Original Article

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Association between Serum Osteopontin Levels and Cardiovascular Risk in Hypothyrodism

Hipotiroidide Serum Osteopontin Düzeyleri ve Kardiyovasküler Risk Arasındaki İlişki

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Abstract

Purpose: Cardiovascular effects of hypothyroidism are well known. Osteopontin (OPN) is a new inflammatory marker which was first isolated from the bone. Flow-mediated dilatation (FMD), a noninvasive technique to measure this endothelium-dependent function, has been used in several clinical studies to show cardiovascular risks. The aim of our study was to assess FMD value in hypothyroidism patients and to investigate whether plasma OPN level is a parameter which can predict cardiovascular risks in this group of patients.
Material and Method: This study included 39 patients who had high levels of thyroid-stimulating hormone (TSH) and 11 healthy euthyroid controls. Plasma TSH, free thyroxine, fibrinogen, high-sensitive C-reactive protein (hsCRP), fasting plasma glucose, total cholesterol (T-chol), low density lipoprotein (LDL), triglyceride and OPN levels were measured at the time hypothyroidism was first detected and after euthyroid state was achieved with levothyroxine treatment. In parallel with these assessments, brachial FMD measurements were also performed.
Results: In hypothyroid patients cardiovascular risk factors such as T-chol, LDL and triglyceride levels were higher than in control group but fibrinogen and hsCRP levels were not different between the groups. OPN levels were similar in patient and control groups, but basal FMD levels were lower in patients with hypothyroidism. After euthyroidism was achieved, OPN levels significantly decreased and FMD levels significantly increased, but a correlation was not detected between these two parameters.
Discussion: Our study did not show a significant correlation between OPN and cardiovascular risk parameters. Further studies are needed to use OPN as a cardiovascular risk marker in hypothyroid patients.
Keywords: Hypothyroidism, osteopontin, flow mediated dilatation, atherosclerosis

Öz

Amaç: Hipotiroidinin kardiyovasküler etkileri iyi bilinmektedir. Osteopontin (OPN), ilk olarak kemikten izole edilmiş olan yeni bir enflamatuvar belirteçtir. Akım aracılı dilatasyon (AAD), çeşitli klinik çalışmalarda kardiyovasküler riski göstermek için kullanılan invaziv olmayan bir tekniktir. Çalışmamızın amacı, hipotiroidi hastalarında AAD'yi değerlendirmek ve bu grup hastalarda kardiyovasküler riski değerlendirmekte plazma OPN düzeyinin bir parametre olarak kullanılıp kullanılmayacağını değerlendirmektir.
Gereç ve Yöntem: Bu çalışma, tiroid uyarıcı hormon (TSH) düzeyi yüksek 39 hasta ve 11 sağlıklı kontrol içermekteydi. Hipotiroidi ilk tespit edildiğinde ve levotiroksin tedavisi ile ötiroidi sağlandıktan sonra, plazma TSH, serbest tiroksin, fibrinojen, yüksek duyarlı C-reaktif protein (hsCRP), açlık plazma glukozu, total kolesterol, düşük yoğunluklu lipoprotein (LDL) kolesterol, trigliserid ve OPN düzeyleri ölçüldü. Bu değerlendirmelere ek olarak brakial arterden AAD ölçümü yapıldı.
Bulgular: Hipotiroidi hastalarında total kolesterol, LDL kolesterol ve trigliserid gibi kardiyovasküler risk faktörleri kontrol grubundan daha yüksekti, ancak fibrinojen ve hsCRP düzeyleri gruplar arasında farklı değildi. OPN düzeyi, hasta ve kontrol grubunda benzerdi fakat, bazal AAD düzeyi hipotiroidi olan hastalarda daha düşüktü. Ötiroidi sağlandıktan sonra, OPN düzeyleri anlamlı derecede azaldı ve AAD düzeyleri anlamlı derecede arttı fakat bu iki parametre arasında korelasyon saptanamadı.
Tartışma: Çalışmamız OPN ve kardiyovasküler risk parametreleri arasında anlamlı bir korelasyon olduğunu gösterememiştir. Hipotiroidi hastalarında bir kardiyovasküler risk belirteci olarak OPN kullanımı için ilave çalışmalar gereklidir.
Anahtar kelimeler: Hipotiroidi, osteopontin, akım aracılı dilatasyon, ateroskleroz

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Introduction

Hypothyroidism is a prevalent hormonal disorder characterized by decreased thyroid hormone. It may develop due to dysfunctions in the thyroid (primary) or pituitary gland (secondary). Primary hypothyroidism is responsible from approximately 99% of all cases (1). In regions where iodine amount is sufficient, the most common cause of hypothyroidism is Hashimoto's thyroiditis. The incidence of hypothyroidism is between 0.5% and 1.9% in women and <1% in men and subclinic hypothyroidism is 3-13.6% in women and 0.7-5.7% in men (2).

Hypothyroidism is known to be an important risk factor for cardiovascular diseases. High blood pressure, high levels of low density lipoprotein (LDL), high-sensitive C-reactive protein (hsCRP), homocystein, plasminogen activator inhibitor-1 (PAI-1) and D-dimer levels in circulation, and endothelial dysfunction are commonly associated with atherogenic risk factors. However, there is not a marker unique to hypothyroidism which can directly show increased cardiovascular risks in hypothyroidism patients (3,4,5,6,7).

Osteopontin (OPN), which is associated with immune system, inflammation and several malignancies, is a new inflammatory marker isolated from bone. Other than osteoblasts and osteoclasts, it is also synthesized in and released from macrophages, T cells, hematopoietic cells, vessel smooth muscle cells, fibroblasts, and myocardial cells. OPN has roles mainly in cellular immunity, cellular migration and infiltration, tissue repair, inflammatory diseases, angiogenesis, apoptosis inhibition, and regulation of extracellular matrix in tumor metastasis. In atherosclerotic plaques, high levels of OPN mRNA and proteins have been reported (8). In a study by Isoda et al. (9) it was found that OPN transgenic mice fed an atherogenic diet developed larger atherosclerotic lesions compared to non OPN transgenic mice. All of these findings suggest a role of OPN in atherosclerosis. In some human studies, blood OPN level was detected to be higher in patients with coronary artery disease than in controls (10).

Endothelial dysfunction, which is a prominent cardiovascular risk factor, is also an important marker. Noninvasive flow-mediated artery dilatation (FMD) technique measures an endothelial function-FMD-from brachial artery and it has been used as an assessment method of cardiovascular risk in several clinical studies (11). In a healthy human, normal FMD is 7-10% of baseline artery diameter. However, in patients with cardiovascular disease, FMD value is 0-5% of baseline value or it cannot be measured. Many studies suggest that endothelial dysfunction detected by FMD may be an independent marker to predict cardiovascular events (12).

The aim of our study was to assess FMD value, which is an atherosclerotic risk marker in hypothyroidism patients, and to investigate whether plasma OPN level is a parameter which can predict cardiovascular risk in this group of patients.

Materials and Methods

This study included premenopausal women between the ages of 20 and 45 years who were admitted to the Başkent University Faculty of Medicine, Department of Endocrinology and Metabolism Diseases and who were detected to have thyroid-stimulating hormone (TSH) elevation for the first time. Patients who had a history of atherosclerotic disease, diabetes mellitus, hypertension, hyperlipidemia, or smoking were excluded. Premenopausal women, whose thyroid function tests and thyroid autoantibodies were normal, were included in this study as euthyroid control group. The study complied with the declaration of Helsinki and was approved by the local research ethics committee. All the subjects gave written informed consent.

At first presentation, weight and height of all subjects were measured. Body mass index (BMI) was measured by dividing weight (in kilograms) by height (in meters) squared (BMI=weight/height^2). In the beginning, after at least 8 hours of overnight fasting, plasma TSH, free thyroxine (fT_4), antithyroglobulin antibody (ATA), anti-thyroidperoxidase antibody (anti-TPO), fibrinogen, hsCRP, fasting plasma glucose, total cholesterol (T-chol), LDL, high density lipoprotein cholesterol (HD), and triglyceride levels were measured. In addition, endothelium-dependent FMD was measured in all patients by high resolution ultrasonography. For OP measurement, plasma samples were preserved at -80 °C.

Then, 1.6-1.8 mcg/kg levothyroxine was introduced to the patients with high TSH levels. At the end of the 3-month follow-up period, when euthyroidism (TSH normalization: 0.4-4.6 µIU/mL) was achieved, all of the initial parameters were reassessed.

Serum TSH levels were measured using the chemiluminescence method with Abbott-Architect analyzer (Chicago, IL, USA) and ATA and anti-TPO levels were measured using the electrochemiluminescence method with Modular E170 analyzer (Roche Diagnostic, Mannheim, Germany).

Plasma fibrinogen levels were measured using the Clauss clotting method with BCT autoanalyzer (Siemens Healthcare Diagnostics, Newark, USA), serum alanine aminotransferase (ALT), T-chol, HDL and triglyceride levels were measured using the original kits of Roche Modular autoanalyzer (Roche Diagnostic GmbH, Mannheim, Germany). LDL levels were measured using Friedewal formula.

hsCRP levels were measured using the particle-enhanced immunonephelometric method with BN II System (Siemens Healthcare Diagnostics, Marburg, Germany).

Complete blood count (CBC) measurement was performed using automated cell counter (Cell-Dyn 3700, Abbott Diagnostics, Abbott Park, IL USA).

Endothelium-Dependent Flow-Mediated Dilatation Measurement

FMD measurements were performed by a trained cardiologist who was blinded to the thyroid function. An Antares US System ultrasound device (Siemens Inc, Mountain, View, CA) and a linear probe which had a multifrequency (4-9 MHz) feature were used for assessment. The subjects laid in the supine position. Right brachial artery was visualized 2-5 cm above the antecubital groove while its long axis was parallel to the probe and the first measurement of brachial artery diameter was made at rest. The measurement was standardized and the distance between the intima of the vessel wall close to the probe and media-adventitia of the vessel

wall away from the probe was measured. In all measurements, B-mode grey scale was used. Just after this measurement, collar of the blood pressure monitor was inflated (50 mmHg above the systolic pressure) for 5 minutes. Diameter measurement (approximately at the same location) was performed again 1 minute after deflation of the collar.

After this procedure, a formula (FMD-difference in artery diameter/ baseline artery diameter) was used to calculate FMD (13).

Osteopontin Measurement

For OPN assessment, blood samples were collected after an overnight fasting and centrifuged plasma was preserved at -80 °C. OPN levels were measured using a commercial ELISA kit which could measure both recombinant and native human OPN (Human OPN assay kit, IBL, Gunma, Japan).

For OPN, sensitivity was 3.3 ng/mL, and intra- and inter-assay variability percentages were <5% and <10%, respectively.

In accordance with the study protocol, the samples were diluted in a ratio of 1/5, injected into pits including OPN antibodies and incubated for 1 hour at 37 °C. After washing, 100 uL signed OPN solution was applied to each pit and kept for 30 m at 4 °C.

After washing, tetramethylbenzidine was used as caloric agent and the measurements were performed with automated ELISA reader (Tecan Sunrise, Salzburg, Austria) which has an absorbance value of 450 nm.

Statistical Analysis

Statistical analysis of the data were made using SPSS 18.0 package program. Categorical variables were summarized as numbers and percentages. Numerical measurements were summarized as mean and standard deviation (SD) (median and minimum-maximum values were provided when necessary). Normal distribution hypothesis of numerical measurements were tested using the Kolmogrov-Smirnov test. Numerical measurements

Table 1. Initial demographic features and laboratory parameters of the patient and the control groups

	The patient group (n=39)	The control group (n=11)	p
Age (years)	35.6±9.9	29.8±8.6	0.09
BMI (kg/m^2)	27.3±4.6	22.8±5.6	0.01
Anti-TPO (0-34 IU/mL)	313 (5-1525)	5.3 (2.8-29)	<0.001
ATA (0-115 IU/mL)	165.5 (10-1246)	10.3(10-120.7)	<0.001
TSH (0.4-4.6 µIU/mL)	19 (6-100)	1.3 (0.4-3.7)	<0.001
fT_4 (9-25 mol/L)	9.0±3.2	13.7±2.1	<0.001
T-chol (125-200 mg/dL)	207.2±53.1	153.7±33.8	0.01
LDL-chol (60-130 mg/dL)	123.8±44.4	88.8±28.3	0.02
HDL-chol (35-80 mg/dL)	51.4±14.8	49.9±10.1	0.75
Triglyceride (55-175 mg/dL)	227 (41-761)	68 (34-123)	0.01
hsCRP (0.0-6.0 mg/L)	3 (2.8-41.8)	3 (0.4-14)	0.08
Fibrinogen (1.8-3.5 g/L)	3.1	2.5	0.23
OPN (ng/mL)	229.7 (129.7-459.1)	194.6 (34.5-457.8)	0.19
FMD (%)	5.0±1.9	6.8±2.5	0.02

BMI: Body mass index, Anti-TPO: Anti-thyroid peroxidase, ATA: Anti-thyroglobulin, fT_4: Thyroxine, TSH: Thyroid stimulating hormone, T-chol: Total cholesterol, LDL-chol: Low density lipoprotein cholesterol, HDL-chol: High density lipoprotein cholesterol, hsCRP: High-sensitive C-reactive protein, OPN: Osteopontin, FMD: Flow mediated dilatation

Table 2. Comparison of pretreatment and 3. month (after euthyroidism was achieved) values of the patient group

	Pretreatment (n=39) (1)	Posttreatment (after euthyroidism was achieved) (2)	p
TSH (µIU/L)	19 (6-100)	2 (0.3-4.4)	p<0.001
T-chol (mg/dL)	207.2±53.1	185.2±41.1	p<0.001
LDL-chol (mg/dL)	123.8±44.4	113.5±35.5	p<0.001
HDL-chol (mg/dL)	51.4±14.8	47.7±10.9	p<0.001
Triglyceride (mg/dL)	227	104	0.11
Fibrinogen (g/L)	3.1±0.8	2.9±0.5	0.63
hsCRP (mg/L)	3 (2.8-41.8)	3 (3-51.4)	0.55
OPN (ng/mL)	229.7 (129.7-459.1)	149.6 (65.9-544)	p<0.001
FMD (%)	5.0±1.9	7.1±2.7	p<0.001

TSH: Thyroid stimulating hormone, T-chol: Total cholesterol, LDL-chol: Low density lipoprotein cholesterol, HDL-chol: High density lipoprotein cholesterol, hsCRP: High-sensitive C-reactive protein, OPN: Osteopontin, FMD: Flow mediated dilatation

were compared between the groups using a t-test if conditions were met and using the Mann-Whitney U test if conditions were not met. Dependent samples t-test was used for pre-and post-comparison of numerical measurements if conditions were met and the Wilcoxon signed-rank test was used if conditions were not met. Correlation between OPN and FMD was analyzed using the pearson correlation coefficient. Statistical significance was accepted as <0.05 in all statistical tests.

Results

This study included 39 patients with high TSH levels and 11 healthy euthyroid control subjects. The patient group consisted of 4 (10.2%) subclinical and 35 (89.8%) hypothyroid patients. After 3 months of follow-up and treatment, control OPN levels were measured in 28 and control FMD values were measured in 30 patients. The mean (±SD) age was similar between the patient and the control groups (35.64±9.98 and 29.81±8.68, respectively) (p=0.09). The mean (±SD) BMI value was higher in the patient group than in the control group (27.3±4.69 vs. 22.8±5.64 kg/m^2, respectively) (p=0.01). Initial data of the patient and the control groups are shown in Table 1.

At the beginning of this study, BMI, T-chol, LDL and triglyceride levels were statistically significantly higher in the patient group than in the control group and the p values were 0.01, 0.01, 0.02, and 0.01, respectively. In addition, FMD values were lower in the patient group, (p=0.02). Fibrinogen, hsCRP and OPN values were similar in the two groups (p>0.05). The mean diastolic blood pressure was also higher in the patient group (74.1±9.8 mmHg vs. 64.5±9.3 mmHg) (p<0.01).

We compared pretreatment (1) and posttreatment (2) (after euthyroidism was achieved) values of several parameters and found that T-chol, LDL, HDL and OPN values significantly decreased and FMD significantly increased. No significant difference was observed in other follow-up parameters (Table 2). Changes in OPN and FMD are schematized in Figure 1 and Figure 2.

Comparison between values of the patient group after euthyroidism was achieved (post treatment) and the initial values of the control group are given in Table 3. T-chol, LDLc and triglyceride levels were higher in the patient group than in the control group even after euthyroidism was achieved.

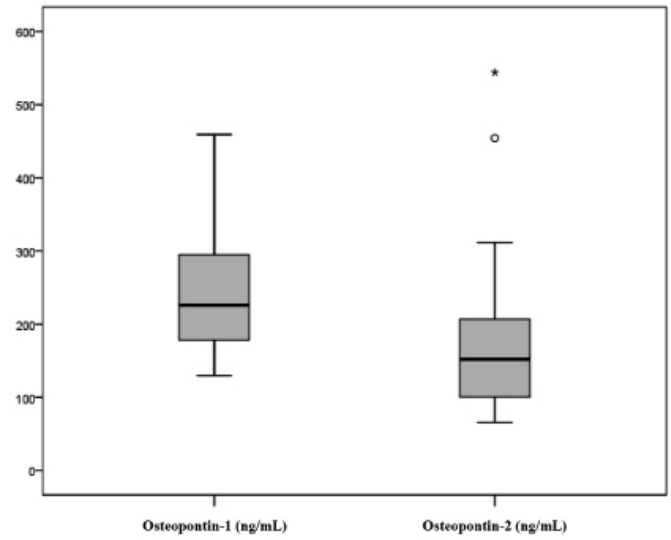

Figure 1. Pretreatment (1) and post treatment (after euthyroidism was achieved) (2) levels of osteopontin in the patient group (p<0.001)

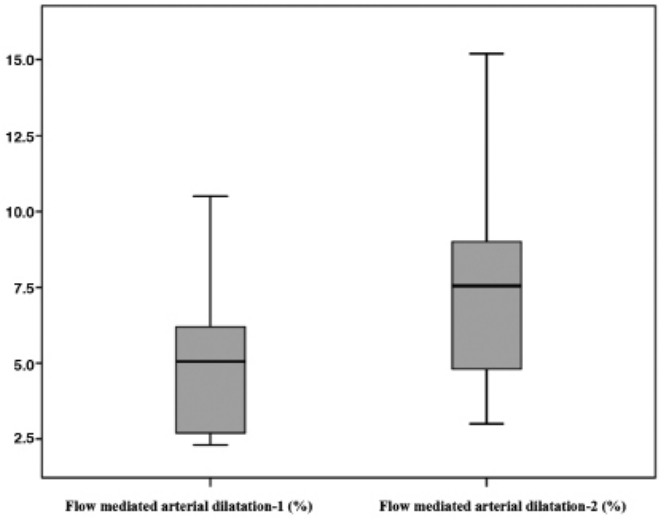

Figure 2. Percentage change in flow mediated arterial dilatation between pretreatment and post treatment (after euthyroidism was achieved) measurements of the patient group (p<0.001)

Table 3. Comparison of follow up parameters of the patient group after euthyroidism was achieved (post treatment) with baseline values of the control group

	Patient group (post treatment) (n=39)	Control group (n=11)	p
TSH (µIU/L)	2 (0.3-4.4)	1.3 (0.4-3.7)	0.27
T-chol (mg/dL)	185.2±41.1	153.72±33.8	0.02
LDL-chol (mg/dL)	113.5±35.5	88.8±28.3	0.04
HDL-chol (mg/dL)	47.7±10.9	49.9±10.1	0.55
Triglyceride (mg/dL)	104 (45-481)	68 (34-123)	0.01
Fibrinogen (g/L)	2.8 (2-4.7)	2.5 (2-30)	0.37
OPN (ng/mL)	149.6 (65.9-544)	194 (34.5-487.8)	0.43
FMD (%)	7.5 (3-15.2)	6.5 (2-10.2)	0.75

TSH: Thyroid stimulating hormone, T-chol: Total cholesterol, LDL-chol: Low density lipoprotein cholesterol, HDL-chol: High density lipoprotein cholesterol, OPN: Osteopontin, FMD: Flow mediated dilatation

In the patient group, we could not detect a correlation between OPN and FMD in pretreatment and posttreatment measurements (pretreatment OPN-FMD; r=-0.17, p=0.29, posttreatment OPN-FMD; r=-0.00, p=0.99).

We tested the correlation between baseline OPN level and pretreatment levels of all the parameters in the patient group and found only a weak negative correlation between diastolic blood pressure and OPN level (r=-0.392, p=0.027). Also, there was no correlation between post-treatment OPN level and baseline or posttreatment levels of all parameters.

After euthyroidism was achieved in the patient group, correlation between percentage change in OPN levels and follow-up parameters were analyzed. Positive correlations were observed between OPN change (%) and baseline T-chol (r=0.55, p=0.005), LDL (r=0.56, p=0.004), fibrinogen (r=0.52, p=0.006), and posttreatment T-chol (r=0.48, p=0.01), and LDL (r=0.44, p=0.02) levels.

Discussion

Epidemiological studies have shown accelerated coronary atherosclerosis in hypothyroidism. Atherosclerotic risk factors associated with hypothyroidism are hypercholesterolemia, hypertension, endothelial dysfunction, hyperhomocysteinemia and increased C-reactive protein level (14).

Previous studies have shown that cholesterol level may be more than 50% or higher in patients with hypothyroidism than that in controls (15). T-chol and LDL levels were correlated with high TSH levels detected in hypothyroidism and this suggested that lipid dysfunction was due to thyroid hormone deficiency (16). In our study, initial T-chol, LDL and triglyceride levels were elevated in accordance with the literature. We also detected significant decreases in T-chol and LDL and nonsignificant decrease in triglyceride levels after achievement of euthyroidism with thyroxine treatment.

A study by Verdugo et al. (17) showed a trend to decrease in HDL levels with thyroid hormone replacement treatment. In another study, hypothyroidism was associated with low HDL level and, after TSH normalization with thyroxine treatment, this level increased (18,19,20). In our study HDL level decreased with thyroxine treatment.

Fibrinolytic activity decreases in subclinical hypothyroidism. In these patients, there is a tendency for thrombosis; in contrast, in hypothyroidism, there is a tendency for bleeding (21). In our study, fibrinogen levels were measured to evaluate clotting system and, no difference was found between hypothyroidism patients and controls. Similarly, fibrinogen levels did not decrease in the patient group after achievement of euthyroidism with thyroxine.

In another study, TSH level was found to be significantly associated with BMI and waist circumference and, BMI increased with increasing TSH levels. Higher BMI values in hypothyroidism patients than in controls in our study may be explained by this fact (22). Although in a study including obese, overweight and normal weight individuals, circulating OPN level was significantly higher in obese and overweight individuals than in normal weight subjects (23), we could not find a positive correlation between BMI and OPN levels. This may be due to small sample size in our study.

The patient and the control groups in our study involved only premenopausal women. According to a study by Cho et al. (24) which investigated the association between serum OPN level and BMD, postmenopausal women had higher serum OPN levels than premenopausal women. We could not evaluate this topic because our study did not involve any postmenopausal woman.

Reza et al. (25) found that OPN was upregulated in several patients with hyperthyroidism and downregulated in hypothyroid patients. While OP was positively correlated with fT_3 and fT_4, it was negatively correlated with TSH and a significant correlation was found between them. The authors suggested that OPN might be useful as a novel prognostic biomarker in patients with impaired thyroid function. We found similar OPN levels in hypothyroid and euthyroid patients. The aforementioned difference could be missed in our study due to small sample size.

In a study on osteoprotegerin (OPG), which is in same class with OPN, it was found that OPG significantly elevated in hypothyroidism and significantly decreased with thyroid replacement. In that study OPG levels were correlated with vascular damage independent from the severity of hypothyroidism (26). In the same study, a positive correlation was detected between TSH and OPG levels but an association was not detected between OPG level and T-chol, LDL and HDL levels. No relationship was detected between baseline levels of serum lipids or changes in the lipid levels and OPG levels after thyroxine treatment. Also no relationship was detected between hemodynamic parameters such as systolic and diastolic blood pressures and OPG levels (26).

In a study involving 409 patients (280 patients with coronary artery disease and 129 patients without coronary artery disease) carotid-femoral pulse wave velocity (PWV) was used as aortic stiffening index and OPN and osteoprotogerinin were used as vascular remodeling and calcification markers. Plasma OPN and OPG were found to be associated with arterial stiffness. The first findings of this study suggested that OPN and OPG were associated with the pathogenesis of atherogenesis in coronary artery disease. In patients with coronary artery disease, higher levels of OPG and OPN and impaired carotid-femoral PWV were detected compared with patients without coronary artery disease. In addition, PWV was found to be associated with OPG and OPN levels. Linear regression analyses showed that plasma OPG and OPN levels increased independent from other cardiovascular risk factors (27). We could not find a significant difference in OPN levels between patients with hypothyroidism and controls, but after treatment of hypothyroidism, plasma OPN levels significantly decreased. FMD values in hypothyroidism patients were lower than in controls. After thyroxine treatment, FMD values significantly increased. However, in contrast to a study by Tousoulis et al. (27) who detected an association between PWV and OPN, we could not detect an association between OPN and FMD.

FMD reflects early phases of atherosclerosis. In hypothyroidism, flow-related, endothelium-dependent vasodilatation has been shown to be impaired like in patients who were at early phases of atherosclerosis. It is not clear whether this alteration

is a direct effect of thyroid hormone deficiency or effect of hypercholesterolemia secondary to hypothyroidism (28). A prospective study by Akcakoyun et al. (29) evaluated endothelial functions in 135 patients who underwent coronary artery stenting under elective conditions with FMD. Measurements were made before and after stenting procedure. FMD was significantly lower in patients who suffered from a vascular event than patients who did not suffer from a vascular event (4.7±1.9% and 6.0±2.0%, respectively, p=0.007) (29). Similar studies by Fathi et al. (30) and Neunteufl et al. (31), showed that low FMD was associated with higher frequency of cardiovascular events. In our study, hypothyroidism patients had lower FMD than controls at the time of diagnosis, as expected. After achievement of euthyroidism with thyroxine treatment, FMD increased significantly approaching euthyroid control group.

As a result, animal studies and experimental studies dominate literature about OPN. OPN use in human studies is for now inadequate. We could not detect a significant correlation between OPN and cardiovascular risk parameters and, therefore, we cannot recommend the use of OPN as a cardiovascular risk marker. Larger scale studies are needed to show the presence of such an association.

Ethics

Ethics Committee Approval: The study complied with the declaration of Helsinki and was approved by the Local Research Ethics Committee, Informed Consent: All the subjects gave written informed consent.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Gülhan Duman, Eda Melek Ertörer, Concept: Gülhan Duman, Eda Melek Ertörer, Neslihan Başçıl Tütüncü, Design: Gülhan Duman, Eda Melek Ertörer, Data Collection or Processing: Gülhan Duman, Emre Bozkırlı, Okan Sefa Bakıner, Eda Melek Ertörer, Analysis or Interpretation: Gülhan Duman, Eda Melek Ertörer, Literature Search: Gülhan Duman, Eda Melek Ertörer, Türkan Mete, Writing: Gülhan Duman, Eda Melek Ertörer, Türkan Mete.

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