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Low Paraoxonase 1 Activity in Diabetes Mellitus as Predictors of Proliferative Diabetic Retinopathy and Macular Edema

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Abstract Purpose: Diabetic retinopathy (DR) is a frequent complication of DM (Diabetes Mellitus) and a leading cause of visual impairments. Serum paraoxonase 1 (PON1) is an antioxidant enzyme that has antiatherogenic properties and plays a role in inhibiting the oxidation of low-density lipoprotein (LDL). We investigate the levels of serum PON1 activity and low-grade inflammatory markers in patients with proliferative DR (PDR) or nonproliferative DR (NPDR); and with or without diabetic macular edema (DME). Methods: We evaluated 91 DM patients and 40 controls. Serum PON1 activity, HbA1c, lipid profile, hsCRP, homocysteine (Hcy), neutrophil to lymphocyte ratio (NLR) levels were compared between patients without DR, with DR and control groups. Patients with DR were evaluated according to PDR or NPDR and the presence of macular edema or not. Results: Patients with DR and without DR, the HbA1c, LDL, NLR, Hcy, hsCRP levels were found significantly higher than controls, while serum PON1 activity was not differ. Serum PON1 activity was found significantly lower in patients with PDR and DME. Conclusion: We found serum PON1 activity was significantly lower in PDR and DME patients. Low PON1 activity may be a risk factor for PDR and DME and can be useful for further evaluation.

Keywords: *Paraoxonase 1, Proliferative Retinopathy, Macular Edema, Diabetes Mellitus*

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1. Introduction

Diabetes mellitus (DM) is characterized by increased risk of microvascular disease. Diabetic retinopathy (DR) and diabetic macular edema (DME) are frequent complications of diabetes and a leading cause of visual impairments in most patients. Multiple risk factors and markers are known for the onset and progression of DR. At any time during the progression of DR, patients with diabetes can also develop DME, which involves retinal thickening in the macular area. Early detection and treatment of DR and DME is recommended to slow the progression of disease [1]. The pathophysiologic mechanism is generally attributed to the adverse effects of chronic hyperglycemia and oxidative stress [2]. The clinical hallmarks of DR include increased vascular permeability, leading to edema, and endothelial cell proliferation. Oxidized and glycated low-density lipoprotein (LDL) has been demonstrated to be cytotoxic to retinal capillary endothelial cells and pericytes. The chronic retinal capillary injury and inflammatory processes results in the development of DR in DM [3].

Paraoxonase is known as a calcium dependent high-density lipoprotein (HDL) associated antioxidant enzyme that catalyzes the hydrolysis of organophosphates and aromatic carboxylic acid esters [4]. Serum paraoxonase 1 (PON1) has been investigated with some diseases such cardiovascular disease, kidney disease, and diabetes. It is believed that PON1 has antiatherogenic properties and plays a significant role in inhibiting the oxidation of LDL both *in vivo* and *in vitro* [5]. Serum PON1 activity is significantly decreased in patients with DM while compared with healthy controls [6].

Increased homocysteine (Hcy) levels have been found in DM patients with vascular microangiopathy [7]. Involvement of Hcy in DM could be mediated through its interactions with PON1 [8]. Increased evidence has suggested that PON1 with Hcy is a risk factor for coronary artery disease [9] and it is implicated in many diseases of

the ocular system, including retinal atherosclerosis [10] and exudative age-related macular degeneration [11].

Neutrophil to lymphocyte ratio (NLR) was defined as a potential predictor of endothelial dysfunction and inflammation and atherosclerotic disorders [12,13].

High sensitivity C-reactive protein (hsCRP) is known as a predictor of atherosclerosis, coronary heart disease and type 2 DM and the high levels were associated with diabetic nephropathy [14]. Furthermore there is evidence that CRP:PON1 ratio may be an index of the risk of developing atherosclerosis [15].

In the current study we aimed to investigate the levels of serum PON1 activity, HbA1c, lipid profile, hsCRP, Hcy, NLR levels in DM patients with and without DR when compared to controls. Also these parameters were compared between patients who had proliferative DR (PDR) and nonproliferative DR (NPDR); and with or without diabetic macular edema (DME).

2. Materials and Methods

2.1. Patients and Design

The study was a prospective, randomized, double-blinded, comparative, and case-control clinical investigation. We evaluated 91 type 2 DM outpatients and 40 controls that admitted to Ankara Numune Research and Education Hospital Endocrinology and Metabolism and Ophthalmology departments between March 2015 and September 2015. Type 2 DM was diagnosed according to American Diabetes Association criteria 2015 [16]. The control group was selected randomly from 40 healthy individuals according to same age, sex and body mass index. None of the DM patients had peripheral neuropathy, nephropathy and macrovascular disease. Sixteen patients were receiving no drug therapy, 44 were receiving insulin, 88 oral hypoglycemic agents (metformin and/or sulphonylurea). None of the patients were using lipid lowering drug therapy. The exclusion criteria of the patients from the study were acute or chronic inflammation, renal and hepatic failure, coronary artery disease, smoking history, alcohol consumption, connective tissue disease and any microvascular DM complications except DR.

Diabetes Mellitus patients were divided into two groups according to whether they had retinopathy status or not. The PON1 activity, HbA1c, lipid profile, hsCRP, Hcy, NLR levels were compared between patients without DR, with DR and control groups. After the evaluation of these three groups, DR patients were divided into four groups according to PDR or NPDR and the presence of macular edema or not.

The study was approved by Ankara Numune Research and Education Hospital local ethics committee and was conducted in accordance with the ethical principles. The authors declare that there is no conflict of interest regarding the publication of this paper.

2.2. Ophthalmological Examination

The retina was assessed after full pupillary dilatation by 0.5% tropicamide and 2.5% phenylephrine hydrochloride, was given twice every 15 minutes and after waiting 45 minutes, in all DM patients. The retinopathy was diagnosed with stereoscopic fundus examination by using slit lamp biomicroscope Volk TransEquator® (Volk Optical, Inc. Australia) high resolution contact lens and color fundus photography. The DRP staging was done by an ophthalmologist without known the laboratory findings. The fundus was visualized using a pan-fundoscopic corneal contact lens. In the suspicion of staging the patients with DR and without DR fundus fluorescein angiography (FFA) was used for confirmation. The requirement for the diagnosis of nonproliferative retinopathy was defined as the presence of one or more microaneurysms or intraretinal hemorrhages or hard exudates; therewithal, proliferative retinopathy was defined as the presence of preretinal hemorrhage or vitreous hemorrhage or diabetic tractional retinal detachment or neovascularization of retina or optic disc in at least one eye.

In fundus examination if there was appearance of focal or cystoid or diffuse thickening and edema in the macula, therewith by screening optic coherence tomography (CIRRUS™ OCT, Carl Zeiss Meditec, Inc.) if the macular thickness measurement was at least 300 micron was accepted as macular edema. In the suspicion of retinopathy and maculopathy the fundus fluorescein angiography was performed to confirm the diagnosis.

2.3. Laboratory Analyses

The venous blood samples were collected between 8.00 and 11.00 a.m. after an 8 hour period of overnight fasting. Serum samples were stored at -80 °C until paraoxonase activity measured at the same day by the same person. Serum paraoxonase (PON1) activity was measured with a commercially available kit (Rel Assay Diagnostics, Turkey, REF No: RL0031, LOT No: JE14028P) by colorimetric method. Paraoxonase based activity was expressed as U/L serum. The coefficient of variation (CV) for individual samples was 5%. The sensitivity of the test was over 98%. Linearity: 0-750 U/L. For measuring fasting blood glucose (FBG), lipids, homocysteine, and high-sensitive CRP levels of fasting venous blood sample was obtained. Complete blood count (neutrophil and lymphocyte) was measured by the hematology analyzer Beckman Coulter LH 750 (Beckman Coulter, Inc., USA). Fasting blood glucose, triglyceride, total cholesterol, HDL, uric acid were measured by enzymatic methods in Beckman Coulter AU 5800 (Beckman Coulter Inc., USA) autoanalyzer. The LDL cholesterol was calculated by using the Friedewald method (LDL = TC - HDL - TG/5.0 (mg/dL). HbA1c was measured by cation exchange HPLC, Arkray ADAMS A1c HA8180 (Arkray Global Business Inc., Kyoto, Japan) with an automatic glycated hemoglobin analyzer. Homocysteine was measured by the chemiluminescent immunoassay (CLIA) method in the IMMULITE 2000 (Siemens Healthcare Diagnostics, UK), immunoassay device.

2.4. Statistical ANALYSIS

Statistical analysis was performed using SPSS for Windows, version 11.5 (SPSS Inc., Chicago, IL, United States). Whether the distributions of continuous variables were normally or not was determined by Kolmogorov-Smirnov test and the Shapiro-Wilk test. Measurements of normally distributed variables are presented as mean ±

standard deviation. Non-normal distributions are also presented as median (minimum-maximum), where applicable. While the mean differences between two groups were compared by Student's t test, One-Way Analysis of variance (ANOVA) was applied to three groups. The median values between two groups were compared by Mann-Whitney U test, while the Kruskal-Wallis test was used for differences between three groups. A p value less than 0.05 was considered statistically significant.

3. Results

The mean age, gender, body mass index in healthy control group and patients with DR and without DR were similar. Mean duration of DM was 8.35±3.8 years (Table 1). Forty-eight of them had DR and 43 did not have. Table 2 shows the PON1 activity and levels of HbA1C (%), standart lipid profile (triglycerides, total cholesterol, HDL, LDL), NLR, uric acid Hcy, and hsCRP in patients with DR and without DR and controls. In DM group patients with DR and without DR, the HbA1c, LDL, NLR, Hcy, hsCRP levels were found significantly higher than healthy control group (p<0.0001, <0.0001, <0.0001, <0.0001, 0.03; respectively). Serum PON1:hsCRP ratio and HDL levels were found significantly lower while compared to controls (p<0.001 and p=0.003; respectively). The serum PON1 activity was not significantly different between the three groups (p=0.112). The PON1:hsCRP ratio appeared to be due to high levels of hsCRP and low levels of PON1. Patients who had PDR and NPDR, also with and without DME were evaluated. The serum HbA1C (%), standart lipid profile, NLR, uric acid Hcy, and hsCRP did not differ between these four groups (p=0.550, 0.787, 0.983, 0.754, 0.879, 0.421, 0.251, 0.581, 0.606; respectively), but the serum PON1 activity was found significantly lower between NPDR and PDR groups (p<0.001). Furthermore patients who had DME the serum PON1 activity and PON1: hsCRP ratio was found significantly lower when compared to those without DME (p<0.001 p<0.001; respectively) (Table 3).

Table 1. Demographical and clinical characteristics of patients and controls

Variables	Controls	Type 2 DM without DR	Type 2 DM with DR
Subjects (n)	40	43	48
Age (years)	51.9±8.5	51.6±8.8	51.5±7.2
Gender			
Female	28 (70.0%)	29 (67.4%)	33 (68.8%)
Male	12 (30.0%)	21 (32.6%)	15 (31.2%)
Height (cm)	164±6.8	163±8.2	164±7.8
Weight (kg)	75±12	79±13	80±14
BMI (kg/m^2)	29.9±5.2	30.2±4.7	29.4±5.1
DM duration (year)	-	8.4±3.2	8.3±4.3

BMI: Body mass index **DM:** Diabetes Mellitus **DR:** Diabetic Retinopathy.

Table 2. Serum concentrations of biochemical parameters in patients with and without diabetic retinopathy and controls

Variables	Controls	Type 2 DM without DR	Type 2 DM with DR	p-value
PON1 (U/L)	152.5 (41-256)	88 (31-362)	72 (27-288)	0.112†
HbA1C (%)	5.2 (5.0-5.5)	7.6 (5.2-14.3)	8.5 (5.1-13.0)	<0.0001^{a,b†}
Triglycerides (mg/dl)	115.5 (66-272)	130 (69-458)	137 (56-322)	0.262†
Total Cholesterol (mg/dl)	201.2±34.11	208.3±43.16	206.9±45.8	0.718‡
HDL (mg/dl)	51 (30-89)	42 (29-72)	45 (26-70)	<0.0001^{a,b†}
LDL (mg/dl)	109.6 ±19.50	127.3±36.05	154.4±34.19	<0.0001^{a,b‡}
NLR	1.5 (0.83-2.99)	2.05(0.70-7.67)	2.33 (0.78-6.11)	<0.0001^{a,b†}
Uric acid (mg/dl)	5.1 (2.7-7.0)	4.75 (2.5-11.6)	5.5 (3.3-13.3)	0.067†
Hcy (umol/L)	8.1 (2.68-22.0)	13.7(6.07-47.8)	14.5 (7.35-40.2)	<0.001 ^{a,b†}
hsCRP (mg/L)	1.9 (0.46-6.14)	3.4 (0.15-16.7)	2.75 (0.25-38.6)	0.03 ^{a,b†}
PON:hsCRP ratio	52.8 (15.1-339.1)	28.(4.41-1720)	36.5 (2.3-224.0)	0.003 ^{a,b†}

† Kruskal-Wallis test ‡ One-Way ANOVA

DM: Diabetes Mellitus **DR:** Diabetic Retinopathy **PON1:** Paraoxonase 1 activity **HDL:** High-density lipoprotein **LDL:** Low-density lipoprotein **NLR:** Neutrophil-lymphocyte ratio **Hcy:** Homocysteine, **hsCRP:** High sensitivite CRP

[a] p<0.05, diabetic patients without retinopathy vs. healthy subjects.

[b] p<0.05, diabetic patients with retinopathy vs. healthy subjects.

Table 3. Serum concentrations of PON1 and biochemical parameters in patients with proliferative and nonproliferative retinopathy and with DME

	Nonproliferative DR (n=24)	Proliferative DR (n=24)	DR with DME (n=17)	DR without DME (n=31)	*p1*	*p2*
PON1 (U/L)	126 (48-288)	59.5 (27-155)	190 (61-288)	62 (27-137)	**<0.001†**	**<0.001†**
HbA1C (%)	8.5±1.80	8.8±1.68	8.05(5.1-13.0)	8.90(6.1-11.0)	0.550‡	0.588†
Triglycerides (mg/dl)	136 (70-312)	138.5 (56-322)	139±70.5	171.2±72.2	0.787†	0.166‡
Total Cholesterol (mg/dl)	197±26.8	210±47.2	192±28.9	213±45.2	0.983‡	0.983‡
HDL (mg/dl)	47±9.06	46.5±6.4	46.8±9.07	46.5±6.47	0.754‡	0.754‡
LDL (mg/dl)	145 (106-258)	155.5 (85-219)	155±42.6	150±30.8	0.879†	0.451‡
NLR	2.59±1.52	2.61 ± 0.80	2.01(1.14-4.36)	2.67(0.78-6.11)	0.421‡	0.937†
Uric acid (mg/dl)	4.8 (3.3-9.5)	5.5 (4.1-9.0)	4.9(3.3-9.5)	5.5(3.8-9.0)	0.251†	0.165†
Hcy (umol/L)	14.4 (8.6-40.2)	16.0 (7.35-26.1)	14.2±3.13	17.4±8.28	0.581†	0.492‡
hsCRP (mg/L)	2.6 (1.0-38.6)	2.7 (0.25-10.3)	2.37(0.85-5.46)	3.05(0.25-38.6)	0.606†	0.659†
PON:hsCRP ratio	47.9 (2.3-175.6)	25.8(5.49-224.0)	54 (14.7-166.6)	21.2 (2.3-224.0)	0.066†	**<0.001†**

† Mann Whitney U test, ‡ Student's t test.

PON1: Paraoxonase 1 activity **DR:** Diabetic Retinopathy **HDL:** High-density lipoprotein **LDL:** Low-density lipoprotein **NLR:** Neutrophil-lymphocyte ratio **Hcy:** Homocysteine, **hs-CRP:** High-sensitivite CRP **DME:** Diabetic macular edema **p1:** Nonproliferative DR vs. Proliferative DR **p2:** DR with DME vs. DR without DME.

4. Discussion

The evidence of animal studies and basic investigations has suggested that oxidative stress and proinflammatory cytokines had a significant role in the pathogenesis of DR [17]. The role of oxidative stress and the pathological pathways leading to DR is not yet completely understood. The role of oxidative stress in diabetes may be through glucose auto oxidation by polyol pathway can increase the production of free radicals [18].

Diabetic macular edema usually occurs because of impairment of blood-retinal barrier that is associated with increased vascular permeability that allows fluid to accumulate within the retina. Disruption of the blood-retinal barrier leads to oxidative stress and inflammatory processes [19]. Systemic inflammatory activity may contribute directly to local retinal changes and results in PDR and DME. Control of the metabolic abnormalities of DM has major effects on the development of diabetic microvascular complications [20]. In the present study we found that PON1 activity is significantly decreased in DR patients with PDR and DME. These results have been confirmed in some investigations [21,22].

Ferretti et al. reported significantly lower PON1 activity in type 1 diabetic patients compared to healthy controls. They hypothesized that the decreased ability of HDL to protect erythrocyte membranes could be related to lipid composition of HDL and low PON1 enzyme activity. It was considered that the risk of diseases related to oxidative damage and lipid peroxidation tend to be greater people with low PON1 activity [23]. The inflammatory state associated with DM could be the cause of the reduced PON1. This could be in the course of changes in lipid metabolism that occur during the acute phase response, the decrease in PON could be another factor linking the acute phase response with increased atherogenesis [24]. Treatment to reduce inflammation could have positive effect on increasing PON1 activity.

Recently it was suggested that PON1 could be used as markers of PDR. Barathi et al. reported that Hcy-thiolactone is positively correlated with Hcy-thiolactonase activity of PON1 in the eye's vitreous in patients with proliferative diabetic retinopathy and macular hole in ex vivo cultured bovine retinal capillary endothelial cells. Vitreous Hcy-thiolactone levels and Hcy-thiolactonase activity were significantly elevated in patients with proliferative diabetic retinopathy, compared with macular hole patients [25]. In accordance with these studies we found significantly higher hsCRP levels and significantly lower PON1 activity was in diabetic patients when compared with controls. Endothelial dysfunction; characterized by an impaired capacity of the arteries to dilate in response to a number of stimuli; has been suggested to be associated with the development of DR and PDR [26,27].

Mackness et al. investigated whether high levels of the inflammatory marker CRP were associated with low levels of anti-inflammatory enzyme PON1, providing a potential mechanistic link between low PON1 and the development of atherosclerosis. The PON1: CRP ratio was associated with the development of vascular complications in diabetes and thought to be a marker of the risk of developing atherosclerosis. [15]. We found PON1: hsCRP ratio was found significantly lower in our patients with DR and DME.

In DME many molecular and physiologic inflammation markers have been developed in the retina. The accumulation and activation of plasma kallikrein, thrombin, and urokinase in the vitreous due to hemorrhage and chronic retinal injury in the diabetic retina may contribute to worsening of retinal inflammation and capillary dysfunction, which lead to retinal ischemia and edema. [28] These results are considered in DME pathogenesis the importance of inflammation tends to be increased. The benefits of intravitreal steroids and antivascular endothelial growth factor agents in the treatment of DME supports that chronic low-grade inflammation may be involved in the pathogenesis [29]. Treatment with dexamethasone intravitreal implant led to significant improvements in both vision and vascular leakage from DME [30]. Among our patients PON1 activity was not differ between DR and controls, but in DR group it found significantly lower in PDR and DME patients. In the English literature limited studies are available about the relationship between PON1 activity and DME. We consider these results are consistent with occurrence of severe inflammation in PDR and DME.

In a recent study, NLR, which is an inflammatory marker, was suggested that while evaluating diabetes patients in terms of DR, higher NLR values may predict the severity of DR [31]. In contrast to this study, no relationship was found between NLR and PDR.

It was also reported that the factors influencing serum levels of PON1 are genetic or environmental, in turn, affect the capacity of HDL to protect LDL from oxidation and, consequently, may be linked to atherosclerosis [32]. PON1 gene therapy may play a role in the management of dyslipidemia in the future. Treatments with statins can modulate expression in vitro of the antioxidant enzyme paraoxonase and is associated with increased serum paraoxonase concentration and activity [33]. *In vivo*, ablation of the PON1 gene is proinflammatory and proatherogenic, while overexpression of human PON1 was found anti-inflammatory and anti-atherogenic [34]. In the future PON1 gene therapy may be useful for prevention of PDR and DME in patients with DM.

The first limitation of our research is the small count of patients included to the study. Therefore, a study with larger number of patients is required to confirm the validity of this observation. The second is that PON1 genotyping, which is more definitive, is not performed in the present study. Larger studies are therefore needed to serve this purpose.

5. Conclusion

Diabetic retinopathy severely affects quality of life for patients with DM by increasing the risk of visual loss. Serum PON1 activity levels tended to decrease in state of high oxidative stress such as DR. In our study, in DR group, the serum PON1 activity was found significantly lower in patients with PDR and DME. These results may be due to severe inflammation and consistent with disease status. We suggest that the lower PON1 activity may be a risk factor for PDR and DME and can be useful for further evaluation.

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