The Relationship between Vitamin D Deficiency and Levels of Serum Endocan and Asymmetric Dimethylarginine as Indicators of Endothelial Dysfunction

D Vitamini Eksikliği ile Endotel Disfonksiyonu Göstergelerinden Serum Endocan ve Asimetrik Dimetilarjinin Düzeyleri Arasındaki İlişki

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Abstract

Aim: Recent studies show that vitamin D deficiency may play a role in the pathogenesis of various diseases, including Alzheimer's disease, Parkinson's disease, multiple sclerosis, diabetes mellitus, and cardiovascular diseases. In this study, we aimed to investigate the relationship between vitamin D levels and levels of serum endocan and asymmetric dimethylarginine (ADMA) as early cardiovascular risk markers in individuals with no history of chronic illness.

Materials and Methods: Two groups of participants were formed: Group 1 consisting of individuals with a vitamin D level <10 ng/ml (n=42) and Group 2 consisting of those with a vitamin D level >30 ng/ml (n=35). The two groups were compared in terms of body mass index (BMI) and levels of serum vitamin D, endocan, and ADMA.

Results: No significant difference was found between the groups in terms of age, sex, and BMI (p=0.67, p=0.69, p=0.052, respectively). The mean serum ADMA level was 104.5±44 μmol/L for Group 1 and 90.42±29 μmol/L for Group 2 (p=0.31). The mean endocan level was 549.5±245 ng/L for Group 1 and 465.99±207 ng/L for Group 2 (p=0.12). There was a significant negative correlation between vitamin D and endocan levels (r=-0.26, p=0.02).

Discussion and Conclusion: No significant difference was observed in participants with low levels of vitamin D. Accordingly, vitamin D deficiency was not found to have a significant effect on endothelial dysfunction.

Keywords: ADMA; endocan; endothelial dysfunction; vitamin D deficiency

Öz

Amaç: Güncel çalışmalar D vitamini eksikliğinin Alzheimer hastalığı, Parkinson hastalığı, multipl skleroz, diyabet ve kardiyovasküler hastalıklar gibi birçok hastalığın oluşumunda rolü olabileceğini göstermektedir. Biz de bu çalışmada kronik hastalık öyküsü olmayan bireylerde D vitamini düzeyi ile erken kardiyovasküler risk belirteçleri olan serum endokan ve asimetrik dimetilarjinin (ADMA) düzeyleri arasındaki ilişkiyi araştırmayı amaçladık.

Gereç ve Yöntemler: İki katılımcı grubu oluşturuldu: D vitamini düzeyi <10 ng/ml olan bireylerden oluşan Grup 1 (n=42) ve D vitamini düzeyi >30 ng/ml olanlardan oluşan Grup 2 (n=35). İki grup beden kitle indeksi (BKİ) ile serum D vitamini, endokan ve ADMA düzeyleri bakımından karşılaştırıldı.

Bulgular: İki grup arasında yaş, cinsiyet ve BKİ bakımından anlamlı fark olmadığı saptandı (sırasıyla p=0,67; p=0,69; p=0,052). Ortalama serum ADMA düzeyi Grup 1 için 104,5±44 μmol/L, Grup 2 için 90,42±29 μmol/L olarak tespit edildi (p=0,31). Ortalama endokan düzeyi ise Grup 1 için 549,5±245 ng/L, Grup 2 için 465,99±207 ng/L idi (p=0,12). Vitamin D düzeyi ile endokan düzeyi arasında anlamlı negatif korelasyon saptandı (r=-0,26; p=0,02).

Tartışma ve Sonuç: D vitamini düzeyi düşük katılımcılarda anlamlı bir farklılık gözlenmemiştir. Buna göre, D vitamini eksikliğinin endotel disfonksiyonuna bir etkisinin saptanamadığı söylenebilir.

Anahtar Sözcükler: ADMA; D vitamini eksikliği; endokan; endotel disfonksiyonu

Received/*Geliş* : 29.09.2019
Accepted/*Kabul*: 30.12.2019

DOI: 10.21673/anadoluklin.626396

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INTRODUCTION

Recent studies show that vitamin D deficiency may play a role in the pathogenesis of various diseases, including Alzheimer's disease, Parkinson's disease, multiple sclerosis, diabetes mellitus, and cardiovascular diseases (1). The vitamin D receptor (VDR) is found in almost all nucleated cells, such as the brain, pituitary, thyroid, breast, heart, liver, kidney, skin, colon and small intestine, prostate gland, and gonads, as well as osteoblasts, mononuclear cells, lymphocytes, and pancreatic islet cells. Increasingly more studies have shown that low levels of vitamin D can cause vascular inflammation and endothelial dysfunction, which is a predictor of future cardiovascular processes, and lead to the formation of foam cells and proliferation of smooth muscle cells, facilitating atherosclerosis. On the other hand, vitamin D replacement is reported to provide an improvement in endothelial function (2,3).

There are different markers that can be used to assess endothelial damage, two of which are asymmetric dimethylarginine (ADMA) and endocan. Nitric oxide (NO), produced by the endothelium, is the most important vasodilator, and during endothelial dysfunction its production is also disrupted. Factors involved in the local NO production include ADMA, which is an endogenous inhibitor of the NO synthase. ADMA levels have been shown to be a strong predictor of endothelial dysfunction (4). Similarly, endocan, a molecule released from endothelial cells, is associated with inflammation and endothelial dysfunction and has been shown to be significantly correlated with other inflammatory parameters (5). However, the relationship between vitamin D levels and endocan as well as ADMA has not been adequately investigated. Accordingly, in this study we aimed to explore the relationship between vitamin D levels and levels of endocan and ADMA as endothelial dysfunction indicators.

MATERIALS AND METHODS

Sample selection

The study was a prospective cross-sectional study, and included volunteers aged between 25 and 65 years who visited our internal medicine outpatient polyclinics for routine health-care examinations. We excluded patients with a history of cerebrovascular incidents, diabetes mellitus, peripheral artery disease, acute and chronic infection, collagen tissue disease, inflammatory bowel disease (Crohn's disease, ulcerative colitis), malignancy, hypo- or hyperthyroidism, chronic liver disease, portal hypertension, anemia due to folic acid or vitamin B12 deficiency, hemolytic anemia, coronary artery disease, ischemic heart disease, uncontrolled hypertension, chronic lung diseases (including COPD, bronchiectasis, asthma, pulmonary hypertension); surgical treatment in the last 6 months; alcoholism and substance abuse; pregnancy and lactation; active bleeding or thrombosis anywhere in the body; and use of corticosteroids, aspirin, clopidogrel, warfarin, beta-blockers, and statin. Those who were severely obese (with a body mass index >35 kg/m^2) and those who recently exercised heavily were also excluded.

Study ethics

The study protocol was approved by the Ethics Committee of the Bezmialem Foundation University. Informed consent was obtained from all volunteers prior to participation in the study.

Biochemical parameters

After 8 hours of fasting, blood samples were taken from the antecubital brachial vein by using a Vacutainer blood collection tube. Then the samples were centrifuged at 3000 xg for 10 min. The sera obtained were transferred to Eppendorf tubes and stored at -80°C in the Bezmialem Foundation University Biochemistry Laboratory until the analyses. On the day of analysis the tubes were brought to room temperature. The ADMA and endocan values in the samples were measured by ELISA on a plate reader (Thermo Scientific Multiskan FC, 2011-06, USA).

All samples were studied at the Biochemistry Department of the Bezmialem Foundation University. Complete blood count (CBC) analysis was performed using the Sysmex XT 1800i instrument (ROCHE-2011, Kobe, Japan). Biochemical analyses were performed with the COBAS 8000 instrument (ROCHE-2007, Tokyo, Japan) and the COBAS-C

system kit. The levels of 25 OH vitamin D3 were analyzed with the kit (ROCHE-2014-34 Mannheim, Germany) and the COBAS E 601 hormone analyzer (ROCHE, 2010, Tokyo, Japan). The thyroid hormone level was measured with Advia Centaur (Advia-2013-Tarrytown, USA) and parathormone with Advia Centaur (2006-Dublin, Ireland).

The procedures for the use of the ADMA ELISA Kit (Eastbiopharm, REF: CK-E11107) and Endocan (ESM-1) ELISA Kit (Eastbiopharm, REF: CK-E11240) were as follows: The samples and kits were brought to room temperature before the work started. Six standards; the resulting stock was obtained by serial dilution of the standard stock. Fifty µl of the standards was added to the antibody-coated microplate wells. Then, 50 µL of Streptavidin-HRP was added to the standards. After pipetting the standard microplate, the serum samples were pipetted in order to give 40 µl of each buffer. Ten µl ADMA-Antibody and 50 µl Streptavidin-HRP were placed on the serum samples, respectively. The microplate was covered and incubated at 37°C for 60 min. After preparing the 30X wash solution contained in the kit, the ELISA plate was washed 5 times at 350 µL in the washer. Fifty µl of Chromogen Solution and Chromogen Solution B were pipetted into all wells, respectively. Incubation was allowed for 10 minutes at 37°C without light. Fifty µl of Stop Solution was added to all wells. The microplate was read at 450 nm absorbance within 10 min. The absorbance of the standards was determined, and a log-log plot was obtained with absorbance in the x-axis and concentration in the y-axis, and the results were expressed in pg/ml.

Statistical analysis

All statistical analyses were performed using the SPSS (Statistical Package for Social Sciences) software. Normal distribution was assessed by the Kolmogorov–Smirnov Z test. Depending on the type and distribution of the variables, descriptive statistics were expressed in mean±standard deviation, number (percentage), and median (minimum–maximum). The t-test and the Mann–Whitney U test were used to compare independent groups with and without normal distribution, respectively. Pearson's chi-squared test was used in the analysis of categorical data. The relationship between normally and non-normally distributed variables was examined by the Pearson correlation coefficient and Spearman correlation coefficient, respectively. $p<0.05$ was considered statistically significant. Statistically significant values are shown in bold in the tables.

RESULTS

A total of 165 volunteers (36 males, 129 females) were evaluated. Of these, only 40 had a vitamin D level >30 ng/ml, which could be considered ideal. The vitamin D levels were between 20 and 30 ng/ml in 22, <20 ng/ml in 103, and <10 ng/ml in 42 individuals.

For the study, two groups of participants were formed: Group 1 (the study group) with severe vitamin D deficiency (vitamin D levels <10 ng/mL) consisted of 42 subjects (32 females, 10 males) and Group 2 (the control group) with normal levels of vitamin D consisted of 35 subjects (28 females, 7 males). The mean participant age was 42.6±9.6 years. The mean participant BMI was 29±5.6 kg/m^2 (30.2±5.2 kg/m^2 for Group 1 and 27.6±5.8 kg/m^2 for Group 2). There was no significant difference between the two groups in terms of sex, age, and BMI (p=0.69, p=0.67, p=0.052, respectively).

Also, there was no significant difference between the two groups in terms of their history of smoking, a risk factor for endothelial dysfunction (p=0.58). Similarly, when the fasting glucose and HOMA-IR values were examined, no statistically significant difference was found. While the LDL-cholesterol levels did not differ significantly, there was a significant difference between the two groups in terms of HDL-cholesterol and triglyceride levels (p=0.03, p=0.008, respectively). As vitamin D levels decreased, HDL-cholesterol levels decreased and triglyceride levels increased (Table 1).

The mean ADMA level was 104.5±44 µmol/L for Group 1 and 90.3±29 µmol/L for Group 2, with no significant difference between the two groups (p=0.31) (Figure 1) (Table 1). Similarly, the mean endocan level was 549.5±245 ng/L for Group 1

Table 1. Intergroup comparison of the patient data

Variables	Group 1 (Mean±SD)	Group 2 (Mean±SD)	*p*	Distribution type
25 $(OH)_2D_3$ (ng/ml)	5.63±2.25	52.99±27.13		Normal
Endocan (ng/L)	549.5±245	465.9±207	0.12	Normal
ADMA (μmol/L)	104.5±44	90.3±29	0.31	Non-normal
HOMA-IR	2.4±1.2	1.9±1	0.15	Non-normal
Glucose (mg/dl)	92.8±10	92.4±8	0.88	Normal
Creatinine (mg/dl)	0.75±0.12	0.78±0.16	0.66	Non-normal
Triglyceride (mg/dl)	131.9±68	101.2±75	**0.008**	Non-normal
HDL-C (mg/dl)	49.1±11.5	54.8±11	**0.034**	Normal
LDL-C (mg/dl)	119.5±32	109.8±27	0.17	Normal
AST (U/L)	18.9±6	17.1±4	0.22	Non-normal
ALT (U/L)	23.6±13	17.8±7	**0.02**	Normal
ALP (U/L)	77.6±24	63.5±16	**0.007**	Normal
Ca (mg/dl)	9.5±0.26	9.5±0.36	0.92	Normal
P (mg/dl)	3.3±0.4	3.5±0.35	0.06	Normal
TSH (uIU/mL)	1.45±0.8	2.16±2	0.09	Non-normal
PTH (pg/ml)	79.16±29	50.4±19	**<0.001**	Normal
CRP (mg/dl)	0.34±0.3	0.34±0.4	0.6	Non-normal
ESR (mm/saat)	12.5±7	12.2±8.1	0.89	Normal

ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; Ca: calcium; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; HDL-C: high-density lipoprotein cholesterol; HOMA-IR: homeostatic model assessment for insulin resistance; LDL-C: low-density lipoprotein cholesterol; P: phosphor; PTH: parathyroid hormone; TSH: thyroid-stimulating hormone

and 465.99±207 ng/L for Group 2, with no statistically significant difference (p=0.12) Serum endocan levels increased as vitamin D levels decreased and there was a significant negative correlation between the two parameters (Figure 2) (Table 1).

We also evaluated the relationship between Ca, P, Mg, ALP, PTH and vitamin D, well known for its effects on the musculoskeletal system and calcium metabolism. There was no statistically significant difference between the groups in terms of Ca, P, and Mg levels (p=0.92, p=0.06, p=0.73, respectively). However, when the serum ALP and PTH levels were compared, a significant difference was found between the groups (p=0.007, p<0.001, respectively). Both ALP and PTH levels increased as vitamin D levels decreased, showing a significant correlation (r=-0.32, p=0.006; r=-0.45, p<0.001) (Table 1).

Also, when the groups were compared in terms of serum AST and ALT levels, it was seen that both AST and ALT levels increased as vitamin D levels decreased. While the serum AST values did not differ significantly, there was a significant difference between the groups in terms of serum ALT levels (p=0.22, p=0.02, respectively). Serum creatinine levels did not differ significantly between the groups (p=0.66) (Table 1).

In order to detect vitamin D deficiency-related changes of inflammatory markers, we also compared the CRP and ESR levels and found no significant difference between the groups (p=0.6, p=0.89 respectively). Similarly, there was no significant difference between the two groups in terms of thyroid-stimulating hormone (TSH) values (p=0.09) (Table 1).

When the correlation between vitamin D levels and serum endocan, ADMA and other parameters was evaluated, there was a significant negative correlation between vitamin D and endocan levels (r=-0.26, p=0.02), vitamin D and HOMA-IR levels (r=-0.2, p=0.047), serum vitamin D and serum triglyceride levels (r=-0.28, p=0.015), serum vitamin D and serum ALT levels (r=-0.23, p=0.04), serum vitamin D and serum ALP levels (r=-0.32, p=0.006),

Table 2. Correlation of vitamin D levels with endocan, ADMA, and other parameters

	r	p
Endocan (ng/L)	-0.26	**0.02**
ADMA (µmol/L)	-0.18	0.1
HOMA-IR	-0.2	**0.047**
Glucose (mg/dl)	-0.09	0.43
Creatinine (mg/dl)	0.1	0.45
Triglyceride (mg/dl)	-0.28	**0.015**
HDL-C (mg/dl)	-0.15	0.2
LDL-C (mg/dl)	-0.16	0.17
AST (U/L)	-0.12	0.3
ALT (U/L)	-0.23	**0.04**
ALP (U/L)	-0.32	**0.006**
Ca (mg/dl)	0.04	0.7
Mg (mg/dl)	-0.19	0.09
P (mg/dl)	0.2	0.08
TSH (uIU/ml)	0.15	0.2
PTH (pg/ml)	-0.45	**<0.001**

ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; Ca: calcium; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; HDL-C: high-density lipoprotein cholesterol; HOMA-IR: homeostatic model assessment for insulin resistance; LDL-C: low-density lipoprotein cholesterol; Mg: magnesium; P: phosphor; PTH: parathyroid hormone; TSH: thyroid-stimulating hormone

and serum vitamin D and serum PTH levels (r=-0.45, p<0.001) (Table 2), while no significant correlation was found between serum endocan and ADMA values (r=0.19, p=0.1).

DISCUSSION AND CONCLUSION

Many studies evaluating various factors that may lead to vitamin D deficiency report that the lack of exposure to sunlight and UV-B radiation, the primary source of vitamin D, is the main cause of the increase in the global prevalence of vitamin D deficiency (6,7). In our study, we found that the rates of vitamin D deficiency and insufficiency were as high as 75% among our subjects. One reason for this could be that the participants were recruited during winter. A study for determining the seasonal fluctuation of vitamin D levels (8) found that the vitamin D deficiency rate among health-care workers was 48.9% in summer and 71.5% in winter, similar to our findings.

The main effect of vitamin D is on the calcium and bone metabolism. For the normal mineralization of the bone, the absorption of calcium and phosphorus is achieved by the effect of active vitamin D. When the vitamin D level falls below a certain threshold, the significant reduction in the intestinal absorption of calcium results in an increase in the PTH release from the parathyroid glands, responsible for the control of the blood and extracellular levels of ionized calcium (9). In our study, we found a significant difference between the two groups in terms of PTH and ALP values, in consistence with the literature. However, there was no significant difference between the calcium and phosphorus levels. Also, there was a negative correlation between vitamin D levels and PTH and ALP, as expected. Our study showed the effects of vitamin D on the bone metabolism, as did previous studies.

The high prevalence of diabetes mellitus in endemic areas where vitamin D levels are low suggests a relationship between vitamin D and diabetes. Studies have shown that vitamin D stimulates the pancreatic insulin production and secretion, and that low levels of vitamin D impair the glucose metabolism (10). An inverse relationship has been reported between all components of human metabolic syndrome and 25 (OH) D levels (11,12). Over the years, decreases in vitamin D levels have been

associated with increased rates of hypertension, obesity, insulin resistance, and glucose intolerance (12). We did not observe a significant difference in HOMA-IR and fasting glucose values in our study; however, when we examined the correlation between vitamin D levels and HOMA-IR, we found a negative correlation. Since our study did not include obese patients, we think that the difference between the two groups is not as clear as we expected.

The relationship between obesity and vitamin D deficiency is also one of the recently popular topics. There have been studies showing that vitamin D regulates the expression of adiponectin, which plays a role in the insulin sensitization, and leptin, which has an important role in appetite control. Vitamin D also inhibits the cytokine release and inflammation of the adipose tissue by inhibiting the NF-κB signaling pathway (11). The role of vitamin D in obesity and the metabolic syndrome is somewhat elucidated in light of these mechanisms. Although obesity is associated with vitamin D levels, there is not sufficient scientific evidence that vitamin D deficiency predisposes to obesity. Although it can prevent obesity to some extent, obese patients cannot be expected to lose weight only with vitamin D replacement. When we compared the groups with high and low levels of vitamin D, we found a remarkable difference between the two groups in terms of BMI, but this difference was not statistically significant, due to the fact that we excluded obese patients while recruiting our study and control groups.

Recent studies have reported that vitamin D deficiency has disruptive effects on endothelial function (13). London et al. showed that endothelial dysfunction and susceptibility to aortic calcification increased in hemodialysis patients with vitamin D deficiency (14). A study in vitamin D receptor-null (VDR-null) mice associated hypertension due to high renin levels and excessive cardiomyocyte stimulation leading to cardiac hypertrophy due to transmission disorder with vitamin D-associated signaling pathway (15). It has also been shown that 1,25 (OH) 2D3 stimulates cardioprotective genes in smooth muscle cells and vascular endothelium. These findings explain the decrease in thrombogenesis and fibrinolysis seen *in vivo* (16).

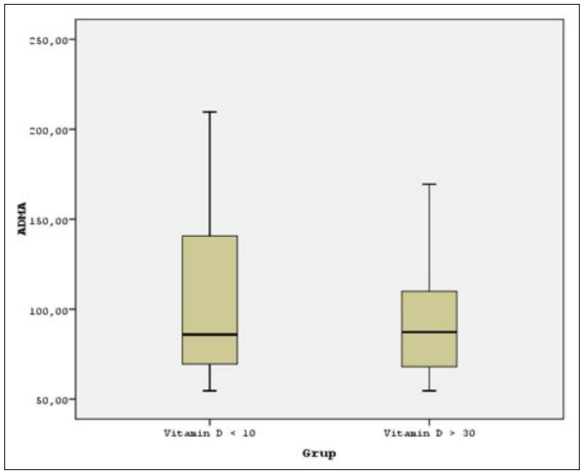

Figure 1. Comparison of ADMA levels according to groups

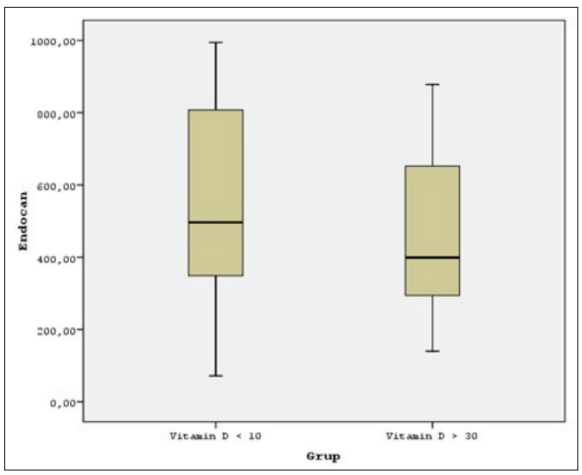

Figure 2. Comparison of endocan levels according to groups

Although animal and observational studies have shown a relationship between low levels of vitamin D and hypertension as well as development of cardiovascular disease (CVD), cardiovascular benefits of vitamin D supplementation have not been adequately demonstrated in randomized controlled trials (17). The association between vitamin D deficiency and CVD incidence seems to be a cause-and-effect relationship, but it is complicated by the fact that low 25-OH D levels may be the result, rather than the cause, of CVDs. People with no CVD who are followed up remotely are more likely to have normal outdoor activities than those with CVDs that require hospitalization. Regular exposure to sunlight allows them to have physiologically normal levels of vitamin D. This suggests that the association between vitamin D and CVDs might be an epiphenomenon (18).

Recently, the role of ADMA in endothelial dysfunction development has also been studied (4,19,20). High plasma levels of ADMA have been associated with an increased risk of cardiovascular events in various patient groups and described as an independent risk factor (19). However, reports on the relationship between ADMA and vitamin D have been contradictory. Ngo et al. (21) found an inverse correlation between vitamin D and ADMA levels in an aging population. Maaty et al. (22) compared patients with coronary artery disease and healthy controls and observed statistically lower levels of vitamin D in the patient group, though with no significant increase in the ADMA and SDMA levels. However, the hs-CRP and NO levels in the group with low vitamin D levels were found to be significantly high. In our study, we did not find statistically significant results in terms of vitamin D and ADMA levels, supporting the data of Maaty et al. Although there is a relationship between vitamin D and the NO system as described by Maaty et al., we also agree that this is not through the ADMA modulation.

When we reviewed the literature, we could not find a previous study focused on the relationship between serum endocan levels and vitamin D insufficiency in a healthy population. However, a study conducted in patients with prominent hypothyroidism reported a negative correlation between endocan levels and free T4 and vitamin D levels, while endocan levels showed a positive correlation with BMI, TSH, anti-thyroid peroxidase and anti-thyroglobulin (23). When we reviewed a recently published study including 38 patients who had a renal transplant at least 1 year before and who had vitamin D deficiency, there was a significant decrease in the endocan levels after vitamin D therapy (24). In our study, there was no significant difference between the groups with low and high levels of vitamin D, but we found a significant negative correlation between vitamin D and endocan levels. The number of our patients and other factors that we did not consider may have contributed to these results.

Similarly, we could not find a previous study exploring the relationship between endocan and ADMA as markers of endothelial dysfunction. In our study, we found no correlation between endocan and ADMA levels. The reason for this might be that ADMA is more likely to act through the NO system and endocan effects via adhesion molecules by different mechanisms, such as the leukocyte migration and release of inflammatory cytokines (5,25).

In conclusion, we showed that Vitamin D affected the bone metabolism, in consistence with previous studies. Increased insulin resistance and high triglyceride and low HDL-cholesterol levels, expected in people with vitamin D deficiency, are congruous with the expected cardiovascular risks. However, serum endocan and ADMA levels, thought to indicate endothelial dysfunction, did not show a significant difference in our participants with vitamin D deficiency. Therefore, our study suggests that there might not be a relationship between vitamin D levels and endothelial dysfunction as seen in CVDs, or that endocan and ADMA could not be adequately used to evaluate that relationship. However, we think that more long-term follow-up studies are needed for more accurate information.

Conflict of Interest and Financial Disclosure

The authors declare that they have no conflict of interest to disclose. The authors also declare that they did not receive any financial support for the study.

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