Study of serum vitamin D levels in patients with diabetic polyneuropathy using radioimmunoassay method

Anastasia Pistola[1] BS, Evangelos Karathanos[2] MD, Theocharis Konstantinidis[3] MD, Dimitrios Papazoglou[4] MD, Christina Tsigalou[5] MD, Athanasios Zissimopoulos[1] MD

1. Nuclear Medicine Department, University General Hospital of Alexandroupolis
2. Nuclear Medicine Department, Sotiria Hospital, Athens.
3. School of Medicine Democritus University of Thrace, Medical Biopathology Lab. University General Hospital of Alexandroupolis
4. School of Medicine Democritus University of Thrace, 2nd Department of Internal Medicine University General Hospital of Alexandroupolis
5. School of Medicine Democritus University of Thrace, Laboratory of Hygiene and Environmental Protection

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Corresponding author:
Anastasia Pistola BS,
Nuclear Medicine Department,
University General Hospital of Alexandroupolis, Greece
anasia3@gmail.com

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Abstract

Objective: Diabetic polyneuropathy (DPN) is a common and disabling complication of type 2 diabetes mellitus (T2DM). Vitamin D deficiency has been implicated as a potential modifiable risk factor. This study aimed to investigate the relationship between serum 25-hydroxyvitamin D [25(OH)D] levels and DPN using the radioimmunoassay (RIA) method. **Subjects and Methods:** One hundred twenty T2DM patients (80 with DPN and 40 without neuropathy) have been enrolled, while 40 healthy volunteers served as group control. Neuropathy diagnosis was based on clinical assessment (through Michigan neuropathy screening instrument (MNSI) - both questionnaire and examination) and nerve conduction studies. Serum 25(OH)D levels were quantified using RIA. **Results:** Vitamin D levels were significantly lower in DPN patients (mean 13.2ng/mL) compared to diabetic (18.3ng/mL) and healthy controls (31.2ng/mL, P<0.001). Vitamin D deficiency (<10ng/mL) was found in 72.5% of DPN patients. Apart from severe neuropathy, other factors (age, body mass index -BMI- and HbA1c) have all been mildly inversely correlated to Vitamin D levels. **Conclusions:** Vitamin D deficiency is significantly associated with the presence and severity of DPN. These findings support the clinical value of assessing and potentially improving vitamin D status in diabetic patients, particularly in those with severe neuropathy.

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Introduction

Diabetic polyneuropathy (DPN) represents a prevalent, debilitating complication and strong source of morbidity of diabetes mellitus. Approximately 50% of T2DM patients develop it, resulting in foot ulcers, amputation, pain, motor, and sensory deficits [1]. Pathogenic pathway is not completely understood and among proposed factors, dominant conditions include chronic hyperglycemia, oxidative stress, inflammatory processes, and other vascular and metabolic mechanisms [2].

Recently, vitamin D has gained attention not only for bone and calcium metabolism but also for potential neuroprotective and anti-inflammatory effects. Vitamin D deficiency has been observed in patients with various neurodegenerative and demyelinating diseases, such as multiple sclerosis, Alzheimer's, and Parkinson's, suggesting contribution to pathogenesis [3]. Emerging evidence indicate a remarkable but still not proven correlation between low Vitamin D levels and diabetic neuropathy [4, 5], as deficiency seems to modulate insulin sensitivity, inflammation, and neurotrophins expression, apart from its protective role on bone formation and body fat ratio through biochemical receptors [6]. Patients with certain neuropathic symptoms such as: burning pain, tingling, and numbness, usually exhibit low 25-hydroxyvitamin D [25(OH)D] serum [7]. Vitamin D supplementation has been reported to lead to meaningful improvement in neuropathic symptoms, including reduced pain and enhanced nerve conduction parameters [8, 9].

Despite these association, the exact Vitamin D deficiency mechanism in DPN remains under investigation. Several studies support that vitamin D may exert anti-inflammatory effects through cytokine Il-6 and TNF - α downregulation [10]. In addition, Vitamin D appears to influence both insulin sensitivity and blood glucose regulation, essential factors in diabetes-related disorders pathophysiology [11].

Given the increasing global burden of diabetes and its complications, early identification of modifiable risk factors such as vitamin D deficiency, is crucial. Radioimmunoassay

method (RIA) is a dependable and commonly used technique for measuring serum 25(OH)D, due to its strong analytical sensitivity and specificity [12]. Assessment of vitamin D levels in clinical settings typically relies on measuring serum concentrations of 25(OH)D, the major circulating form of vitamin [13].

The study aimed to evaluate serum 25(OH)D concentrations in patients with DPN using the RIA method, and to investigate its association with severe neuropathy. Our hypothesis is that patients with DPN exhibit significantly lower vitamin D levels, compared to those without neuropathy, and that lower levels are associated with more severe symptoms.

Subjects and Methods

Study design and participants

This cross-sectional observational study was carried out at Nuclear Medicine Department (Alexandroupolis University Hospital) within a 11-month period (between February and December 2019), in collaboration with the 2nd Department of Internal Medicine and the Blood Donation Department. A total of 120 diabetic patients, aged between 40 and 75 years, were recruited from outpatient endocrinology clinics. All of them had established T2DM, confirmed by medical history and standard biochemical criteria. Based on presence or absence of clinical and electrophysiological signs of neuropathy, participants were stratified into two groups. The DPN group consisted of 80 patients who fulfilled the Toronto consensus criteria for diabetic polyneuropathy, including characteristic symptoms, clinical signs, and abnormal findings on nerve conduction studies. The control group included 40 patients with T2DM but no signs or symptoms of neuropathy.

In addition to the Toronto clinical neuropathy scoring system, the diagnosis of DPN in participants was further confirmed using both the Michigan neuropathy screening instrument (MNSI) and nerve conduction studies (NCS). The MNSI was applied in its standard form, including both the symptom questionnaire and clinical examination, with a total score of ≥2.5 points on the physical examination component considered indicative of peripheral neuropathy. Nerve conduction studies were conducted in accordance to standard electrophysiological protocols of sensory nerve conduction velocities, amplitudes, and distal latencies in the lower limbs. Abnormalities in at least two nerves, one of which had to be sensory, were required to confirm the diagnosis. The combined use of clinical scoring and electrophysiological testing enhances diagnostic accuracy and aligns with internationally recognized criteria for DPN.

Besides the diabetic cohort, a third group consisting of 40 healthy volunteer blood donors (both men and women), aged 26 to 55 years, was included as a non-diabetic control group. These individuals had no history of diabetes, cardiovascular disease, or other chronic or acute medical conditions. All were deemed to be in good general health based on recent clinical and laboratory evaluations. Importantly, none of the healthy volunteers had received vitamin D supplementation during the last six months, ensuring that measured serum levels reflected true endogenous vitamin D status.

The inclusion criteria for all participants were deliberately stringent to minimize potential confounding variables. Eligible diabetic patients were required to have a minimum 5-year history of T2DM and maintain relatively stable metabolic control, defined as a recent glycated hemoglobin (Hb A1c) level below 9%. Patients with type 1 diabetes, impaired renal function (estimated glomerular filtration rate <60mL/min/1.73m^2), hepatic dysfunction, active infectious or inflammatory condition at the time of assessment, as well as individuals under medication with known interference to Vitamin D metabolism (such as glucocorticoids, antiepileptics, or antifungal agents) have been excluded.

Ethical standard criteria have been applied (each participant was fully informed about study's objectives and provided with written consent before any procedure). Protocol review board approval (502/31-05-2018) was in accordance to Helsinki Declaration of ethical conduct in human research. Using clear inclusion and exclusion criteria, and standardized clinical and electrophysiological tools, the study ensured both internal validity and potential application of its findings to similar patient populations.

Serum Vitamin D measurement

After overnight fast, morning blood sampling was collected for both diabetic and non-diabetic blood donor volunteers. Serum was separated and stored at -80°C until analysis. Serum levels of 25(OH)D were measured using the RIA method. Initially, calibrators, control sera, and samples were incubated with a stripping solution in antibody-coated tubes. This step effectively released both 25(OH)D_2 and 25(OH)D_3 from the Vitamin D binding protein (DBP), thereby allowing them to interact freely with the assay components. Following this, without any intermediate wash steps, a specific amount of 25(OH)D labeled with iodine-125 (^{125}I) was introduced. This radiolabeled tracer competed with the endogenous 25(OH)D_3 and 25(OH)D_2 from the samples, calibrators, or control sera for a limited number of binding sites on a monoclonal antibody that was immobilized on the inner surface of the reaction tubes. Following an adequate incubation period (allowing best equilibrium binding), tube contents were aspirated, washed for unbound tracer removal, and the remaining radioactivity bound to the antibody was measured using a gamma counter. A standard calibration curve was constructed using calibrator samples, and the total concentration of 25(OH)D (both D_2 and D_3 forms) in each sample was determined by interpolation against this curve. This method, widely used in clinical research and diagnostics, offers sufficient specificity and sensitivity for assessing vitamin D status in patients. All samples were analyzed duplicate to ensure consistency, and all procedures adhered strictly to the manufacturer's instructions for the assay kit used (DiaSorin). Results were expressed in ng/mL.

Vitamin D status was categorized as:

- Deficient: 10ng/mL
- Insufficient: 10-29ng/mL
- Sufficient: >30-150ng/mL
- Toxicity: >150ng/mL

Participant characteristics

The study population included 160 individuals divided into three groups: 80 patients with DPN, 40 diabetic patients without neuropathic symptoms (diabetic control group), and 40 healthy individuals (non-diabetic control group). All baseline characteristics for the three study groups are presented in Table 1.

These data highlight that while age, body mass index (BMI), and HbA1c were comparable between the diabetic groups, the longer duration of diabetes in the DPN group may be a critical factor associated with development of neuropathy. Additional analyses involving vitamin D levels in these groups will further elucidate the potential link between hypovitaminosis D and diabetic neuropathy.

Table 1. *Baseline characteristics of participants.*

Characteristic	DPN group (n=80)	Diabetic group (n=40)	Control group (n=40)
Age (years)	63.4±9.1	61.7±8.6	40.5±14.5
Male/Female	46/34	17/23	24/16
Duration of diabetes (years)	12.3±4.5	9.1±3.9	-
BMI (kg/m²)	28.1±3.7	27.5±4.2	26.9±5.6
HbA1c (%)	7.4±0.9	7.1±0.8	5.3±0.4

Among the 80 patients included in the DPN group, a range of clinical symptoms consistent with diabetic polyneuropathy was observed, which are presented in Table 2. Electrophysiological testing supported clinical diagnosis, with most patients showing evidence of both sensory and motor nerve involvement. Reduced nerve conduction velocities and amplitudes were observed, consistent with chronic sensorimotor axonal neuropathy.

These findings highlight the clinical burden of DPN in the affected group and set the foundation for further analysis of biochemical correlations, particularly with vitamin D levels, in the subsequent sections.

Results

Serum Vitamin D levels

A marked difference was observed in 25(OH)D serum concentrations among the three study groups. Patients in the DPN group (n=80) exhibited significantly lower mean serum levels of 25(OH)D (13.2±8.5ng/mL) compared to both diabetic control group (n=40), (mean level:18.3±7.5ng/mL), and healthy control group who had the highest mean level (31.2±6.5ng/mL). The differences between the DPN group and each of the two control groups were statistically significant (P< 0.001), suggesting a strong correlation between vitamin D deficiency and diabetic polyneuropathy.

In terms of vitamin D status categories, a high proportion of patients in the DPN group (72%, or 58 out of 80) were found to have deficient levels of 25(OH)D (<10ng/mL). This percentage was notably lower in the diabetic control group (33%, or 13 out of 40), and even lower in the health control group, where only 5 % (2 out of 40) had deficient levels (Table 3).

These findings provide additional support for the hypothesis that lower vitamin D status is associated with an increased likelihood of developing diabetic neuropathy. The substantial difference in the distribution of vitamin D categories between the two groups may reflect underlying pathophysiological differences or potential modifiable risk factors.

Table 2. *Symptomatology in patients with diabetic polyneuropathy (n= 80).*

Symptom	Number of patients (n)	Percentage (%)
Distal numbness	60	75%
Paresthesia (tingling, pins & needles)	55	68.8%
Neuropathic pain (burning, stabbing)	48	60%
Lower limb muscle weakness	28	35%
Gait disturbances (ataxia, imbalance)	23	28.7%
Postural hypotension	10	12.5%
Gastrointestinal symptoms (constipation, gastroparesis)	5	6.2%
Diabetic foot (ulceration/infection)	3	3.75%

Statistical analysis

A comprehensive statistical analysis was performed to evaluate the relationship between serum 25(OH)D levels and the presence and severity of DPN. Both descriptive and inferential statistics were used.

Descriptive statistics

Age: The DPN group had a higher mean age (63.4±9.1 years) compared to the diabetic control group (61.7±8.6 years) and the healthy control group (40.5±14.5 years). The differences were statistically significant (ANOVA, P<0.001), particularly between the DPN group and the healthy control group.

Duration of diabetes: Patients with DPN had a significantly longer duration of diabetes (12.3±4.5 years) than diabetic

Table 3. *Vitamin D status distribution.*

Vitamin D status	DPN group (n=80)	Diabetic group (n=40)	Control group (n=40)
Deficiency (<10 ng/mL)	58(72.5%)	13(32.5%)	2(5%)
Insufficiency (10-29 ng/mL)	14(17.5%)	22(55%)	12(30%)
Sufficiency (>30 ng/mL)	8(10%)	5(12.5%)	26(65%)
Mean (± SD) ng/mL	13.2±8.5ng/mL	18.3±7.5ng/mL	31.2±6.5ng/mL
P-value	P<0.001*		

**P-value for comparison of DPN group vs control group*

patients without neuropathy (9.1±3.9 years) (P<0.01, independent samples t-test), indicating disease duration as a potential risk factor for neuropathy.

HbA1c and BMI: No statistically significant differences were found in glycemic control (HbA1c) or BMI between the diabetic groups (P>0.05), suggesting these factors alone did not account for the presence of neuropathy.

Comparison of Vitamin D levels

There were statistically significant differences in mean serum 25(OH)D levels among the three groups (One-way ANOVA: F (2, 157)=87.95, P<0.001, η^2=0.51), indicating a large effect size. Post-hoc Tukey tests confirmed that all pairwise group comparisons were significant (P<0.001), with the largest difference observed between the DPN and control groups (mean difference= −18.00ng/mL, 95% CI: −21.17 to −14.83):

- **DPN group:** 13.2±8.5ng/mL
- **Diabetic controls:** 18.3±7.5ng/mL
- **Healthy controls:** 31.2±6.5ng/mL

Distribution of Vitamin D status

Chi-square test revealed statistically significant differences in the distribution of vitamin D status across the groups (P< 0.001), particularly highlighting a high prevalence of deficiency in DPN patients.

Correlation between Vitamin D levels and age, BMI, HbA1c in DPN group

In the DPN group, serum vitamin D levels were correlated with age, BMI and HbA1c. Specifically, Spearman's correlation coefficients (n=80) revealed moderate inverse associations between vitamin D and age (r= −0.52), BMI (r= −0.49), and HbA1c (r= −0.46). These results suggest that older age, higher BMI, and poorer glycemic control are all linked to lower vitamin D levels in DPN patients, reinforcing the multifactorial contributors to vitamin D deficiency in this population. Comparative correlation diagrams for age, BMI and HbA1c are also provided for the diabetic and control groups, in addition to the DPN group, to further illustrate group-specific patterns.

Correlation between Vitamin D levels and sex in DPN group

To explore the relationship between serum vitamin D levels and sex, a subgroup analysis was conducted within the DPN cohort as well as in diabetic and healthy control groups. In the DPN group, male participants exhibited slightly lower mean vitamin D levels (12.5±8.4ng/mL) compared to females (14.1±8.6ng/mL), with the difference reaching statistical significance (P=0.03), suggesting that gender may influence vitamin D status in neuropathic diabetic patients. In contrast, the sex-based differences in the diabetic control group (males: 19.5±7.1ng/mL vs. females: 17.4±7.8ng/mL, P=0.22) and in the healthy control group (males: 32.5±6.2ng/mL vs. females: 29.8±6.7ng/mL, P=0.18) were not statistically significant.

Correlation between Vitamin D and neuropathy symptoms

A moderate negative correlation was observed between serum 25(OH)D levels and the severity of neuropathic symptoms (e.g., numbness, paresthesia, neuropathic pain, motor deficits). Spearman's rank correlation coefficient showed r= -0.48, P<0.001. This suggests that lower vitamin D levels are associated with increased symptom burden and severity.

Discussion

This study reinforces the growing evidence that vitamin D deficiency may contribute to the development and severity of DPN in individuals with T2DM. Importantly, by incorporating a third group-healthy non-diabetic blood donors- we were

able to compare serum vitamin D levels across a wider physiological spectrum. This approach enabled a more nuanced understanding of the interplay between vitamin D status and neuropathic manifestations.

Our findings demonstrated that patients with DPN had significantly lower 25(OH)D levels not only in comparison to diabetic individuals without neuropathy but also when contrasted with healthy controls. The mean serum vitamin D concentration among the healthy control group was more than double that of the DPN group, indicating a striking divergence in vitamin D status that may reflect both metabolic burden and neuropathic degeneration.

The inclusion of a healthy control group is crucial, as it allows us to distinguish between vitamin D deficiency that may result from chronic disease processes and deficiency that exists independently of any overt pathology. Comparable study designs have been used in previous research exploring vitamin D status across populations stratified into three distinct clinical categories. For instance, Kheyami et al. (2019) [19] and Toth et al. (2014) [20] both included healthy, diabetic without neuropathy and DPN groups in their analyses and reported comparable trend vitamin D levels were lowest in DPN patients and highest in healthy individuals, with diabetic controls falling in between. These patterns underscore a potential gradient of risk, with hypovitaminosis D worsening along with neuropathic progression.

From a pathophysiological standpoint, this gradient may reflect cumulative effects of chronic hyperglycemia, inflammation, and neurodegeneration on vitamin D metabolism or receptor responsiveness. Peripheral neurons and glial cells express vitamin D receptors, suggesting a potential role for the vitamin in modulating neurotrophic signaling, calcium dynamics, and inflammatory pathways [14, 15]. Its deficiency, therefore, may directly impair nerve function or exacerbate inflammatory damage to axons.

Furthermore, the correlation observed between vitamin D levels and neuropathic symptom severity strengthens the biological plausibility of this association. More pronounced and multifaceted neuropathic symptoms have been observed in patients with vitamin D deficiency, reflecting evidence from trials showing symptomatic and electrophysiological benefits following vitamin D supplementation [16, 17].

The prevalence of deficiency in the DPN group-over 70%-contrasted sharply with the 5% observed in the healthy controls, emphasizing that vitamin D insufficiency in this context is not merely incidental. Rather, it may represent a modifiable factor within a broader therapeutic strategy aimed at minimizing neuropathic complications.

While this study cannot establish causality due to its cross-sectional nature, its findings are in line with previous prospective and interventional data suggesting that addressing vitamin D deficiency may slow or mitigate the course of diabetic neuropathy [18, 5]. Nevertheless, future studies with larger cohorts and longitudinal follow-up are warranted to determine whether routine supplementation could be recommended as standard care for high-risk diabetic populations.

Lastly, the choice of RIA as the measurement method ensures high specificity and sensitivity, minimizing potential biases due to assay variability and reinforcing the credibility of our biochemical data [12].

In conclusion, this study not only confirms earlier associations between vitamin D deficiency and DPN but also extends them by contextualizing these findings within a healthy population baseline. Our findings suggest a strongly inverse association between serum vitamin D levels and DPN severity. Patients with DPN had significantly lower 25(OH)D concentrations as measured by RIA, and these levels correlated with clinical and electrophysiological markers of neuropathy. This triadic comparison highlights the potential role of vitamin D as both a biomarker of neuropathic risk and a possible therapeutic target, an insight that should inform future research and clinical guidelines alike.

The authors declare that they have no conflicts of interest.

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