Abstract

Aim: Discovery of paraoxonase natural substrate, homocysteine thiolactone, shed more light on the protective role of paraoxonase and toxicity of homocysteine. Since homocysteine thiolactone and paraoxon were hydrolyzed at different sites in the PON protein, the aim of this study was to investigate the Hcy-thiolactonase (HTase). This study was undertaken to ascertain whether low the Hcy-thiolactonase activity is associated with paraoxonase activity and to clarify its relation with ox-LDL and total plasma homocysteine levels in coronary artery disease.

Material and Methods: Forty-six subjects undergoing coronary angiography for suspected coronary artery disease were included. Depending on angiography results, 14 subjects with normal coronary arteries according to Gensini scoring were selected as a control group. Serum homocysteine thiolactonase and paraoxonase activities were measured spectrophotometrically. Homocysteine, ox-LDL levels were measured with ELISA methods.

Results: A significant decrease in HTase activity and a significant increase in ox-LDL levels were observed in patients compared with controls (p=0.040, p=0.037, respectively). Homocysteine levels and paraoxonase activity did not show any statistically significant difference between groups. Positive correlations between HTase and paraoxonase activities were observed in study groups (rs=0.742, p=0.004 for control, rs=0.494, p=0.01 for patient).

Discussion: HTase activity decreased in coronary artery disease in spite of unchanged paraoxonase activity and is associated with a higher level of ox-LDL. N-homocysteinylation of HDL changes the properties of apolipoprotein, which could affect the enzymatic activities. When considering a relationship between HTase activity and HDL levels, correlation observed in our study confirms a possible consequence of low PONs homocysteine thiolactonase activity.

Keywords
Homocysteine; Homocysteine Thiolactone; Paraoxonase; Oxide LDL; Gensini score
Introduction
Homocysteine, a sulphur-containing non-protein amino acid, is an intermediate product in the metabolism of dietary methionine (Met). S-Adenosyl methionine (SAM) participating in transmethylation, transsulfuration, and aminopropylation is both a universal methyl donor for all transmethylation reactions and precursor of decarboxylated-adenosylhomocysteine which is aminopropyl donor for polyamine synthesis. Methionyl donation yields S-adenosylhomocysteine (SAH) which is the sole source of homocysteine in mammals. S-Adenosylhomocysteine hydrolase (SAHase; EC 3.3.3.1) rapidly breaks down SAH to adenosine and homocysteine entering the remethylation and transsulfuration pathways. In all normal mammalian cells, homocysteine is remethylated to methionine by methionine synthase (EC 2.1.1.13), an enzyme that requires 5-methyltetrahydrofolate and vitamin B_{12}. Alternative remethylation of homocysteine was shown in the liver and kidney of rats. Betaine–homocysteine methyltransferase (BHMT; EC 2.1.1.5) using betaine as a methyl donor catalyzes this reaction, in which BHMT metabolizes approximately 25% of this non-protein amino acid. The transsulfuration pathway is present only in the liver, pancreas, kidney, and small intestine, metabolizes homocysteine into cysteine via two-step enzymatic reactions catalyzed by cystathionine β-synthase (CBS; EC 4.2.1.22) and cystathionine γ-lyase (CSE; EC 4.4.1.1), both requiring pyridoxal phosphate as a cofactor. Disturbances in Hcy metabolism result from genetic enzyme defects or deficiency of cofactor(s) lead to accumulation of Hcy [1]. Numerous studies have demonstrated that hyperhomocysteinemia is associated with clinical manifestations such as atherothrombotic diseases, pregnancy complications, neurodegenerative disorders and cancer [2-5]. Trials and meta-analysis assessing the clinical effectiveness of homocysteine (Hcy)-lowering interventions showed that supplementation of B vitamins effectively reduces homocysteine levels, but it is uncertain whether supplementation has beneficial effects on cardiovascular outcomes [5,6]. So, to clarify the mechanism of homocysteine toxicity it is important to predict effective treatment methods.

Homocysteine is also metabolized to homocysteine thiolactone as a result of the error-editing activity of methionyl-tRNA synthetase in protein synthesis. Hcy thiolactone synthesis is low under normal metabolic conditions, but it is enhanced when Hcy levels are increased due to a reduction in its transmethylation and/or transsulfuration reactions. In vivo both human and mice studies confirmed the hypothesis that the Hcy-thiolactone synthetase in protein synthesis. Hcy thiolactone synthesis is low as a result of the error-editing activity of methionyl-tRNA synthetase (EC 3.1.8.1). The paraoxonase (PON) family consists of three enzymes (PON1, PON2, and PON3). PON1 and PON3 are synthesized by the liver and secreted into the blood circulation where they become associated with HDL particles, while PON2 is an intracellular enzyme. While all isoenzymes of paraoxonase exhibit HTase activity, PON1 has paraoxonase, arylesterase, and HTase activities and also inhibits low-density lipoprotein (LDL) oxidation and increases macrophage-associated cholesterol efflux [9]. There are several studies in which paraoxonase activity was measured in patients with various diseases however, HTase activity may have novel clinical significance. While the paraoxonase and arylesterase assays only reflect the activity of PON1, the HTase assay, measured with its natural substrate, reflects that of PON1, PON2, and PON3 activities [10-12]. There was a limited study on HTase activity in pathologies, so this study was undertaken to ascertain whether the low the Hcy-thiolactonase activity is associated with paraoxonase activity and to clarify its relation with ox-LDL and total plasma homocysteine levels in coronary artery disease.

Material and Methods
Study population
A total of 46 subjects undergoing coronary angiography for suspected coronary artery disease (CAD) at the University Hospital, Department of Cardiology were included in this study. Referral for angiography was made by the patient’s attending physician. CAD angiographic severity was evaluated with a Gensini Scoring system. A Gensini score > 0 was considered as CAD. After coronary angiography, 14 subjects with normal coronary arteries (NAC) according to Gensini scoring were selected as a control group, 32 subjects with CAD were included in the patient group. Patients with a history of acute coronary syndrome, malignant disease, thyroid function disorders, and history of bay-pass surgery were excluded from the study. Potential confounding factors were measured and included age, smoking, alcohol consumption, total blood cholesterol levels, LDL-cholesterol, HDL-cholesterol and triglycerides, body mass index (BMI), blood pressure. The study protocol was approved by the Local Ethical Committee of the Hospital in accordance with the ethical standards of the Helsinki Declaration (Protocol no. 2009/042) and all subjects gave written informed consent.

Blood sampling
Venous blood samples were collected in a vacutainer tube containing EDTA for plasma and containing STS® gel and clot activator for serum in the overnight fasting state and centrifuged at 3000xg for 10 min. All samples were stored at -80°C until the measurement of homocysteine thiolactonase, paraoxonase, homocysteine, and ox-LDL.

**Serum homocysteine thiolactonase activity assay**
Commercially available assay kit (Alfresa Auto HTase; Alfresa Pharma Corporation, Japan) was used for serum HTase activity measurements. This colorimetric method depends on the hydrolysis of lactone ring of substrate γ-thiobutyrolactone with homocysteine thiolactonase and reaction of free thiols with Ellman’s reagent, 5,5′-dithiobis(2-nitrobenzoic acid).

**Serum paraoxonase activity assay**
Paraoxon (O,O-diethyl-O-p-nitrophenylphosphate; Sigma Chemical Co.) was used as a substrate for paraoxonase activity assay and the initial rate of substrate hydrolysis to p-nitrophenol was measured. Absorbance increase was monitored in the assay mixture containing 2 mmol/L CaCl₂ and 5.5 mmol/L
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paraoxon and 50 μL serum in the Tris–HCl buffer (100 mmol/L, pH=8.0) at 412 nm at 25 °C. Enzyme activity was calculated from the molar extinction coefficient 17.100 M⁻¹cm⁻¹. One unit of paraoxonase activity is defined as 1 nmol of 4-nitrophenol formed per minute under the above assay conditions [13].

Measurement of plasma ox-LDL levels
Plasma ox-LDL levels were measured by using sandwich ELISA method according to manufacturer instruction (ox-LDL ELISA kit, Immunodiagnostic, Bensheim, Germany).

Measurement of Plasma Homocysteine Levels
Plasma total homocysteine levels were measured using enzyme immunoassay method according to manufacturer instruction (Axis Homocysteine EIA kit, Axis- Shield Diagnostics, Dundee, United Kingdom).

Other parameters were measured in the routine biochemistry laboratory of Kırıkkale University Hospital.

Statistical analysis
The statistical analyses were performed with SPSS 10.0 (SPSS Inc., Chicago, IL, USA). Distributions of variables were tested using the Kolmogorov- Smirnov test. Data are presented as median (25th to 75th interquartile range, IQR) for variables that were not normally distributed. A comparison between the two groups was performed with the Mann-Whitney U test. The association between the variables was investigated with the Spearman's rank correlation (rs). A p-value of <0.05 was considered to be statistically significant.

Results
A total of 46 subjects participated in this study. Thirty-two of them were included in the patient group according to Gensini Scores on coronary angiography, the rest of them were grouped as control. The demographic characteristics of the subjects were given in Table 1.

The mean age of the patient (19 male, 13 female) and control (6 male, 8 female) groups were 64.09±9.52 and 52.64±10.23 years, respectively (p=0.002). There was no statistically significant difference between patient and control groups in terms of BMI, systolic, and diastolic blood pressures, fasting glucose, LDL-cholesterol, triglyceride and uric acid levels. While the mean of creatinine and Hs-CRP were significantly higher in patients (1.02±0.31 and 4.82±4.44 mg/dL, respectively) than in controls (0.84±0.22 and 1.99±2.11 mg/dL, respectively), the mean of total cholesterol and HDL-cholesterol were significantly lower (181.25±42.22, 41.29±10.55 mg/dL for patients; 205.86±32.94, 45.41±10.86 mg/dL for controls).

A significant decrease in HTase activity and a significant increase in ox-LDL levels were observed in patients compared with controls (p=0.040, p=0.037, respectively). Total homocysteine levels and paraoxonase activity did not show any statistically significant difference between groups (Table 2). There were positive correlations between HTase and paraoxonase activities in total study groups (rs=0.533, p=0.0001) (Figure 1). This relation was stronger in controls (rs=0.742, p=0.004) than that in patient group (rs=0.494, p=0.01). HTase activity also correlated with triglyceride levels in patient group (rs=0.390, p=0.049) and correlated with HDL levels in control group (rs=0.581, p=0.037).

### Table 1. Demographic and Biochemical Characteristics of Groups

|                        | Patients (n=32) | Controls (n=14) | p     |
|------------------------|----------------|----------------|-------|
| Age (years)            | 63.0 (59.0-73.0) | 50.5 (44.0-62.0) | 0.002 |
| Male/Female            | 19/13          | 6/8            |       |
| Gensini Score          | 15.75 (13.25-25) | -              | 0.551 |
| BMI (kg/m²)            | 26.75 (25.65-28.96) | 26.40 (25.21-29.40) | 0.580 |
| Systolic Blood Pressure (mmHg) | 130.0 (125.0-140.0) | 132.5 (117.5-141.5) | 0.675 |
| Diastolic Blood Pressure (mmHg) | 80.0 (75.0-85.0) | 80.0 (67.5-86.5) | 0.675 |
| Fasting Glucose (mg/dL) | 115.50 (95.75-142.25) | 100.00 (92.00-125.25) | 0.247 |
| Total cholesterol (mg/dL) | 178.50 (150.25-197.50) | 199.50 (175.00-232.00) | 0.046 |
| HDL-Cholesterol (mg/dL) | 38.10 (34.02-47.75) | 48.00 (43.92-52.25) | 0.738 |
| LDL-Cholesterol (mg/dL) | 112.20 (85.95-125.75) | 121.35 (106.55-142.62) | 0.071 |
| Triglyceride (mg/dL)   | 139.00 (101.50-205.75) | 152.50 (116.25-216.25) | 0.676 |
| Creatinine (mg/dL)     | 1.00 (0.84-1.06) | 0.81 (0.69-0.96) | 0.028 |
| Uric Acid (mg/dL)      | 5.62 (4.91-6.39) | 5.00 (4.00-6.12) | 0.197 |
| Hs-CRP (mg/dL)         | 3.20 (1.70-5.90) | 1.23 (0.70-2.32) | 0.007 |

Values are represented as median (IQR)

### Table 2. Homocysteine thiolactonase, paraoxonase activities and homocysteine, oxide-LDL levels in the patient and control groups

|                        | Patients (n=32) | Controls (n=14) | p     |
|------------------------|----------------|----------------|-------|
| HTase (U/ml)           | 131.47 (85.81-164.34) | 177.48 (124.89-216.92) | 0.040 |
| Hcy (μmol/l)           | 16.4 (14.2-19.3) | 16.5 (13.7-17.6) | 0.824 |
| PON (U/ml)             | 102.0 (54.2-149.1) | 77.9 (40.1-151.9) | 0.738 |
| Ox-LDL (mg/ml)         | 131.1 (62.7-505.2) | 75.2 (54.2-142.2) | 0.057 |
| Ox-LDL/Total Cholesterol | 0.79 (0.28-2.79) | 0.42 (0.17-0.55) | 0.011 |
| HTase/HDL              | 3.35 (1.94-4.11) | 4.02 (2.97-4.42) | 0.198 |

Values are represented as median (IQR)

![Figure 1](image-url) . The Correlation between Homocysteine Thiolactonase (HTase) and Paraoxonase (PON) Levels in the study population (rs=0.533, p=0.0001)
Discussion

Traditional risk factors cannot explain all types of the coronary artery diseases. Many studies, either observational or randomized, and evidence from meta-analysis explored that elevated homocysteine concentrations increased the risk of atherosclerotic vascular diseases. Like homocysteine, ox-LDL is an important and independent risk factor for cardiovascular diseases.

The reaction of Hcy-thiolactone, a novel Hcy metabolite, with proteins impairs their functions; these proteins acquire cytotoxic, proinflammatory, properties, which are suggested as responsible mechanisms for Hcy-associated pathologies [14]. Pro-atherogenic effect of Hcy may exert leading to homocysteinylatiion and preventing the antioxidant properties of HDL [15]. PON1 carried on HDL in the circulation having paraoxonase, arylesterase and HTase activities is an important player in HDL’s athero-protective role. There are several studies in which paraoxonase activity measured in patients with various diseases [12,16,17] however, HTase activity may have novel clinical significance. HTase activity modulates the susceptibility of LDL to homocysteinylation, glycation, and also oxidation. It has been shown that homocysteinylatiion of LDL by HTL leads to LDL aggregation and increases uptake of LDL by cells [18]. Gurda et. al. examined the effects of Hcy-thiolactone, N-Hcy-protein, and Hcy on gene expression of vascular cells and showed that Hcy-thiolactone and N-Hcy-protein lead to changes in gene expression of different metabolic pathways associated with CVD [19]. Hassan et. al demonstrated that lower activity of HTase and mild elevations (>13.5 μmol/l) in plasma Hcy levels increased the mortality and had a negative effect on long-term survival in patients undergoing percutaneous coronary intervention (PCI) [20]. Previous studies, except study performed by Domaglo et. al. [21], in a patient with diabetes [12,22], renal transplant [23] showed decreased HTase activity. In our study, plasma homocysteine levels and PON paraoxonase activity did not show any significant differences in study groups but, in line with the literature, decreased HTase activity was observed in patients with coronary artery disease compared with subjects having normal coronary artery. Because homocysteine thiolactone and paraoxon are able to be hydrolyzed at different sites in the PON1 protein, such modification may have different effects on the two activities of PON enzyme. In addition, N-homocysteinylatiion of HDL changes the properties of apolipoprotein, which could affect enzymatic activities. Our results also showed that lower HTase activity associated with a higher level of ox-LDL. The Ox-LDL is thought to be a mixture of heterogeneous modified particles having many pro-inflammatory and pro-atherogenic properties. Considerable data suggest that increased levels of Ox-LDL autoantibodies have diagnostic and prognostic value for evaluation of the presence or extent of atherosclerosis [24]. Tsimikas et. al. demonstrated that Ox-LDL significantly elevated after ACS and suggested that circulating Ox-LDL markers reflect plaque disruption and oxidative stress in ACS [25].

Conclusion

HTase activity decreased in coronary artery disease in spite of unchanged paraoxonase activity and is associated with a higher level of ox-LDL. N-homocysteinylatiion of HDL changes the properties of apolipoprotein, which could affect the enzymatic activities. When considering a relationship between HTase activity and HDL levels, correlation observed in our study confirms a possible consequence of low PONs homocysteine thiolactonase activity.

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