European Review for Medical and Pharmacological Sciences 2022; 26: 7046-7052

Severe vitamin D deficiency is associated with endothelial inflammation in healthy individuals even in the absence of subclinical atherosclerosis

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Abstract. – OBJECTIVE: Vitamin D has beneficial effects, some of which involve the cardiovascular system. No study to date has investigated the association between serum endocan levels, as a biomarker of endothelial inflammation, and vitamin D levels in the absence of subclinical atherosclerosis detected by carotid intima-media thickness (CIMT) in healthy individuals.

PATIENTS AND METHODS: Subjects were categorized into three groups based on vitamin D levels according to Endocrine Society guidelines. Mean CIMT was calculated from six measurements on two scans. Statistical significance was set at $p < 0.05$, and all testing was two-sided.

RESULTS: The concentration of serum endocan was 802.8 ± 411.4 ng/L in the group with the lowest serum vitamin D level, 454.8 ± 334.3 ng/L in the mild/moderately low serum vitamin D level group, and 269.4 ± 180.2 ng/L in the group with normal serum vitamin D levels ($p < 0.01$). Receiver operating characteristics curve analysis revealed that a serum vitamin D concentration of 7.5 ng/mL had a 97% sensitivity and 81% specificity for the prediction of serum endocan level greater than 270 ng/L, which could be an indicator for endothelial inflammation.

CONCLUSIONS: Demonstrating that vitamin D deficiency can cause endothelial damage in the early period of atherosclerosis without the development of clinical cardiovascular disease will have a pivotal role in the prevention of cardiovascular mortality and morbidity.

Key Words:
Endocan, Vitamin D deficiency, Atherosclerosis, Endothelial inflammation.

Introduction

Vitamin D plays an important role in calcium metabolism through the regulation of calcium absorption from the intestine, and vitamin D deficiency has been associated with all-cause mortality, cardiovascular mortality, and heart failure1[1,2]. Recent studies[3,4] indicate that low levels of vitamin D are a global problem. The lowest necessary serum level of 25-hydroxy vitamin D has been recommended to be 20-30 ng/mL, according to the consensus summit[5] and to a study[6] in which the analysis was extended to cover possible advantages of vitamin D for several health outcomes.

Low levels of vitamin D are clearly related not only to higher incidences of cancer[7] and immune dysfunction[8], but also to the occurrence of cardiovascular diseases[9,10], hypertension[11], and metabolic syndrome[12], and independently associated with impaired reperfusion in myocardial infarction patients undergoing reperfusion with primary coronary angioplasty[13]. Preclinical and clinical investigations revealed positive effects of vitamin D on fibrinolysis, lipid metabolism, thrombosis, and endothelial functions[14]. Jointly, these findings strongly suggest that 25-hydroxy vitamin D has beneficial effects, some of which involve the cardiovascular system.

Serum endothelial-specific molecule-1 (endocan) is a biomarker that has been found to be associated with endothelium-related events such as endothelial dysfunction, hypertensive end-organ damage, pulmonary embolism, acute myocardial infarction, and diabetic vascular complications[15-18]. No study to date has investigated the association between vitamin D levels and serum endocan levels as a biomarker of endothelial inflammation in the absence of subclinical atherosclerosis in healthy individuals. Our study aimed at investigating the relationship between serum

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endocan levels and vitamin D levels in healthy COVID-19-naive individuals.

Patients and Methods

Study Design

This case-control study was carried out in the Department of Internal Medical Sciences, Faculty of Medicine, Istanbul University. Approval was obtained from the Medical Research Ethics Committee of the Faculty of Medicine at Istanbul University. The study abided by the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. All participants provided written informed consent.

Study Population

A total of 328 healthy volunteers aged 18-45 years were screened from March 2019 to April 2022. 81 participants who did not have a previous COVID-19 infection, who did not smoke during their lifetime, and who had a carotid intima-media thickness (CIMT) below 0.9 mm were included in the study. Volunteers were assigned to one of three groups, according to their vitamin D levels: < 10 ng/mL, 10-20 ng/mL, and > 20 ng/mL.

Data Collection

The demographic data (age and sex), laboratory findings, and CIMT values of participants were collected by two investigators (T.C. and S.C.A.). All data were independently reviewed and entered into the computer database by two analysts (N.S. and A.M.).

Serum Endocan Measurement

In order to estimate the serum levels of endocan, 10 mL of peripheral venous blood were drawn into plain blood collection tubes without any additives, immediately immersed in melting ice, and allowed to clot for 1 hour before centrifugation (1,700 g at 4°C for 10 min). Serum was stored at -80°C until analyzed, and samples were thawed only once. Endocan levels were measured using an enzyme-linked immunosorbent assay (Endocan ELISA kit, Bioassay Technology Laboratory, Shanghai, China). Measurements of other biochemical parameters were performed using standard laboratory techniques.

Vitamin D Measurement

All blood samples were collected during winter (November-February) to avoid seasonal variations. Blood samples were stored at -70°C within 30 min of collection. Serum vitamin D concentrations were measured using an automated Vitamin D2-D3 HPLC Analyzer (Zivak Technologies 25-OH Vitamin D2-D3 HPLC analysis kit). Subjects were assigned into one of three groups: severely vitamin D deficient (< 10 ng/mL), mildly/moderately vitamin D deficient (< 20 ng/mL), and vitamin D sufficient (≥ 20 ng/mL), according to guidelines by the Endocrine Society[19].

Measurement of Carotid Intima-Media Thickness

Carotid intima-media thickness (CIMT) was measured according to the regular method described previously[20]. All healthy volunteers were in the supine position. Longitudinal scanning was performed from the common carotid artery to the bifurcation point. CIMT was measured from the far wall of the internal carotid artery within 10 mm proximal to the bifurcation. Three points were measured, which were synchronized with R wave peaks on the ECG to avoid possible errors resulting from variable arterial compliance. Mean CIMT was calculated from six measurements on two scans.

In healthy adults, CIMT ranges from 0.25 to 1.5 mm[21], and values > 1.0 mm are often regarded as abnormal[22].

Statistical Analysis

Descriptive statistics were obtained for all study variables. Continuous variables were compared using ANOVA testing. Continuous data were expressed as mean (SD) values. Categorical data were expressed as proportions. The Pearson correlation coefficient and Spearman rank correlation coefficient were used for linear correlation analysis. Error bar graph was used to show mean serum endocan levels according to vitamin D levels. Data were analyzed using SPSS version 26.0 (IBM Corp., Armonk, NY, USA). For all the statistical analyses, $p < 05$ was considered significant and all testing was two-sided.

Results

Basic Characteristics, Laboratory Findings, and Imaging Modality of The Study Group

The mean age of the study group was 29.8 ± 8.4 years. There was no statistically significant difference in terms of age and gender between the groups with severe vitamin D deficiency, mild/moderate vitamin D deficiency, and sufficient vitamin D level (Table I). There was no statistically

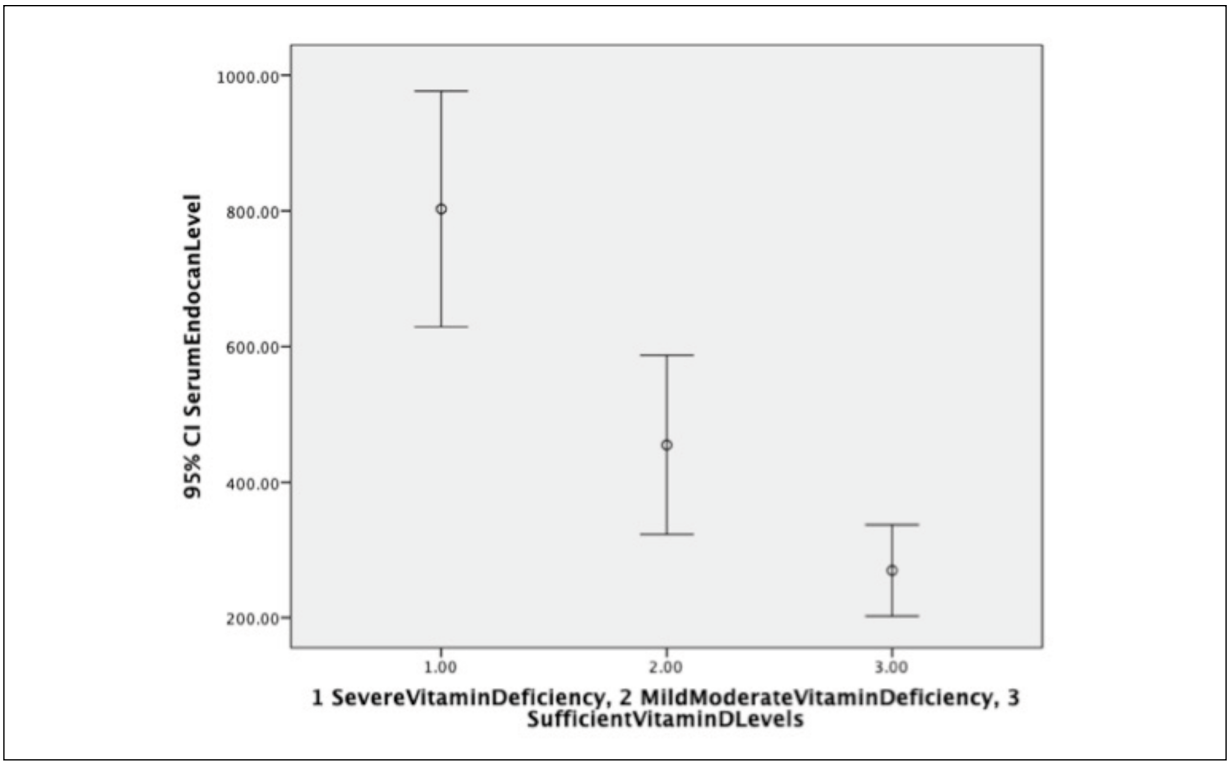

Figure 1. Error bar graph showing mean serum endocan levels according to the vitamin D levels.

significant difference between the groups in terms of serum lipid levels. Serum C-reactive protein (CRP) levels, which is an inflammation marker, were also similar between groups (Table I). There was no statistically significant difference between serum creatinine, fasting blood glucose, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) levels. The concentration of serum endocan was 802.8 ± 411.4 ng/L in the group with the lowest serum vitamin D level, 454.8 ± 334.3 ng/L in the mild/moderately low vitamin D level group, and 269.4 ± 180.2 ng/L in the group with normal serum vitamin D levels. There was a statistically significant difference between the groups ($p < 0.01$, $p = 0.01$, $p < 0.01$; Table I, Figure 1). Carotid intima-media thickness was similar between all groups ($p = 0.22$, $p = 0.99$, $p = 0.93$; Table I).

Correlations Between Serum Endocan Levels and Laboratory Findings

Correlations between serum endocan levels, age, CIMT, and serum vitamin D, C-reactive protein (CRP), and total cholesterol levels are shown in Table II. There was only a moderate negative correlation between serum endocan level and serum vitamin D level (r = -0.52, $p < 0.01$; Table II).

Serum Endocan Levels Stratified according to Serum Vitamin D Levels

The concentration of serum endocan was 802.8 ± 411.4 ng/L in the group with the lowest serum vitamin D level, 454.8 ± 334.3 ng/L in the mild/moderately low vitamin D level group, and 269.4 ± 180.2 ng/L in the group with normal serum vitamin D levels. There was a statistically significant difference between the groups ($p < 0.01$, $p = 0.01$, $p < 0.01$; Table 1, Figure 1). In the receiver operating characteristics (ROC) curve analysis, we found that 7.5 ng/mL serum vitamin D level had a 97% sensitivity and 81% specificity for the prediction of serum endocan level greater than 270 ng/L, which could be an indicator for endothelial inflammation (Figure 2).

Discussion

In our study, serum endocan levels were found to be higher in the group with the lowest vitamin D level, and endocan levels were lower in the group with sufficient vitamin D. In many studies[15-18], serum endocan concentration was shown as an important biomarker in endothelial inflammation, and our study is the first to demonstrate a close relation-

Table I. Comparison of basic demographic data, laboratory findings, and imaging modality of healthy volunteers according to serum vitamin D concentration.

Variables	Total n=81	Group 1 Severe Vitamin D Deficiency, < 10 ng/mL	Group 2 Mild/Moderate Vitamin D Deficiency, ≥10 ng/mL, < 20 ng/mL	Group 3 Sufficient Vitamin D, ≥ 20 ng/mL	Multiple comparison *p*-value (Group 1-2, 1-3, and 2-3)
Age (years)	29.8±8.4	27.2±7	27.8±7.6	33.6±8.9	p=0.93, p=0.95, p=0.91
Male (%)	30.9%	29.2%	25.9%	36.7%	p=0.66, p=0.52, p=0.66
Laboratory Findings					
Triglyceride (mg/dL)	83.3±36.3	86.7±37	76.5±34.8	88.6±38.5	p=0.41, p=0.52, p=0.95
Total cholesterol (mg/dL)	170.9±37.5	172.9±35.2	156.4±23.6	188.2±52.4	p=0.14, p=0.87, p=0.19
CRP (mg/L)	0.67±0.39	0.7±0.37	0.74±0.45	0.59±0.36	p=0.72, p=0.33, p=0.17
Creatinine (mg/dL)	0.7±0.13	0.7±0.15	0.7±0.13	0.7±0.11	p=0.83, p=0.73, p=0.64
Glucose (mg/dL)	85.6±10	86±8.1	83.3±10.4	87.3±11	p=0.85, p=0.24, p=0.22
ALT (U/L)	17.7±10.3	18±10.1	17.15±10.6	18±10.4	p=0.52, p=0.17, p=0.69
AST(U/L)	20±18.3	18.9±5.7	17.6±4.8	23.2±5.6	p=0.74, p=0.37, p=0.78
Endocan (ng/L)	489.3±380.4	802.8±411.4	454.8±334.3	269.4±180.2	p<0.01, p=0.01, p<0.01
Imaging Modality					
Carotid intima-media thickness (mm)	0.58±0.11	0.59±0.09	0.56±0.11	0.58±0.11	p=0.22, p=0.99, p=0.93

Endocan = endothelial specific molecule-1; CRP = C-reactive protein; ALT = alanine amino transferase; AST = aspartate amino transferase; CIMT = carotid intima-media thickness.

ship between serum endocan levels and low serum vitamin D levels, even without the presence of subclinical atherosclerosis in healthy individuals.

Vitamin D is a lipid-soluble vitamin and an important component of bone and mineral metabolism[23]. It is also a hormonal factor modulating cardiovascular function[24,25]. It has been shown to prevent endothelial dysfunction and inflammatory processes[26] that represent the pathophysiological basis of atherosclerosis[27]. In fact, vitamin D deficiency has been associated with a higher mortality and an enhanced cardiovascular risk in large registries[28,29]. The interest in the role of vitamin D on thrombotic risk has become even more relevant after the identification of vitamin D receptor (VDR) on platelets[23,30], indicating that the function of platelets may also be affected by vitamin D concentrations in serum. To date, few studies examining the association between vitamin D level and platelet activation have been reported. A previous study found that platelet aggregation induced by adenosine diphosphate (ADP) was decreased in patients treated with calcitriol[31]. Furthermore, vitamin D level has been negatively correlated with mean platelet volume and associated with the increased incidence or risk of acute coronary syndrome, hypertension, and stroke in patients with stable coronary artery disease[32]. Increased platelet aggregation and thrombosis risk have been demonstrated in *VDR*-null mice[33]. Accordingly, a low concentration of vitamin D may promote platelet aggregation.

In our study, we found that a serum vitamin D concentration of 7.5 ng/mL had a 97% sensitivity and 81% specificity for the prediction of serum endocan concentration greater than 270 ng/L, which can be a predictor of endothelial inflammation. In previous studies, 270 ng/L serum endocan concentration was found to be a cut-off for endothelial pathologies and poor clinical outcomes[34].

Table II. Correlations between serum endocan levels, age, carotid intima-media thickness (CIMT), and serum vitamin D, C-reactive protein (CRP), and total cholesterol levels.

		Age	CIMT	Vitamin D	CRP	Total Cholesterol
Endocan	Correlation Co-efficient (r)	- 0.08	0.1	- 0.52	- 0.02	- 0.1
	p-value	p = 0.57	p = 0.48	$\mathbf{p < 0.01}$	p = 0.89	p = 0.48

Endocan = endothelial specific molecule-1; CRP = C-reactive protein, CIMT = carotid intima-media thickness.

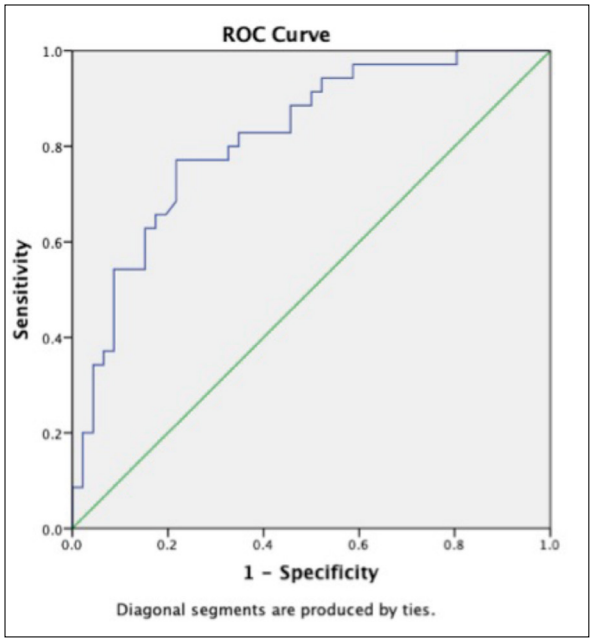

Figure 2. The receiver operating characteristics (ROC) curve analysis of serum vitamin D level for prediction of serum endocan level greater than 270 ng/L.

To emphasize, clinicians should be aware of endothelial pathologies in patients or even in healthy subjects with vitamin D levels below 7.5 ng/mL. In addition, in a separate clinical study, it would be appropriate to investigate whether there is a decrease in serum endocan level that accompanies vitamin D replacement.

Endocan is a biomarker that has been found to be a predictor of endothelial inflammation and pathologies such as atherosclerosis, diabetic vasculopathy, vascular invasion in malignancies, new vascular formation, and vascular thrombosis. Additionally, endocan is a diagnostic and prognostic biomarker in acute coronary syndrome and acute pulmonary embolism[15-18]. Serum endocan levels are increased in diseases with vascular involvement or invasion[35,36]. A study showed that there was a significant correlation between endocan messenger RNA (mRNA) levels and intratumoral microvessel density, mRNA of vascular endothelial growth factor, and vascular or venous invasion[35]. A previous study also demonstrated that serum endocan levels correlated positively with both markers of inflammation and were associated with all-cause mortality and cardiovascular outcomes in patients infected with COVID-19[34,37]. Therefore, serum endocan level increases in the presence of COVID-19 infection and influences mortality and cardiovascular outcomes, independent of conventional and nonconventional risk factors. In light of those results, one of the exclusion criteria of our study was a history of COVID-19 infection at any time, as it is an independent cause of high plasma endocan levels[37].

Our study had some limitations and strengths. Since COVID-19 infection causes endothelial damage in the short and long term, people who had contracted COVID-19 were excluded to minimize any confounding factors. In addition, volunteers using tobacco were excluded from the study, as tobacco use may cause elevated endothelial inflammation markers due to its similar effects as COVID-19 infection. The prevalence of tobacco uses and past COVID-19 infection among healthy volunteers has unfortunately limited the number of subjects who can be included in the study compared to the number of people screened. Instead of a limitation, we see the selection of suitable, healthy volunteers as a strength of our study. Another strength is that all healthy volunteers included in the study had normal CIMT, providing objective data that clinical atherosclerosis had not yet begun in participants.

Conclusions

Demonstrating that vitamin D deficiency can cause endothelial damage in the early period without the development of clinical cardiovascular disease will have a critical role in the prevention of cardiovascular mortality and morbidity in patients with a high risk of cardiovascular disease, especially in patients with a high risk of early atherosclerosis.

Whether vitamin D replacement has a place in the prevention of cardiovascular events will be determined by future studies with long-term follow-up.

Conflict of Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethics Approval

Approval was obtained from the Medical Research Ethics Committee of the Faculty of Medicine at Istanbul University. The study abided by the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Informed Consent

All participants provided written informed consent.

Availability of Data and Material

Data and material are available and can be sent up on request.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

Authors' Contributions

S.E., M.K. and N. S.: Conceptualization, Methodology, Software. S.E. and M.K.: Data curation, Writing- Original draft preparation. T.C., S.C.A., M.A., H.A., S.G., Y.C. and M.K.: Visualization, Investigation. S.E. and T.T.: Supervision A.M.: Software, Validation S.E. and M.K.: Writing-Reviewing and Editing.

Acknowledgements

We would like to thank all healthy volunteers who were involved in our study. M.K. accepts full responsibility for the accuracy and the integrity of the data.

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