: metformin; diabetes *mellitus*, Type 2; vitamin B12 deficiency; homocysteine; methylmalonic acid

CORESPONDENCE/*CORRESPONDÊNCIA*

Isabel Inácio (ORCID: 0000-0002-5777-5136)
Serviço de Endocrinologia
Centro Hospitalar do Baixo Vouga
Av. Artur Ravara
3810-501 Aveiro
Portugal.
E-mail: isabelmrinacio@gmail.com

> INTRODUCTION

Metformin is the first-line therapy for the treatment of type 2 diabetes (T2D). [1] Nevertheless, metformin has side effects, being associated with vitamin B12 (B12) deficiency by a report from the 2016 Diabetes Prevention Program Outcomes Study (DPPOS). [2] Since 2017, the American Diabetes Association (ADA) Standards of Medical Care in Diabetes states that the long-term use of metformin may be associated with biochemical B12 de-

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Resumo

Introdução: O uso prolongado de metformina pode estar associado à deficiência de vitamina B12 (B12). Homocisteína (Hcy) e ácido metilmalónico (MMA) são biomarcadores mais sensíveis, sendo este último mais específico. Este estudo teve como objetivo estimar a deficiência de B12 em doentes portugueses com diabetes tipo 2 (T2D) sob tratamento com metformina de acordo com os níveis de B12, Hcy e MMA e avaliar a correlação com a duração da diabetes, exposição e doses de metformina, anemia e neuropatia.
Métodos: Estudo transversal de doentes portugueses com T2D sob metformina (MET) versus um grupo controlo não medicado com metformina (nMET). Foi realizado um perfil analítico abrangente incluindo B12, Hcy e MMA. A neuropatia foi avaliada através do *Michigan Neuropathy Screening Instrument* (*MNSI*).
Resultados: Um total de 56 doentes foram avaliados (64,3% do sexo masculino) com duração mediana da T2D de 12,0 (9,0 - 17,8) anos. O grupo MET foi composto por 40 e o nMET por 16 doentes. Os doentes com MET apresentaram níveis de B12 significativamente mais baixos [329,5 (246,5 - 455,0) *vs.* 485,0 (335,0 - 580,0), p = 0,035]. A prevalência de deficiência de B12 (B12 ≤ 211 pg/mL) foi de 12,5% no grupo MET e 6,3% no nMET (p = 0,662). Os níveis medianos de MMA foram superiores em doentes com doses maiores (≥ 2000 mg) de metformina (18,2 *vs.* 6,1 g/L, p = 0,033), e correlacionaram-se positivamente com a duração da T2D (r=0,439, p=0,010) e duração do tratamento com metformina (r = 0,454, p = 0,026). Não foram encontradas correlações estatisticamente significativas com hemoglobina ou scores do *MNSI*.
Conclusões: Doentes portugueses medicados com metformina apresentaram níveis mais baixos de B12. Apesar do reduzido tamanho da população do estudo, os nossos resultados sugerem a necessidade do rastreio periódico da deficiência de B12 em doentes com T2D sob metformina em doses diárias mais altas (≥2000 mg) e, eventualmente, com o uso adicional do biomarcador MMA.

Palavras-chave: metformina; diabetes *mellitus* tipo 2; deficiência de vitamina B12; homocisteína; ácido metilmalónico

ficiency and periodic measurement of B12 levels should be considered in metformin-treated patients, especially in those with anemia or peripheral neuropathy. [3] The latest version of the ADA guidelines continue to support this recommendation based on the 2016 DPPOS study and a post-hoc analysis of a recent randomized control trial. [1,2,4]

The prevalence of B12 deficiency with metformin use has been reported to be 4.3% to 30%, varying with race, metformin doses and the use of different biomarkers or distinct standard values. [2,5-7]

There are several biomarkers of B12 deficiency. The ADA recommends periodic measurement of B12 levels, without making strong recommendations, although isolated serum B12 measurement has low sensitivity and specificity. [4,8,9] Homocysteine (Hcy) and methylmalonic acid (MMA) are more sensitive biomarkers, the latter being more specific. [4,9-11]

Moreover, there is no recommendation on how often this screening should be performed in the ADA guidelines, as well as the best form of prevention and treatment. Additionally, it remains unknown to what extent the effect of metformin on B12 may contribute to the development of clinically relevant endpoints, such as neuropathy and anemia. [12] Despite evidence supporting metformin-induced vitamin B12 deficiency and its associated complications, the monitorization of B12 levels is still seldom performed in clinical practice. [2,8,13-15]

To date, only one retrospective study has evaluated the prevalence of B12 deficiency among Portuguese patients with T2D. B12 deficiency was reported in 24.7% of patients. However, in this study by Bello *et al.*, the authors point out as study limitations the non-inclusion of more reliable biomarkers of B12 deficiency (such as Hcy and MMA) and that no correlation was established between laboratory parameters and clinical data. [16]

Thus, the aims of our study were to estimate B12 deficiency in Portuguese patients with T2D taking metformin according to the serum levels of B12, Hcy and MMA and examine the correlation with diabetes duration, metformin exposure and doses, anemia and neuropathy.

> MATERIALS AND METHODS

This cross-sectional observational study was carried out in Endocrinology Department of Centro Hospitalar do Baixo Vouga (CHBV) in Portugal, from february to december 2018. The sample included Portuguese adult patients (≥ 18 years) with T2D with ≥ 1 year of diagnosis. We excluded patients with of gastric and/or intestinal surgery, known malabsorption syndromes, vitamin B12 supplementation in the 3 months prior to enrolment, pernicious anemia, vegetarians, vegans or pregnant women. After analysis of 115 eligible patients, 56 T2D patients were included in this study (Figure 1). Patients were divided into 2 groups: those taking metformin (MET) and a control group not taking metformin (nMET).

Demographic and clinical data was obtained from medical appointments and medical records, and included age, gender, diabetes duration, ethnic and socioeconomic data (ethnicity, literacy, low income, living alone, degree of dependence), presence of microvascular [dia-

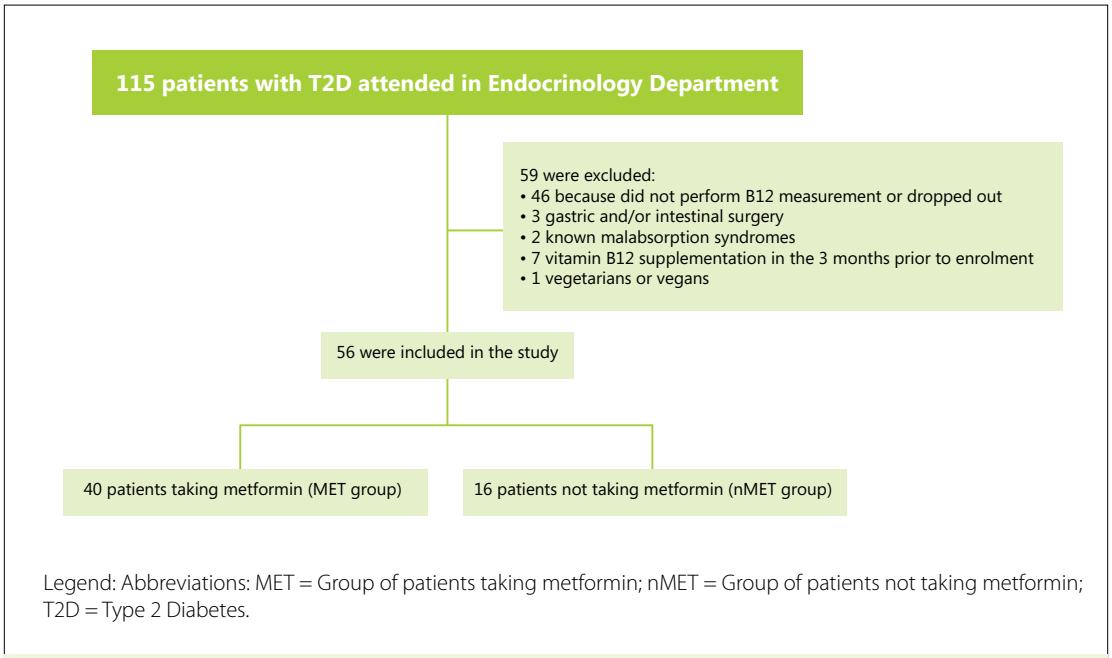

Figure 1 - Recruitment and participants flowchart.

betic retinopathy, nephropathy and peripheral neuropathy (PN)] and macrovascular disease [cerebrovascular disease, ischemic heart disease and peripheral arterial disease (PAD)], smoking habits, dietary history, use of acid-blocking drugs and calcium or vitaminic supplements, recent transfusions, comorbidities and other possible causes of neuropathy [toxins or neurotoxic medications (alcohol, chemotherapy), hypothyroidism, kidney disease, malignancies (multiple myeloma, bronchogenic carcinoma), human immunodeficiency virus (HIV), chronic inflammatory demyelinating neuropathy, inherited neuropathies, or vasculitis]. Peripheral neuropathy was assessed by the validated portuguese version of the Michigan Neuropathy Screening Instrument (MNSI). [15] Physical examination also included height, weight and blood pressure.

Metformin users had the starting date and initial and current dose collected.

Laboratory investigations included blood count, peripheral blood smear (PBS), HbA1c, renal and liver function, estimated glomerular filtration rate (eGFR), TSH, FT4, measurement of B12, folate, Hcy and MMA. Sample collection was requested, but not performed or supervised by the authors and consequently some parameters were not determined for all subjects. Low vitamin B12 was defined as ≤ 211 pg/mL, and borderline-low levels were defined as between 212 and 246 pg/mL inclusive, according to the values provided by the manufacturer. Low folate was defined as ≤ 5.38 ng/mL, according to the manufacturer.

Elevated homocysteine was defined as ≥ 15 µmol/L and elevated MMA as > 32 ug/L, also according to the values provided by the manufacturer. Anemia was defined as hemoglobin < 12 g/dL or hematocrit <36% (females) and hemoglobin < 13 g/dL or hematocrit < 40% (males).[2,18]

The study has been approved by the local Ethics committee at CHBV and is performed according to the Declaration of Helsinki. All participants gave written informed consent.

The statistical analysis was performed using IBM SPSS Statistics®, version 23.0. As the Kolmogorov-Smirnov test did not show a normal distribution, continuous variables were expressed as median values (25-75 percentiles). Categorical variables were displayed as frequencies using the Fisher's exact test. For continuous variables the appropriate non-parametric (Mann-Whitney) test was used. Spearman correlation analyses were used to study associations between variables. Statistical significance was considered if $p < 0.05$.

> RESULTS

A total of 56 patients were evaluated: 40 patients taking metformin (MET) and 16 controls not taking metformin (nMET). The patients were between 38 to 83 years of age, and most patients included were male (n = 36, 64.3%).

The demographic and clinical characteristics of these patients are shown in Table I. No patient had use of calcium supplements that could decrease B12 deficiency prevalence, recent transfusions that could modify A1C, or causes of neuropathy other than diabetes such as chemotherapy, history of multiple myeloma, bronchogenic carcinoma, HIV, chronic inflammatory demyelinating neuropathy, inherited neuropathies or vasculitis.

Comparing MET to nMET group, in the MET group the median B12 levels were sufficient but significantly lesser, and the prevalence of B12 deficiency (12.5 *vs.* 6.3%, p = 0.442) and borderline-low vitamin B12 levels (25.0 *vs.* 6.3%, p = 0.107) were greater but nonsignificant (Table II). Although there were no statistically significant differences, MMA elevation (> 32ug/L) was greater in MET (23.1 *vs.* 14.3%), but Hcy elevation (≥ 15 µmol/L) alone and combined with MMA elevation was lesser (42.9 *vs.* 66.7%; 12.1 *vs.* 14.3%, respectively).

MET patients were exposure to metformin for 10.0 (5.0 - 15.0) years, with a median initial daily dose (n = 19) of 1000 (1000-2000) mg and a median current daily dose (n = 40) of 2000 (2000-2500) mg.

Median MMA levels were greater in patients with larger doses (≥ 2000 mg) of metformin compared with lesser doses (< 2000 mg) (18.2 *vs.* 6.1 g/L, p = 0.033), with no statistically significant difference according to duration of metformin use. Median B12 and homocysteine levels were not significantly different when assessed for duration and current daily dose of metformin (Table III). No statistically significant differences were found between median B12, Hcy or MMA levels with current daily metformin dose of ≥ 1000 mg, compared with lesser dose (< 1000 mg).

Median MMA levels were correlated with T2D duration (Spearman's rho = 0.439, p = 0.010) and exposure to metformin (rho = 0.454, p = 0.026). Median Hcy levels were correlated with hematocrit (rho = -0.308, p = 0.042), creatinine (rho = 0.678, p ≤ 0.001) and urea (rho = 0.568, p ≤ 0.001).

No statistically significant correlations were found between median B12, Hcy or MMA levels with age, doses of metformin, A1C, folate, hemoglobin, hematocrit, mean corpuscular volume or MNSI scores.

We found a significantly higher prevalence of anemia in the nMET group. However, a higher mean corpuscular volume and macrocytosis was detected in the peripheral blood smear in the MET group, although not statistically significant. The MNSI scores by section or global were not different between the MET and nMET groups (Table I).

In addition, the patients of nMET group had significantly higher prevalence of diabetic nephropathy and use of acid-blocking drugs, and tended to have lower folate levels although within the normal range (Table I and II).

> DISCUSSION

The prevalence of B12 deficiency in the population with T2D under treatment with metformin described in the literature is heterogeneous, due to the use of different biomarkers (vitamin B12, hcy and/or MMA), methodologies and cut-off levels. It is difficult to evaluate the accurate prevalence of B12 deficiency due to the absence of a gold standard. [17,19] Nevertheless, the prevalence of vitamin B12 deficiency found in our study is consistent with previous reports (4.3 to 30.0%). [2,6,7] According to our knowledge, only one retrospective study has estimated the prevalence of B12 deficiency among Portuguese patients with T2D using metformin. The study included 627 patients with T2D treated with metformin and they found that B12 deficiency was present in 24.7% of their population. [16]

By measuring MMA or Hcy levels, we found more patients with B12 deficiency than by measuring B12 alone. This is expected, because Hcy and MMA are more sensitive biomarkers. [9] In spite of the well documented superior sensitivity of MMA compared to Hcy, [4,9,10] in our population, a higher prevalence of vitamin B12 deficiency was detected based on serum elevated Hcy levels (42.9%), compared to elevated serum MMA (23.1%). This can be justified by the fact that a smaller number of patients measured MMA, compared to Hcy.

Somewhat paradoxically, Hcy elevation alone and combined elevation of Hcy and MMA was higher in percentage in nMET than in MET group, but no significant difference was found. In nMET group, the patients had significantly higher prevalence of diabetic nephropathy and tended to have lower folate levels although within the normal range. This can be interpreted considering that homocysteine is also increased in kidney failure and folate deficiency. [9,10,19,20] Furthermore, these findings reiterate the greater specificity of elevated MMA level for B12 deficiency and, despite the small sample size of this study, it suggests that MMA could be a better follow-up marker than Hcy in patients with diabetes, especially with impaired kidney function and other interfering conditions. [9,10,19]

Previous studies reported an association between low serum B12 and metformin treatment. [4,21] In our study, patients taking metformin had significantly lower levels of B12.

Several studies have shown that daily metformin doses

Table I - Demographic and clinical characteristics of the study population.

	MET	n	nMET	n	*p* value
Age, years	60.5 (53.3-72.0)	40	70.0 (57.5-73.0)	16	0.220
Male, n (%)	27 (67,5%)	40	9 (56,3%)	16	0.540
Diabetes duration, years	12.0 (9.0-15.0)	40	14.0 (8.5-23.8)	16	0.408
BMI, kg/m^2	31.6 (27.7-34.6)	40	30.7 (27.0-35-0)	16	0.911
Caucasians, n (%)	40 (100.0%)	40	16 (100.0%)	16	-
Literacy, years	6.0 (4.0-9.0)	39	8.0 (4.0-10.5)	12	0.911
Low income, n (%)	11 (28.2%)	39	4 (30.8%)	13	0.999
Living alone, n (%)	10 (26,3%)	38	4 (30,8%)	13	0.734
Degree of dependence, n (%)		39		15	0.065
Autonomous	27 (69.2%)		6 (40.0%)		
Partially dependent	12 (30.8%)		9 (60.0%)		
Totally dependente	0		0		
Alcohol consumption, n (%)	25 (64.1%)	39	9 (56.3%)	16	0.761
Current smoker, n (%)	4 (40.0%)	40	1 (6.3%)	16	0.999
Dyslipidemia, n (%)	29 (74.4%)	39	16 (100.0%)	16	0.026
Hypertension, n (%)	33 (84.6%)	39	15 (93.8%)	16	0.660
Systolic Blood Pressure, mmHg	134.5 (126.0-153.8)	40	141.5 (128.0-158.5)	16	0.549
Diastolic Blood Pressure, mmHg	77.0 (71.0-87.0)	40	73.0 (66.5-93.0)	16	0.457
Cardiovascular disease, n (%)		40		16	0.774
Cerebrovascular disease	5 (12.5%)		2 (12.5%)		
Ischemic heart disease	10 (25.0%)		7 (16.0%)		
Peripheral arterial disease	10 (25.0%)		8 (50.0%)		
Diabetic retinopathy, n (%)	11 (27.5%)	40	8 (50.0%)	16	0.129
Diabetic nephropathy, n (%)	16 (40.0%)	40	14 (87,5%)	16	0.002
Peripheral neuropathy ‡– MNSI, n (%)					
Score A ≥ 3	11 (31.4%)	35	6 (46.2%)	13	0.667
Score B ≥ 2	30 (90.9%)	33	9 (90.0%)	10	0.668
Score A ≥ 3 or Score B ≥ 2	30 (90.9%)	33	11 (91.7%)	12	0.714
Dairy/meat/fish not daily, n (%)	3 (7.9%)	38	1 (7.7%)	13	0.734
Acid-blocking drugs†, n (%)	9 (22.5%)	40	9 (56.3%)	16	0.025
Vitaminic supplements, n (%)	1 (2.6%)	39	0	16	-
Periodontal disease, n (%)	13 (34.2%)	38	1 (7.7%)	13	0.082
Hypothyroidism, n (%)	2 (5.0%)	40	2 (12.5%)	16	0.570
Anemia*, n (%)	6 (15,4%)	39	7 (43,8%)	16	0.037

Legend: Data are presented as median (25-75 percentiles) or number (%), unless otherwise specified. Abbreviations: BMI = Body mass index; H2 blocker = Histamine 2 receptor blocker; MET = Group of patients taking metformin; MNSI = Michigan Neuropathy Screening Instrument; nMET = Group of patients not taking metformin; PPI = Proton pump inhibitor.
*Anemia was defined as hemoglobin < 12 g/dL or hematocrit < 36% (females) and hemoglobin < 13 g/dL or hematocrit < 40% (males). † Acid-blocking drugs included H2 blocker or PPI. ‡ In Portuguese patients, the best cut-off for sensitivity and sensitivity of the MNSI to discriminate between absence and presence of diabetic neuropathy is ≥ 3 for section A and is ≥ 2 for section B.(15)

Table II - Laboratory parameters of the study population.

	MET	n	nMET	n	*p* value
Fasting glucose, mg/dL	135.0 (71.0-167.0)	39	134.0 (98.0-187.0)	15	0.839
A1C, %	7.5 (6.7-8.7)	40	7.5 (6.3-8.8)	16	0.842
Hemoglobin, g/dL	15.0 (13.2-16.2)	39	13.3 (11.2-15.1)	16	0.013
Hematocrit, %	45.8 (41.0-48.0)	39	40.9 (35.0-46.8)	16	0.035
Mean Corpuscular Volume, fL	94.3 (89.5-98.5)	39	93.7 (90.1-99.0)	16	0.999
Macrocytosis at PBS, n (%)	3 (7,7%)	39	1 (6,7%)	15	0.999
Neutrophils hypersegmented at PBS, n (%)	0 (0%)	39	0 (0%)	15	-
Total cholesterol, mg/dL	152.0 (133.3-177.0)	40	147.5 (136.3-169.5)	16	0.420
HDL-cholesterol, mg/dL	41.1 (36.8-48.7)	40	39.6 (31.2-46.8)	16	0.452
LDL-cholesterol, mg/dL	94.5 (76.3-113.5)	40	78.0 (61.8-94.3)	16	0.029
Triglyceride, mg/dL	143.0 (98.5-183.3)	40	151.5 (107.8-204.3)	16	0.360
AST, IU/L	21.0 (19.0-28.0)	39	22.0 (19.0-29.0)	16	0.802
ALT, IU/L	27.0 (20.0-32.5)	40	26.0 (18.5-36.5)	16	0.657
Creatinine, mg/dL	0.9 (0.8-1.1)	40	1.8 (1.3-2-3)	16	<0.001
Urea, mg/dL	46.3 (38.7-55.1)	40	92.9 (73.7-109.7)	16	<0.001
eGFR <60 mL/min/1.73m^2	8 (20.0%)	40	13 (86.7%)	15	<0.001
uACR ≥30mg/mg Cr, n (%)	19 (48.7%)	39	7 (70.0%)	10	0.199
TSH, mU/L	1.9 (1.2-2.6)	39	1.7 (1.0-2.5)	16	0.572
T4L, ng/dL	1.2 (1.1-1.2)	40	1.1 (1.0-1.3)	14	0.867
Vitamin B12, pg/mL	329.5 (246.5-455.0)	40	485.0 (335.0-580.0)	16	0.035
Folate, ng/mL	7.8 (6.4-10.0)	40	6.6 (5.3-8.4)	16	0.063
Homocysteine (Hcy), µmol/L	14.2 (12.2-17.5)	35	20.4 (11.9-22-7)	9	0.171
Methylmalonic acid (MMA), µg/L	14.5 (6.8-30.5)	26	8.2 (3.8-21.2)	7	0.495
B12 Deficiency (≤ 211 pg/mL), n (%)	5 (12.5%)	40	1 (6.3%)	16	0.442
Combined low and borderline B12 levels (≤ 246 pg/mL), n (%)	10 (25.0%)	40	1 (6.3%)	16	0.107
Hcy ≥ 15 µmol/L, n (%)	15 (42.9%)	35	6 (66.7%)	9	0.184
MMA > 32 µg/L, n (%)	6 (23.1%)	26	1 (14.3%)	7	0.531
Hcy ≥ 15 µmol/L and MMA > 32 µg/L, n (%)	4 (12.1%)	33	1 (14,3%)	7	0.639

Legend: Data are presented as median (25-75 percentiles) or number (%), unless otherwise specified. Abbreviations: eGFR = estimated glomerular filtration rate; Hcy = Homocysteine; MET = Group of patients taking metformin; MMA = Methylmalonic acid; MnMET = Group of patients not taking metformin; PBS = Peripheral blood smear.

and duration of metformin treatment increase the risk of B12 deficiency. [2,5,20-22] Some studies also report that patients with B12 deficiency had longer diabetes duration. [6,16] Our study showed that larger doses and longer duration of metformin use is associated with greater MMA levels. Our findings indicate that only median MMA levels, not B12 or Hcy, were significantly higher in patients with daily doses ≥ 2000 mg of metformin, which is consistent with data of a recent meta-analysis. [21] Previously it was shown that patients treated with daily metformin doses of ≥1,000 mg had a higher risk of B12 deficiency, [22] but in contrast, in our population, this risk

Table III - Median Vitamin B12, Homocysteine and Methylmalonic Acid Levels according to Duration of Metformin Use and Current Daily Dose of Metformin in MET group.

	Duration of metformin use			Current daily dose of metformin		
	<4 years	≥4 years	*p* value	<2000 mg	≥2000 mg	*p* value
Vitamin B12, pg/mL	235.5 (189.0-459.0)	347.5 (254.0-441.0)	0.169	410.0 (356.5-452.0)	307.0 (242.5-451.0)	0.348
Hcy, µmol/L	15.5 (12.1-21.6)	14.0 (12.5-17.3)	0.513	16.4 (12.4-22-1)	14.2 (11.6-16.6)	0.586
MMA, µg/L	7.3 (6.8-22.6)	13.9 (7.4-34.9)	0.414	6.1 (3.3-6.5)	18.2 (8.5-31.8)	0.033

Legend: Data are presented as median (25-75 percentiles). Abbreviations: Hcy = Homocysteine; MET = Group of patients taking metformin; MMA = Methylmalonic acid.

seems to be higher only with doses from 2000 mg. Also, only median MMA levels, not B12 or Hcy, were positively correlated with T2D duration and duration of metformin treatment. Altogether, these results suggest that it is important to account not only for treatment duration but also the metformin dose in vitamin B12 deficiency screening protocols. [5]

Clinically, B12 deficiency could lead to altered mental status, megaloblastic anemia, and neurological damage. Peripheral neuropathy caused by B12 deficiency may be confused with diabetic peripheral neuropathy or may contribute to its aggravation, and permanent neurological damage may occur if not early diagnosed and treated. [22]

As in a recent meta-analysis, [21] we found no statistically significant associations between metformin use and anemia parameters or MNSI scores. Also, a recent narrative literature review did not identify a causal relationship between the development or worsening of peripheral neuropathy and the use of metformin, since the existing evidence is scarce. [23] However, in a previous study, there was a greater prevalence of anemia at 5 years in patients with T2D treated with metformin, compared to placebo. [2] In the present study, the high prevalence of anemia in the nMET group can be explained by the higher frequency of diabetic nephropathy in this group. In other study, the increase of serum MMA in metformin users was associated with a significant worsening of a validated clinical neuropathy score. [4]

The strengths of this study were to evaluate three different biomarkers of B12 deficiency (vitamin B12, Hcy and MMA) which allowed a wider and more comprehensive assessment of B12 deficiency. In addition, it included an extensive panel of demographic, clinical and analytical parameters, and applied a validated and specific neuropathy scale for the study population (the Portuguese version of MNSI). We evaluated correlations between laboratory parameters and clinical data. In addition, our study was designed for participants with DM2 in a single ethnic population.

There are some limitations worth mentioning. The limited sample size hinders data interpretation. This fact can justify the absence of significant differences between the groups for Hcy and MMA. Moreover, some patients did not perform all parameters, mainly only 44 and 33 patients performed Hcy and MMA, respectively, restricting the possibility to efficiently compare their levels between groups. Other limitations are the cross-sectional observational nature of the study and its relatively short duration.

> CONCLUSIONS

We demonstrated that patients with T2D under metformin had lower median B12 levels. B12 deficiency was frequent in this Portuguese population, ranging from 12.1 to 42.9% according to the biomarker evaluated. Median MMA levels were higher in patients taking daily doses of metformin of 2000 mg or more, and were positively correlated with T2D duration and exposure to metformin. Our data suggests the need for routine monitoring for B12 deficiency in patients with T2D, probably with the additional use of more sensitive biomarkers as MMA and in metformin users at higher daily doses (≥ 2000 mg). Further prospective and quality studies are needed to identify the risk factors for B12 deficiency using different biomarkers and the real contribution to the development of anemia and neuropathy in patients with T2D using metformin. <

Department and institution where the work was performed/*Departamento e instituição onde o trabalho foi realizado:*

Endocrinology Department, Centro Hospitalar do Baixo Vouga, Aveiro, Portugal/*Departamento de Endocrinologia, Centro Hospitalar do Baixo Vouga, Aveiro, Portugal.*

Ethics approval and consent to participate/*Aprovação ética e consentimento para participar:*

This study was approved by the local Ethics committee and was performed according to the Declaration of Helsinki. All participants gave written informed consent/*Este estudo foi aprovado pela comissão de ética local e foi realizado de acordo com a Declaração de Helsínquia. Todos os participantes assinaram termo de consentimento.*

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The authors declare that they have no conflicts of interests/*Os autores declaram não ter conflitos de interesses.*

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