Evaluation of Calciferol, Cobalamin, and Stromelysin-1 in Patients with Diabetic Peripheral Neuropathy due to Type-2 Diabetes Mellitus

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ABSTRACT

Objective: To evaluate the relationship between calciferol (vitamin D), cobalamin (vitamin-B12), and Stromelysin-1 (MMP-3) circulating levels in patients with diabetic peripheral neuropathy (DPN), patients with DM type 2 (T2DM) without neuropathy, and healthy control groups.
Study Design: Cross-sectional descriptive study.
Place and Duration of Study: Department of Internal Medicine, Namik Kemal University of Medicine, Tekirdag, Turkey, between November 2020 and February 2022.
Methodology: Healthy, age, and gender matched volunteers who were admitted to the hospital for a check-up with no health problem constituted the control group (n=30). Cases diagnosed with T2DM (n=30) and those with DPN (n=30) comprised the experimental group. Stromelysin-1, calciferol, and cobalamin levels were analysed from blood samples from all groups using enzyme-linked immunosorbent assay (ELISA) with a commercial kit. Tukey's Honest Significant Difference (HSD) test was performed after one-way analysis of variance (ANOVA) for intergroup comparisons. Alpha significance level was accepted as <0.05.
Results: There were significant differences in terms of the stromelysin-1, calciferol, and cobalamin levels of both the T2DM and DPN groups compared to healthy volunteers. These differences were statistically significant (p=0.00). There was a very weak negative correlation between stromelysin-1 and calciferol (p=0.972, r=-0.007) and a weak negative correlation between cobalamin and stromelysin-1 (p=0.062, r=-0.345) in DPN patients, without statistical significance.
Conclusion: Serum stromelysin-1 expression may be related to DPN progression in diabetic patients and may be a potential marker in DPN. Calciferol and cobalamin levels may also be important in the development of DPN.

Key Words: *Calciferol, cobalamin, Diabetic peripheral neuropathy, Stromelysin-1.*

INTRODUCTION

Diabetes mellitus (DM) is a common disease in the world. Half of the patients with DM are affected by a microvascular complication, diabetic peripheral neuropathy (DPN). It can lead to skin ulcers, infections, and serious foot problems such as bone and joint pain in extremities and amputation.[1]

The signs and symptoms of peripheral neuropathy often get worse at night, with numbness, loss of pain-sensation of heat, a tingling or burning sensation, sharp pain or cramps, and increased sensitivity to touch. The pain mechanisms thought to underlie this pathology are still not fully known. Hyperglycaemia, reduced blood flow, hypoxia, hypoxia-induced pro-angiogenesis, and proinflammatory responses may act in the pathogenesis of DPN.[2] Moreover, proinflammatory cytokines and also, cobalamin and calciferol deficiencies are thought to play a role in DPN pathogenesis.[3]

Matrix metalloproteinases (MMPs) are a large group of zinc endopeptidases that perform an important role in neuro-inflammation by the segmentation of the extracellular matrix (ECM) proteins, chemokines and proinflammatory cytokines.[4] MMPs have 20 members, but MMP-9 and MMP-2 are more studied

components of the group. Stromelysin-1(MMP-3) activates MMP-9. Therefore, Stromelysin-1 is also important like MMP-9, but there are few studies about it.[5] MMP inhibitors have been developed to target different kinds of inflammation-related diseases like oncological diseases, arthritis, atherosclerosis, and periodontitis. Moreover, MMP-9 inhibition can reduce neuropathic pain in the early phase.[6,7] Since, the mechanism of the disease is not fully known and there is no radical or clear pharmacological treatment protocol, patients' quality of life is negatively affected all over the world putting an extra burden on the health economy every year.[8]

Uncovering the pathogenesis of DPN and its relationships with risk factors may accelerate the development of novel treatment models. The aim of this study was to determine the serum stromelysin-1, calciferol, and cobalamin levels in patients with diabetic peripheral neuropathy, patients with T2DM without neuropathy and healthy control groups, and evaluate the relationship between calciferol, cobalamin, and Stromelysin-1 (MMP-3) in the development of DPN.

METHODOLOGY

This study was conducted between November 2020 and February 2022 with ethical approval from the Local Ethics Committee of the Faculty of Medicine at Istanbul Medipol University, Turkey. Additionally, permissions were obtained from all patients and healthy volunteers by informed consent forms. All experimental procedures conformed to the ethical standards of the responsible committee on human experimentation and with the declaration of Helsinki.

DPN diagnosis with neurological history and examination is effective and reliable, rarely supported by neurophysiological, and quantitative sensory tests. So, when diagnosing the cases with DPN, findings on their history of clinical symptoms, neurological examination findings, and sometimes electrophysiological, quantitative sensory, and autonomic function tests were evaluated altogether. In this study, the diagnosis of DPN was made with respect to 2017 American Diabetes Association's diabetic neuropathy position statement.[9]

However, DPN diagnosis is principally a clinical one and electrophysiological testing is not essential, to clarify the diagnosis and to exclude other neuropathy types, EMG was also made to each patient stating typical symptoms with positive physical examination findings, in our study 10-g monofilament testing. The patients without typical symptoms and any physical examination findings, DPN is excluded as it was suggested in this literature.

Patients with type 1 DM, cardiovascular or cerebrovascular disease, liver or kidney failure, malignancy, autoimmune disease, acute or chronic infection, or history of trauma, surgery, or pregnancy were also excluded. With concern that the MMP levels of healthy control volunteers and all cases might be affected, data from patients using pharmaceutical or food supplements containing hyaluronic acid, chondroitin sulphate, collagen, or similar substances for orthopaedic complaints were excluded. In addition to these, patients using any anticoagulant

medication such as heparin were excluded from the research. Moreover, patients using antithrombotic agents that might affect the endothelial plasminogen activator inhibitor or plasminogen activator inhibitor-1, also known as serpin E1, were also excluded. Patients who did not take any non-steroidal anti-inflammatory drugs in the last 15 days and were diagnosed with T2DM and DPN were included.

Analyses were done in at least three repetitions to avoid experimental errors. Samples taken from the cases were numbered and coded. This allowed the researcher performing the experimental analysis and the one performing the statistical evaluation to be blind to the data.

All groups were determined to include 18 females and 12 males. The group consisting of healthy volunteers who were admitted to the hospital for a check-up was called the control group (n=30). The control group was created to match the age, gender, and body mass index (BMI) of the cases in the other two groups.

The experimental group was divided into two subgroups. The first group consisted of patients diagnosed with T2DM but not DPN (n=30). The second group consisted of T2DM patients with DPN (n=30).

Blood samples from all groups were collected between 8:00 and 9:00 a.m., after a 30-minute period of rest. The serum was centrifuged at 2000×g, 4°C for 15 minutes and all samples were stored at -80°C until the analysis.

Fasting blood glucose (FBG) and cobalamin were analysed using an automated analyser (Cobas e8000; Roche Diagnostics, Tokyo, Japan). Serum vitamin-D [$25(OH)D_3$] was measured using an Agilent 1260 Infinity LC coupled to an Agilent 6460 Triple Quadrupole Mass Spectrometer with Agilent JetStream (AJS, USA). The results are reported in ng/ml. Serum stromelysin-1 concentrations were measured using the Thermo Fisher Scientific Human MMP-3 enzyme-linked immunosorbent assay (ELISA) kit. The sensitivity of the commercial kits was 0.0005 ng/mL with a range of variation of 0.06-4.00 ng/mL.

The Minitab (22 version) Software was used for data evaluation. The analyses were carried out at 95% confidence interval. For descriptive statistics, the findings were given as percentage (%), minimum (min), maximum (Max), or Mean±SD.

For multiple independent variables and groups, the one-way Analysis of Variance (ANOVA) test was used to evaluate how these independent variables interacted among themselves and to investigate the effects of these interactions on the dependent variable, F value was calculated to compare the amount of systematic variance in data with non-systematic variances. When the number of samples and variances were equal, Tukey's Honest Significant Difference (HSD) test was used for comparisons between groups after ANOVA. Pearson's correlation test was used to determine whether there was a linear correlation between two numerical measurements and their direction and intensity. The results are given as correlation coefficient (r). The alpha significance value was accepted as <0.05 for all statistical analyses.

Table I: Comparison of biochemical parameters of healthy volunteers, T2DM and DPN cases.

Variable	Group	Mean±SD	Grouping*
FBG (mg/dl)	Healthy, Control (n=30)	91.90±3.74	C
	T2DM (n=30)	210.90±91.40	A
	DPN (n=30)	155.30±64.90	B
HbA1c (%)	Healthy, Control (n=30)	5.27±0.218	C
	T2DM (n=30)	9.20±2.11	A
	DPN (n=30)	8.05±2.42	B
Stromelysin-1 (ng/ml)	Healthy, Control (n=30)	136.48±48.61	C
	T2DM (n=30)	251.30±75.60	B
	DPN (n=30)	493.56±5.60	A
Calciferol (ng/ml)	Healthy, Control (n=30)	20.85±3.66	A
	T2DM (n=30)	11.97±7.61	B
	DPN (n=30)	16.60±12.22	AB
Cobalamin (ng/ml)	Healthy, Control (n=30)	606.50±79.70	A
	T2DM (n=30)	248.50±93.10	B
	DPN (n=30)	252.20±87.20	B

*From A to C, A is the best and C is the worst; n is the number of samples; SD is standart deviation; FBG (F=25.34; p<0.000); HbA1c (F=35.51; p<0.000), Stromelysin-1 (F=368.58; p<0.000), Calciferol (F=8.06; p<0.001) and Cobalamin (F=168.24; p<0.001).

RESULTS

The ratio of females to males was 1.5 (F: 18; M: 12) in all groups. Regarding the demographic data, the mean age of the control group (n=30) was observed to be 57.67±15.55 years.

For the experimental groups, the mean ages were 56.50±6.07 years in the T2DM group (n=30) and 58.17±8.00 years in the DPN group (n=30). The mean BMI value of the control group was found to be 29.69 (kg/cm^2, overweight). For the experimental groups, mean BMI values were 29.98 (kg/cm^2) in the T2DM group and 32.03 (kg/cm^2) in the DPN group.

The mean ages of diabetes were 5.6±4.42 years in the T2DM group and 3.93±2.49 years in the DPN group (Table I).

Regarding the correlation test findings, there was a very weak negative correlation between calciferol and stromelysin-1 (r=-0.007; p=0.972). Also, a weak negative correlation was observed between cobalamin and stromelysin-1 (r=-0.345, p=0.062). There was a weak positive correlation between calciferol and cobalamin (r=0.076, p=0.691). However, all of these correlations were not statistically significant (p>0.05).

DISCUSSION

Diabetic neuropathy is known to cause pathologies such as extremity infections, ulcers, and subsequent amputation and so leads to high rates of morbidity.[10,11] MMPs play a key role in maintaining the balance between ECM synthesis and decompo-sition. MMPs are associated with several physiological and pathophysiological processes, including T2DM.[7] In fact, more importantly, some studies highlight that cobalamin usage reduces symptoms in DPN patients with normal B-12 serum levels. Calciferol deficiency on its own has been reported to be an independent risk factor for the development of peripheral neuropathy in T2DM patients.[3]

Khalaf et al. found a high prevalence of vitamin-B12 deficiency in T2DM cases but emphasised that there was no correlation between vitamin deficiency and the rate of DPN diagnosis.[12]

Also, a cross-sectional study was performed with 162 DM patients (122 have DNP) by Alveres et al. who found negative correlation between DNP and the plasma level of vitamin-B12.[13]

In another placebo-controlled double-blind study, 90 patients with T2DM with both peripheral and autonomic DPN were randomised to an active 44 patients and 46 patients control group were evaluated. While vitamin-B12 levels increased, neurophysiological parameters, sudomotor functions, and pain scores improved.[14]

There are studies reflecting the calciferol and DPN relationship also. In their study, Shillo et al. report a correlation between low calciferol levels and DPN.[15] In another study, 861 patients with T2DM were recruited and they suggested that calciferol deficiency is an independent risk of DPN and it may be marker of DPN.[16] In a study by Xiaohua et al., 483 patients were recruited. They suggested that DPN and also inflammatory cytokines are associated with severe calciferol deficiency.[17]

The present findings in the study demonstrated that both calciferol and cobalamin levels were lower in cases with DPN (vitamin-D = 16.60±12.22/vitamin-B12 = 252.20±87.20) and T2DM without DPN (vitamin-D = 11.97±7.61/; /vitamin-B12 = 248.50±93.10) when compared to healthy volunteers (Vitamin-D: 20.85±3.66/vitamin-B12: 606.50±79.70), with statistical significance (p<0.05).

Factors such as the time after the first diagnosis of T2DM, HbA1C levels, and smoking are associated with neuropathy in the adult age group, suggesting even higher rates in young adults over 30 years of age. However, it has been reported that there is no statistically significant difference between patients with and without neuropathy in terms of BMI, smoking and/or alcohol use, and cholesterol levels. Moreover, there is evidence that FBG levels are the most important factor determining neuropathy.[18] The data from the present research was compat-ible with the literature in this context. There was a statistically significant correlation between FBG and DPN (p<0.05), but no significant correlation between BMI and DPN (p>0.05).

MMPs have been reported to have a crucial role in pathophysio-logical mechanisms at the cellular level in the development of DPN and especially MMP-9 and MMP-2 have been investigated in the laboratory environment in experimental animal models or in cell cultures.[7]

Again, one study that discussed molecular mechanisms associated with MMP-mediated diabetic neuropathic pain and various endogenous, natural, and synthetic MMP inhibitors[18] has emphasised that MMPs have a key role in remodelling ECM. They reported that the abnormal MMP activity, which leads to ECM abnormality, is triggered in pathological conditions.[19] The authors highlighted that hyperglycaemia is a strong stimulus regulating MMP expression in the central and peripheral nervous system, among oxidative stress and inflammation.[19] Stating that MMP-mediated ECM abnormality accelerates neuropathic pain in diabetic patients, they emphasised that both central nerves and peripheral nerves may become hypersensitive *via* MMP.[19]

It is a scientific fact that DPN affects many systems and organs, particularly the autonomic nervous system.[20] It is characterised by persistent inflammation *via* a significant amount of pro-oxidative substances and proinflammatory cytokines such as interleukin (IL)-1β, IL-6, and TNF-α or other inflammatory mediators such as nerve growth factor, surfactant protein, nitric oxide, and prostaglandin-E2.[20]

However, there are only five studies in the literature with high evidence levels where vitamin-D and vitamin-B12 are evaluated together in DPN cases, there is no study discussing these together with MMP-3. Here, for the first time in the literature, stromelysin-1, calciferol, and cobalamin levels were evaluated together in blood samples from humans. Thus, no commercial cell lines or animal tissues were used. The research was conducted on serum obtained from blood tissue taken from humans. This was another strength of this study. The number of patients included was relatively small. Also, the study was a single-centre, cross-sectional clinical study. These may limit the generalisation of results. Exploring the pathophysiology of DPN and its relations with affecting factors, also new early diagnostic markers may help to inhibit the progression of the disease. According to our results, stromelysin-1 may play role in DPN pathogenesis. Moreover, serum cobalamin and calciferol deficiency seems to be related to the development of DPN. New prospective and multicentre studies, with greater sample size are necessary to confirm these results.

CONCLUSION

When compared with healthy volunteers, T2DM and DPN cases were found to have statistically significant (p<0.05) higher FBG, HbA1c, and stromelysin levels and decreased calciferol and cobalamin levels. Stromelysin-1 had a very weak negative correlation with calciferol and a weak negative correlation with cobalamin. Moreover, there was a weak positive but not significant correlation between calciferol and cobalamin.

ETHICAL APPROVAL:
The study protocol was reviewed and approved by the Institutional Ethics Committee at Istanbul Medipol University.

PATIENTS' CONSENT:
All participants signed the informed consent before enrolment in the study.

COMPETING INTEREST:
The authors declared no competing interest.

AUTHORS' CONTRIBUTION:
BB: Collected and analysed data and wrote the manuscript.
IY: Analysed data and wrote the manuscript.
NK: Searched literature and analysed data.
BEB: Designed the study and agreed to be accountable for all aspects of the work.
NEK: Developed the theoretical framework.
HO: Review, editing, and supervision.
All the authors have approved the final version of the manuscript to be published.

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