Evaluation of Oxidative Stress, the Activities of Paraoxonase and Arylesterase in Patients With Subclinical Hypothyroidism

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Introduction: In subclinical hypothyroidism (SH), serum lipid and lipoprotein concentrations are frequently changed. Compared with the healthy population, the levels of oxidized low-density lipoprotein cholesterol are higher, and the levels of high-density lipoprotein cholesterol are lower. In patients with SH, the mechanism of atherosclerosis may beattributed to the lipid abnormalities. There is evidence showing that oxidation plays an important role during the process of atherosclerosis. Inthis study, we evaluated the activity of paraoxonase (PON) and arylesterase (ARE) in patients with SH and investigated their relation with oxidative stress.

Methods: The study enrolled 25 patients with SH and 20 sex- and age-matched healthy controls. The patient group and the control group were compared in terms of the activity of PON and ARE and the oxidative stress index.

Results: Between 2 groups, no significant difference was found in terms of age, sex, serum levels of total cholesterol, low-density lipoprotein, and high-density lipoprotein. In the SH group, the activity of PON was significantly lower than that observed in the control group ($P < 0.05$). Arylesterase activity also was significantly lower in the group with SH, compared with the control group ($P < 0.05$). Oxidative stress index was found to be significantly higher in the patient group, compared with the healthy subjects ($P < 0.01$). Oxidative stress index showed a strong positive correlation with the levels of thyroid stimulating hormone in all cases ($r = 0.60$, $P < 0.01$).

Conclusions: The activity of PON and ARE were significantly decreased, and oxidative stress was increased in patients with SH. Lower activities of these 2 biomarkers indicate increased oxidative damage in SH. Atherosclerosis in SH can be attributed to increased oxidative stress.

Key Words: subclinical hypothyroidism, paraoxonase, arylesterase, atherosclerosis, oxidative stress

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Subclinical hypothyroidism (SH) is the most commonly seen thyroid dysfunction and is defined as the presence of high levels of thyroid stimulating hormone (TSH) when the values of free triiodothyronin (fT3) and free thyroxin (fT4) are within reference range. Thirty percent of the patients with SH may exhibit symptoms suggesting thyroid hormone deficiency. Most of the complaints of the patients are dry skin, weakening of memory and thinking, weakness of muscles, fatigue, muscular cramps, swollen eyes, cold intolerance, constipation, and deep voice.[1,2] Definitive insights about the therapeutic modalities are still lacking.

Subclinical hypothyroidism is accompanied by left and right ventricular systolic and diastolic dysfunction, increased atherosclerosis, and myocardial infarction risk. In patients with SH, mechanism of atherosclerosis may be attributed to the lipid abnormalities as well as direct effects of thyroid hormone on blood vessels.[1,3–5]

In thyroid disorders, serum lipid and lipoprotein concentrations are commonly changed. Compared with the healthy population, serum levels of low-density lipoprotein (LDL) cholesterol are higher, and those of high-density lipoprotein (HDL) cholesterol are lower.[1,3–5] High-density lipoprotein prevents the oxidative changes in LDL. In several studies, it has been reported that paraoxonase 1 (PON 1), which is an antioxidant enzyme present in the structure of HDL, contributes to the protective effect of HDL, by preventing the LDL oxidation.[6,7] Paraoxonase 1 is associated with the apolipoprotein A-1 (apoA-1) and apolipoprotein J (apo J) proteins of HDL.[8] Apolipoprotein A-1 is the structural protein of HDL and stabilizes the linking of PON 1 to the HDL phosphoriles by N-terminal ending. It was suggested that lower PON 1 serum activity despite the normal levels of HDL, would lead to a decreased protective effect of HDL against the oxidation of LDL and, thereby, to an increased incidence of atherosclerosis. Paraoxonase 1 is an ester hydrolase that has both arylesterase (ARE) and PON activities.[9] As PON, for whom the physiological substrates have not been defined yet, catalyzes the hydrolysis of the organophosphate compounds frequently used for the production of insecticide and sarin, which are important for the toxicological studies and for the in vivo xenobiotic metabolism studies. On the other hand, after the determination of the presence of PON in the structure of HDL in the plasma, the studies investigating the physiological functions of PON are gradually increasing. The importance of PON for the cardiovascular physiology, the relation of PON with lipid and lipoprotein metabolism, its potential anti-atherogenic effect, and the antioxidant characteristics against peroxidative damage are extensively investigated.

Patients with SH are suggested to exhibit atherosclerosis and oxidative stress. Paraoxonase and ARE are known to be markers ofinflammatory state. Our aim was to evaluate the activities of PON and ARE in patients with SH and to investigate the status of serum total antioxidants and total oxidative status to see whether there is a relation between PON/ARE activity and oxidative stress. Moreover, we aimed to determine whether PON/ARE activity and total antioxidant status contribute to the development of atherosclerosis in the patients with SH.

MATERIALS AND METHODS

This study was prospectively designed. The study protocol was approved by the local ethics board of our hospital. Before performing any procedure, all the patients were extensively informed about the study and signed an "informed consent form."

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From the patients between 18 and 65 years that were admitted to our hospital for various reasons, the patients with SH for whom medical history, physical examination, and clinical and laboratory investigations were performed; who were newly diagnosed; and who had not received medical therapy before were enrolled. Our study group consisted of 25 patients with SH (37 ± 11 years, 72% female subjects) and 20 age- and sex-matched healthy subjects (35 ± 9 years, 60% female subjects).

The exclusion criteria were defined as follows: age younger than 18 or older than 65 years, morbid obesity (body mass index [BMI], >35 kg/m^2), hyperlipidemia, hypertension, diabetes mellitus, malignancy, hepatic disease, acute or chronic infections, renal failure, autoimmune disease, cardiovascular disease, anemia, and smoking.

According to the criteria of the biochemistry laboratory of the hospital, normal serum levels were considered as follows: TSH: 0.27 to 4.20 mIU/mL, fT3: 1.80 to 4.60 pg/mL, fT4: 0.93 to 1.70 ng/dL. Cases with SH were defined as having serum TSH level above 4.20 mIU/mL and fT4 level within the reference range. Blood samples were collected from all patients after an 8- to 12-hour fasting period. For the blood samples for which the serum part would be separated, the tubes with gel and red cap were used. To separate the serum and the plasma, the blood taken into biochemistry tubes were centrifuged at 3000 rpm for 10 minutes. Serum samples were aliquoted and then stored at –80°C.

Systolic and diastolic arterial blood pressures of all the cases were measured using an aired manometer from the brachial artery after a resting period of at least 5 minutes at lying position before the examination. Body weight was measured using a calibrated balance at 0.1 kg sensitivity with light clothes and without shoes. Height measurements were performed in standing position without shoes, with a sensitivity of 0.01 m. BMI was calculated by dividing the body weight by the square of the height in meter (kg/m^2).

Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using a formula defined by Matthews.

HOMA-IR = (fasting insulin level (microU/mL) * Fasting glucose level (mmol/dL) / 22.5.

Measurement of Total Oxidant Level (Total Oxidant Status)

In the serum samples, total oxidant status (TOS) levels were investigated using a fully automated method developed by Erel.[10] According to this method, serum oxidants oxidize the complex of ferrous ion-o-dianisidine to ferric ion. Again, the existing molecules of glycerol accelerate the oxidation reaction. Ferric ions form a colored complex with xylenol orange in an acidic environment. The intensity of the color, which is related to the amount of the oxidants present in the serum sample, is measured at 37°C and at a wave length of 530 nm with spectrophotometry. As the calibration of the measurement is done using hydrogen peroxide, the results of the measurement are expressed as micromolar hydrogen peroxide equivalent in 1 L (μmol H_2O_2 Equiv/L).

Measurement of Total Antioxidant Capacity

In the serum samples, total antioxidant capacity (TAC) levels were investigated using a fully automated method developed by Erel.[11] According to this method, as a strong biological radical, $ABTS^+$ cation radicals are formed. 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) enters to a reaction with peroxidase, and H_2O_2 and is oxidized to blue-green $ABTS^+$ cation radicals. Antioxidants of the serum sample convert the blue-green $ABTS^+$ cation radicals to the uncolored ABTS form and, thereby, decrease the amount of environmental $ABTS^+$ cation radicals. The change of absorbance at 37°C and 660 nm will be associated with the total antioxidant capacity of the serum sample. As the calibration of the measurement was performed using stable antioxidant standard solution called Trolox Equivalent (an analogue of vitamin E), the results of the measurement are expressed as μmol Trolox Equiv/L.

EVALUATION OF OXIDATIVE STRESS INDEX

Oxidative stress index was used as an indicator of the oxidative stress grade in the patient and control groups. Calculation of OSI using the kits for TAC and TOS was performed according to the formula cited below:

OSI (Arbitrary Unit) = [TOS (μmol H_2O_2 equivalent/L) / TAC (μmol Trolox equivalent/L)] × 100.

MEASUREMENT OF PAROXONASE ACTIVITY

Paraoxonase 1 activity of the serum samples was examined using fully automated method developed by Rel Assay Diagnostics (Mega Tıp, Gaziantep, Turkey). According to this method, paraoxonase activity is measured in medium without NaCl (basal paraoxonase activity) and with NaCl (salt-stimulated paraoxonase activity). Hydrolysis of the paraoxone (diethyl-p-nitrophenylphosphate) is monitored with the follow-up of the increase of absorbance at 37°C and 412 nm. The amount of p-nitrophenol resulting from the hydrolysis is calculated using the molar absorption coefficient 17,000 $M^{-1}cm^{-1}$ (at pH 8). Net value with enzymatic activity is calculated by subtracting the basal activity value from salt-stimulated activity value. The results are expressed as unit per liter, which is equal to the hydrolysis of 1 micromole substrate in 1 minute and 1 liter.

MEASUREMENT OF PAROXONASE ARYLESTERASE ACTIVITY

Paroxonase arylesterase activity of the serum samples was measured using fully automated method developed by Rel Assay Diagnostics (Mega Tıp, Gaziantep, Turkey. According to this method, phenylacetate is used as a substrate for the measurement of arylesterase activity, and with the hydrolysis of phenylacetate, phenol and acetic acid are formed. The resulting phenol joins to 4-aminoantipyrine and potassium ferricyanide and is measured with colorimetric method. Arylesterase enzyme activity is calculated from 4000 $M^{-1}cm^{-1}$, which is the molar absorption coefficient of the resulting colored complex. The results are expressed as unit per liter, which is equal to the hydrolysis of 1 micromole phenylacetate in 1 minute and in 1 liter.

STATICTICAL ANALYSIS

Statistical analysis was performed using SPSS (Statistical Package for Social Sciences) for Windows 11.5 software. The variables were expressed as SD and mean or median. The means were compared using Student T test, and median values were compared using Mann Whitney U test. Data were compared with each other using Spearman correlation test. The incidences were compared using χ^2 and Fisher exact tests. In this analysis, $P < 0.05$ was considered as significant.

RESULTS

Clinical and demographic characteristics of the patients and the control group are presented in Table 1. Demographic data of the study group also is shown in Figure 1. Figure 2 shows the comparison of the thyroid function tests of the patients and the control group in our study. The value of TSH in study

TABLE 1. Clinical and Demographic Characteristics of the Study and the Control Groups

	Patients (n = 25)	Controls (n = 20)	*P*
Age (yr)	37 ± 11	35 ± 9	<0.05
Total cholesterol (mg/dL)	184 ± 35	172 ± 27	<0.05
LDL (mg/dL)	112 ± 29	102 ± 22	<0.05
HDL (mg/dL)	50 ± 11	51 ± 10	<0.05
Triglyceride (mg/dL)	107 ± 37	85 ± 40	<0.05
Paraoxonase (U/L)	135 (82–195)*	190 (124–355)*	<0.05
Arylesterase (U/L)	232 ± 33	255 ± 40	<0.05
TAC (μmol Trolox equivalent/L)	2.5 ± 0.2	2.3 ± 0.2	<0.05
TOS (μmol H_2O_2 equivalent/L)	5.9 (5.4–7.4)*	3.9 ± 0.8	<0.05
OSI	239 (194–303)*	174 ± 41	<0.05
Glucose (mg/dL)	85 ± 7	93 ± 4	<0.05
Urea (mg/dL)	23 ± 4	25 ± 5	<0.05
Creatinine (mg/dL)	0.7 ± 0.1	0.8 ± 0.07	<0.05
BMI (kg/m^2)	26 ± 2	23 ± 3	<0.05
HOMA-IR	1.8 ± 0.8	1.5 ± 0.5	<0.05
Diastolic blood pressure (mm Hg)	71 ± 8	71 ± 6	<0.05
Systolic blood pressure (mm Hg)	111 ± 12	116 ± 9	<0.05
Heart rate (beats/min)	76 ± 8	77 ± 7	<0.05

*Median values between 25%–75% confidence interval.

group was significantly higher than the control group.(6.09 ± 1.41 mIU/mL, 1.92 ± 1.00 mIU/mL, respectively; $P > 0.0001$). The value of fT3 and fT4 was not significantly different between the groups (fT)3: study group 3.09 ± 0.60, control group 3.09 ± 0.31; $P = 0.98$; fT4: study group 1.31 ± 0.53, control group 1.26 ± 0.14; $P = 0.71$).

Paraoxonase activity was significantly decreased in patients with SH, compared with the control group ($P < 0.05$). In the patient group, paraoxonase showed a positive correlation with total oxidant level ($r = 0.40$, $P = 0.04$), but there was no correlation with HDL ($r = 0.18$, $P = 0.37$).

Arylesterase activity was markedly impaired in the group with SH, when compared with the control group ($P < 0.05$). Arylesterase showed a moderate positive correlation with serum HDL level in healthy subjects ($r = 0.49$, $P < 0.05$) and a slight positive correlation with serum triglyceride level in SH patients ($r = 0.41$, $P < 0.05$). Additionally, ARE showed a moderate positive correlation with BMI in SH patients ($r = 0.58$, $P < 0.05$) and a slight positive correlation with serum glucose level in all cases ($r = 0.35$, $P < 0.05$).

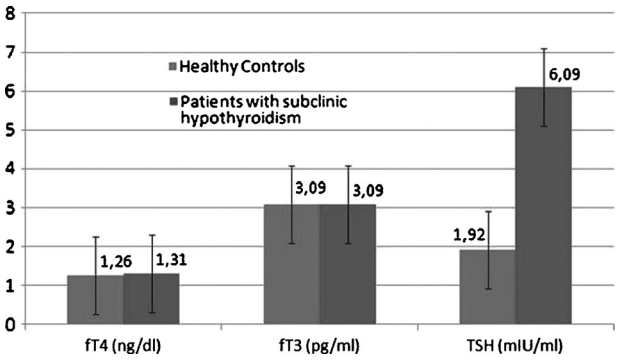

FIGURE 1. The thyroid function tests of patients and controls. Serum fT3, fT4, and TSH levels of the patients with SH and the control group (fT)3: free triiodothyronine; fT4: free tetraiodothyronine; TSH, thyroid stimulating hormone).

Oxidative stress index was significantly increased in patients with SH, compared with the control group ($P < 0.05$). There was a strong positive correlation between OSI and serum TSH in all subjects ($r = 0.60$, $P < 0.05$). Oxidative stress index exhibited a strong positive correlation with TOS levels in all cases ($r = 0.94$, $P < 0.05$), whereas a moderate negative correlation with serum glucose level was obtained ($r = –0.52$, $P < 0.05$).

Total oxidant level was significantly increased in patients with SH, compared with the control group ($P < 0.05$), and a strong positive correlation was observed between TOS and serum TSH level ($r = 0.69$, $P < 0.05$). There also was a moderate negative correlation between TOS and serum glucose level in all cases ($r = –0.56$, $P < 0.05$).

Total antioxidant capacity was increased in patients with SH, compared with the controls ($P < 0.05$; Table 1). Total antioxidant capacity showed a moderate positive correlation with serum urea level in the control group ($r = 0.54$, $P < 0.05$) and a slight positive correlation with heart beats per minute in the SH group ($r = 0.41$, $P < 0.05$) and with serum TSH levels in all cases ($r = 0.43$, $P < 0.05$). Moreover, TAC showed a slight positive correlation with triglyceride levels in all cases ($r = 0.44$, $P < 0.01$) and a moderate positive correlation with BMI in all cases ($r = 0.46$, $P < 0.01$).

Figure 2 shows the comparison of PON activity, ARE activity, TAC, TOS, and OSI values of the patients with SH and the controls.

DISCUSSION

Subclinical hypothyroidism is the most commonly encountered thyroid dysfunction.[12] In patients with SH, the mechanism of atherosclerosis may be attributed to lipid abnormalities.[1,3–5] Whereas some studies revealed higher levels of serum cholesterol in patients with SH, some of them observed no difference in terms of serum lipid levels between SH patients and healthy controls.[13–15] Subclinical hypothyroidism is a risk factor for

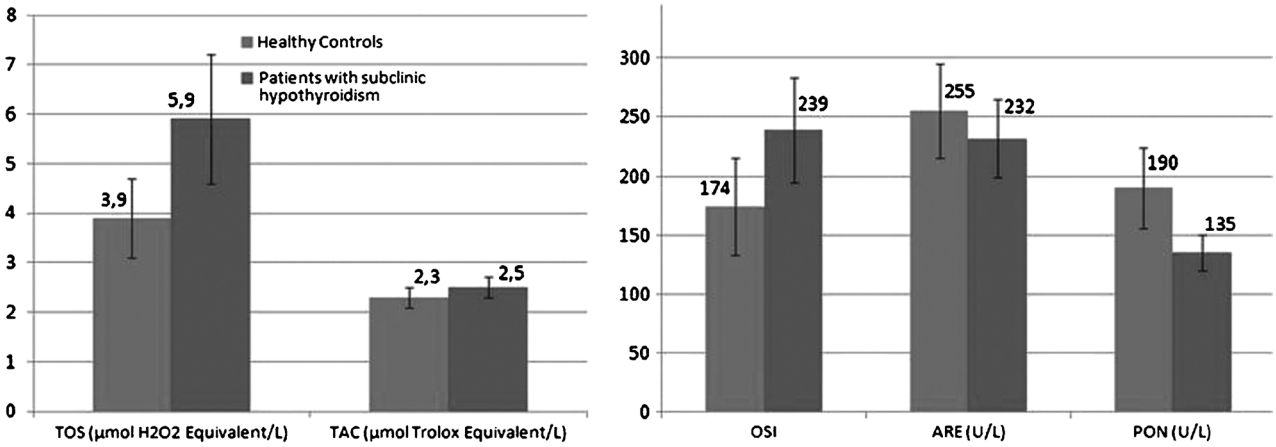

FIGURE 2. The thyroid function tests of the patients and the control group. Comparison of paraoxonase activity, arylesterase activities, TAC, TOS, and OSI values between the patients with SH and the healthy cases. Serum levels of PON, ARE, OSI, TAC, and TOS of the patients and the control group.

atherosclerosis because of increased levels of LDL cholesterol and decreased levels of HDL cholesterol.[1,3–5] In some studies, it was revealed that normal serum level of TSH has an inverse effect on the levels of serum lipoproteins.[16–18] An increase of 1 mIU/mL in the level of serum TSH increases serum total cholesterol concentration by 0.09 mM (3.5 mg/dL) in women and by 0.16 mM (6.2 mg/dL) in men.[16] Because of these lipid abnormalities, SH may be considered as a risk factor for atherosclerosis.[19,20] In Rotterdam study,[21] a strong correlation was found between SH and atherosclerotic cardiovascular diseases, in the postmenopausal women, independent from classical risk factors as smoking, hypertension, hypercholesterolemia, and diabetes mellitus. In the study performed by Başkol et al.,[22] it was suggested that lipid peroxidation plays an important role in the pathogenesis of atherosclerosis in hypothyroidism. Protective effect of HDL against LDL oxidation was documented in several studies before. Paraoxonase 1, an antioxidant enzyme found in the structure of HDL, contributes to the protective effect of HDL by preventing the oxidation of LDL.[6,7] Durrington et al.,[23] in the study in which they aimed to realize the hypothesis that PON 1 is responsible to destruct the lipid peroxides before their accumulation on LDL, observed that purified PON 1 is effective in the prevention of the lipid peroxidation of LDL. In the study performed by Aviram et al.,[24] they observed that PON 1 alone has a greater effect for the prevention of LDL oxidation. In several studies, it was shown that HDL-dependent PON prevents not only LDL oxidation but also HDL oxidation.[25] This effect is related to the ability of PON to hydrolyze the lipoprotein-mediated peroxides. It was suggested that a low PON1 serum activity despite the normal HDL levels would lead to a decrease in the protective effect of HDL against LDL oxidation and, thereby, to an increase in the incidence of atherosclerosis.

During the acute phase reaction, a significant decrease was observed in the PON 1 activity.[26] Ayub et al.[27] reported that serum PON 1 activity and concentration were decreased within 2 hours after the onset of the symptoms of myocardial infarction. However, the level of PON 1 remained unchanged during 42 days after myocardial infarction, although the acute phase reaction was over. These data support the idea that PON 1 might play a role in the atherosclerotic process.

In our study, we documented that serum PON 1 activity was significantly decreased in patients with SH, compared with the control group. In the study performed by Başkol et al.,[22] PON 1 activity was found to be lower in the pretreatment patients compared with the control group and posttreatment cases of hypothyroidism. Posttreatment mean PON 1 activity for hypothyroidism showed a significant increase but still remained significantly lower compared with the control group. In contrast to our results, Millionist et al.[28] observed that PON 1 activity was similar among the patients with SH and the control group. Azizi et al. documented that[29] patients with hyperthyroidism and hypothyroidism showed a lower PON 1 activity compared with the control group.

In our study, PON 1 did not show any correlation with HDL in the group with SH. In the study performed by Jayakumari and Thejaseebai,[30] although PON1 activity was positively correlated with HDL cholesterol in healthy controls, in the presence of coronary artery disease, they showed a negative correlation. Similar to our results, Şentürk et al.[31] observed no correlation between PON 1 activity and HDL levels in the patients with acute coronary syndrome.

Paraoxonase 1 is an ester hydrolase with both arylesterase and paraoxonase activities. Polymorphism has a determining effect on its ability to prevent the LDL oxidation. In the present study, serum arylesterase activity was found to be significantly lower in patients with SH, suggesting development of atherosclerosis. Contrast to our results, in the study conducted by Coria et al.,[32] no marked difference on ARE activity was obtained among women with obvious hypothyroidism, SH, and euthyroidism. However, Gamboa et al.[33] observed impairment in PON and ARE activities in patients with chronic heart disease.

Paraoxonase 1 enzyme prevents lipid oxidation by hydrolyzing lipoprotein-derived peroxides and hydrogen peroxide, which are main reactive oxygen species produced by arterial wall cells during atherogenesis.

Thyroid hormones are major factors involved in oxidative metabolism.[34] However, the knowledge about the mechanism by which hypothyroidism influence the oxidative stress is limited and conflicting. In the study performed by Resch et al.,[35] it was shown that both hyperthyroidism and hypothyroidism are associated with increased oxidative stress. In the study performed by Sarandöl et al.,[36] increased oxidative stress was observed in SH. Similarly, Erdamar et al.[37] revealed an increase in reactive oxygen species and impairment in antioxidant system in patients with hyperthyroidism and hypothyroidism. In the animal study performed by Venditti et al.,[38] the rats with hypothyroidism showed

an increased predisposition for oxidative stress. In our study, we found significantly increased oxidative stress in patients with SH, compared with the control group ($P < 0.01$).

Whereas total antioxidant capacity provides information about all antioxidants existing in the organism, malonylaldehyde is a lipid peroxidation marker used in the increased oxidative stress-related lipid peroxidation. In the present study, we found significantly increased total antioxidant capacity in our patients. Supporting our results, Torun et al.[39] denoted higher values of malonylaldehyde in patients with hypothyroidism and SH.

Subclinical hypothyroidism is characterized by inflammation and increased prevalence of cardiovascular events.[40] Subclinical hypothyroidism accelerates atherogenesis through modification of atherosclerotic risk factors and direct effects on vessels.[41] Oxidative stress markers can be used to determine the oxidative status and atherosclerotic process in patients with SH. We demonstrated in the current study that PON 1 and ARE activities were significantly decreased and that oxidative stress was significantly increased in patients with SH, indicating oxidative damage. According to current evidence, absolute beneficial effect of thyroid replacement therapy in patients with SH is still a debate. Nevertheless, prediction of atherosclerosis, based on oxidative status and inflammatory markers and prevention of cardiovascular events, is of particular importance. It can be suggested that PON 1 and ARE activities may be used for determination of the therapeutic response and during the follow-up. Large, randomized, controlled studies with greater patient samples are warranted.

We were not able to perform coronary angiography to our patients. The major limitation of this study was exclusion of coronary artery disease, based on symptoms, physical examination, and electrocardiography. The other points were small number of patients; significantly higher BMI in the patient group; and exclusion of coronary artery disease in our groups in terms of symptoms, anamnesis, and electrocardiogram.

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