

# Evaluation of platelet indices and pro-inflammatory cytokines in type 2 diabetic patients with retinopathy

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## SUMMARY

**OBJECTIVE:** The aim of this study was to investigate whether platelet parameters and pro-inflammatory cytokines associated with platelet activation could be surrogate markers of the diabetic retinopathy stages in type 2 diabetic patients.

**METHODS:** This prospective case-control study included 108 type 2 diabetes mellitus patients and 48 healthy controls. After fundoscopic examination, patients were divided into three groups: no retinopathy, nonproliferative diabetic retinopathy, or proliferative retinopathy. Platelet selectin, interleukin-1alpha, and interleukin-6 values were measured by the enzyme-linked immunosorbent assay method. Homeostatic Model Assessment for Insulin Resistance formula was used to assess insulin resistance in patients.

**RESULTS:** Mean platelet volume was lower and interleukin-1alpha was higher in the patients compared to the healthy controls ( $p=0.046$  and  $p<0.001$ , respectively). In addition, a positive correlation between the platelet distribution width and HbA1C levels was observed in the patients ( $r=0.334$ ,  $p<0.001$ ).

**CONCLUSION:** In the studies evaluating the utility of platelet indices and the associated cytokines in diabetic retinopathy, there is a need for the standardization of the measurements. All medications that can affect platelet activation should be taken into consideration.

**KEYWORDS:** Diabetic retinopathy. Platelet activation. Interleukin-1alpha. Interleukin-6. p-selectin.

## INTRODUCTION

Nearly 4% of the world's population has diabetes mellitus (DM) and half of these patients have varying degrees of diabetic retinopathy (DR)<sup>1</sup>. Nonproliferative diabetic retinopathy (NPDR) and proliferative retinopathy (PDR) are the two main stages of DR. Microaneurysms, retinal hemorrhages, soft and hard exudates, and venous beading are the common features of NPDR. PDR is characterized by neovascularization, vitreous hemorrhages, fibrovascular bands, and tractional retinal detachment<sup>2</sup>. Functional changes in platelets also play roles in DR pathogenesis<sup>3</sup>.

Platelets mediate the leukocyte recruitment during inflammation<sup>4</sup>. Large platelets are more reactive in the inflammatory processes<sup>5</sup>. Platelet selectin (p-selectin) is a structural molecule that leads platelets to interact with leukocytes and the damaged vessel wall<sup>6,7</sup>.

Diagnostic strategies with the possibility of therapeutic intervention can be developed by identifying practical and objective new biochemical markers that may be associated with the development of DR. In the present study, we compared platelet indices, serum p-selectin, interleukin-1alpha (IL-1 $\alpha$ ) and IL-6 levels, and insulin resistance in type 2 diabetic patients with retinopathy and healthy controls. We also aimed to delineate whether these parameters could be surrogate markers for DR stages.

## METHODS

### Study population

The patients admitted to the internal diseases and ophthalmology outpatient clinics between February 2008 and October 2008 were

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enrolled in the study. A study group of 108 type 2 diabetic patients with retinopathy (56 females and 52 males) and 48 nondiabetic healthy controls (29 females and 19 males) were the subject (Table 1). The local ethics committee of our institute approved the study (06.07.2007/1491-454-07). Participants with severe organ failure, any malignancies, chronic inflammatory and autoimmune diseases, thrombocytopenia ( $<100,000/\text{mm}^3$ ), severe anemia and leukopenia ( $\text{Hb}<9 \text{ g/dl}$ , leukocyte count  $<3,000 \text{ mm}^3$ ), any hematological disease, and history of cerebrovascular accident (CVA) and coronary heart disease were excluded from the study. In addition, patients taking corticosteroids, nonsteroidal anti-inflammatory drugs, and anticoagulant drugs which can potentially affect mean platelet volume (MPV) were excluded from the study.

### Study design

After overnight fasting, 5 mL of venous blood samples were drawn from each participant, and samples were collected in a tube with ethylenediaminetetraacetic acid (EDTA) for complete blood count assessed with CELL-DYN Sapphire auto-analyzer (PN9231319A Abbott, USA). Plasma for p-selectin, IL-1 $\alpha$ , and IL-6 was separated after centrifugation of the blood sample with EDTA at 5,000 g for 10 min at 30°C, and the supernatant plasma was stored at -40°C.

The IL-1 $\alpha$  level was determined using ELISA kits (Bender MedSystems, catalog number: BMS243/2) by following the

instructions of the manufacturer. IL-6 level was determined using ELISA kits (BioSource Immunoassay, catalog number: KHC0061: 1 plate, KHC0062: 2 plates, KHC0061: 5 plates). Bender MedSystems ELISA kits (catalog number: BMS219/3) were used for the measurement of p-selectin.

To overcome the interobserver variability, the indirect stereoscopic retinal examinations of all the diabetic patients were carried out by the same ophthalmologist. Fundus photography and fundus fluorescein angiography procedures were also applied. The “Early Treatment Diabetic Retinopathy Study” (ETDRS) classification was used for the staging of DR in the patients<sup>8,9</sup>. At the end of fundoscopic examinations, the patients were classified into three groups: no DR, NPDR, and PDR groups.

For the assessment of insulin resistance in both groups, HOMA-IR (Homeostatic Model Assessment for Insulin Resistance) calculation was used:  $\text{HOMA-IR} = \text{Fasting plasma insulin (U/mL)} \times \text{fasting blood glucose (mg/dL)} / 405$ . HOMA-IR values of  $\geq 2.7$  were accepted as the presence of insulin resistance<sup>10</sup>.

### Statistical analysis

Data analysis was performed using MedicReS E-PICOS AI Smart Biostatistics Software® (version 21.3; New York, NY, USA). Descriptive statistics were reported as mean and standard deviation for continuous variables, whereas ratios (%) were used for categorical variables. The continuous and categorical

**Table 1.** Clinical characteristics and laboratory results of all participants.

		Type 2 diabetics		Healthy controls		p
		n (%)		n (%)		
Gender	Male	52 (48.15)		19 (39.58)		0.321
	Female	56 (51.85)		29 (60.47)		
	n	Mean $\pm$ SD	N	Mean $\pm$ SD	p	
Age (years)	108	54.18 $\pm$ 9.61	48	50.04 $\pm$ 8.93	0.012	
DM duration (years)	108	8.02 $\pm$ 8.34				
HOMA-IR	106	5.55 $\pm$ 6.37	48	2.66 $\pm$ 1.43	<0.001	
HbA1C (%)	108	8.63 $\pm$ 2.08				
PLT (bin/mm <sup>3</sup> )	108	268.30 $\pm$ 72.34	48	290.85 $\pm$ 60.32	0.061	
MPV (fL)	108	7.70 $\pm$ 1.34	48	8.06 $\pm$ 0.88	0.046	
PDW (%)	106	16.02 $\pm$ 1.32	48	16.15 $\pm$ 0.84	0.438	
PTC (%)	106	0.20 $\pm$ 0.06	48	0.23 $\pm$ 0.04	<0.001	
IL-1 $\alpha$ (pg/mL)	108	1.97 $\pm$ 1.16	48	1.23 $\pm$ 0.70	<0.001	
IL-6 (pg/mL)	100	5.86 $\pm$ 4.52	48	6.87 $\pm$ 3.66	0.178	
p-Selectin (ng/mL)	105	15.78 $\pm$ 11.05	47	13.76 $\pm$ 13.51	0.333	

SD: standard deviation; DM: diabetes mellitus; HOMA-IR: Homeostatic Model Assessment for Insulin Resistance; LDL-K: low density lipoprotein-cholesterol; PLT: platelet; MPV: mean platelet volume; PDW: platelet distribution width; PTC: plateletcrit; IL-1 $\alpha$ : interleukin-1alpha; IL-6: interleukin-6; p-selectin: platelet selectin.

variables were compared by Student's t-test and the chi-square test, respectively. Pearson's correlation test was used to reveal any relationship between continuous variables. The confidence level of 95% was determined for statistical significance ( $p < 0.05$ ).

## RESULTS

The demographic and clinical characteristics of the patients and the control groups are presented in Table 1. The average ages of the patients and the controls were different ( $54.18 \pm 9.61$  and  $50.04 \pm 8.93$  years, respectively,  $p = 0.012$ ). The patient group had a higher HOMA-IR index compared to the control group ( $5.55 \pm 6.37$  and  $2.66 \pm 1.43$ , respectively,  $p < 0.001$ ).

There was no significant difference between the patients and the controls in terms of the platelet counts and the platelet distribution width (PDW) ( $p = 0.061$  and  $p = 0.438$ , respectively). MPV was lower in the type 2 DM patients than in the controls ( $7.7 \pm 1.34$  and  $8.06 \pm 0.88$ , respectively,  $p = 0.046$ ). Platecrit (PTC) level was also lower in the patient group ( $p < 0.001$ ). The IL-1 $\alpha$  level was higher in the patients than in the controls ( $1.97 \pm 1.16$  and  $1.23 \pm 0.70$ , respectively,  $p < 0.001$ ). IL-6 and p-selectin levels were not different between the two groups (Table 1).

There were positive correlations between p-selectin and MPV ( $r = 0.246$ ,  $p = 0.011$ ) and between PDW and glycated hemoglobin (HbA1C) ( $r = 0.334$ ,  $p < 0.001$ ) in the patients (Table 2). No statistically significant difference was found between the patients with ( $n = 11$ ) and without DR ( $n = 36$ ), and statistical analysis did not reveal any difference between the patients with NPDR and PDR.

## DISCUSSION

Since platelet indices are cheap and easily interpreted laboratory tests, they have attracted clinicians so far. Although there

are controversies about the diagnostic and prognostic utilities of these tests, they can provide valuable information about several critical conditions<sup>11</sup>.

In this study, we aimed to evaluate the platelet indices, pro-inflammatory cytokines, p-selectin, and insulin resistance in type 2 DM patients and compared them with the healthy controls. In addition, correlations to DR stages were evaluated.

The differences between the patients and healthy controls were not statistically significant except for serum IL-1 $\alpha$  level, which was higher in the patients. Statistical analyses also did not reveal any correlation between the stages of DR and the other investigated parameters. According to our results, the platelet count and PTC and MPV values were lower in type 2 diabetics. Although most of the studies declared higher MPV values in type 2 diabetic patients<sup>11,12</sup>, reports revealing no difference between these patients and controls or reports with lower values in DM patients also exist<sup>13-15</sup>.

The wide range of normal values is an important limitation in clinical studies, and they cannot be standardized among the laboratories. Anticoagulants, the interval between venipuncture, and the laboratory analysis can also influence platelet size. Using EDTA instead of citrate as an anticoagulant can result in a 30% increase in MPV<sup>16</sup>. Blood/citrate solution at a concentration of 0.12 mol/L (4/1) was previously proposed as an ideal anticoagulant for MPV measurement<sup>17</sup>. All these factors limit the usefulness of MPV.

Drugs can influence platelet activation, so the value of the platelet indices may be different depending on the treatment options<sup>11-13</sup>. In terms of the medications, insulin and/or oral antidiabetic drugs that lower blood glucose and acetylsalicylic acid (ASA) and/or clopidogrel that inhibits platelet function could have altered the results<sup>14,15</sup>. ASA has direct antiplatelet activity. Statins also decrease the MPV values<sup>18</sup>.

**Table 2.** Correlations between p-selectin, HbA1C, platelet indices, and the pro-inflammatory cytokines in the patients with type 2 diabetes mellitus.

	P-selectin			HbA1C		
	N	r	p	N	r	p
p-Selectin (ng/mL)				105	-0.035	0.726
IL-1 $\alpha$ (pg/mL)	105	0.023	0.816	108	0.164	0.089
IL-6 (pg/mL)	97	0.068	0.508	100	-0.041	0.682
MPV (fL)	105	0.246	0.011	108	0.072	0.461
PDW (%)	103	-0.121	0.222	106	0.334	<0.001
PTC (%)	103	0.049	0.625	106	0.141	0.151
HOMA-IR	105	0.082	0.404	108	-0.045	0.645
HbA1C (%)	105	-0.035	0.726			

p-Selectin: platelet selectin; MPV: mean platelet volume; PDW: platelet distribution width; PTC: platecrit; IL-1 $\alpha$ : interleukin-1alpha; IL-6: interleukin-6; HOMA-IR: Homeostatic Model Assessment for Insulin Resistance.

Insulin therapy is likely to reduce insulin resistance and prevent osmotic changes due to hyperglycemia. In a previous study, diabetics were categorized according to the treatments and the MPV value was the lowest in the insulin treatment group, having the best glycemic control<sup>19</sup>.

In our study, as stated in previous reports, a positive correlation was found between PDW and HbA1C levels<sup>20,21</sup>. An increase in PDW indicates heterogeneity in platelet morphology. Osmotic changes due to hyperglycemia could lead to an increase in PDW, and hyperglycemia can also induce inflammation, which can explain the heterogeneity<sup>5</sup>.

P-selectin is located in the alpha granules of platelets and endothelial cells. The expression of p-selectin increases inflammation and helps the adhesion of platelets, endothelial cells, and neutrophils. P-selectin indicates an increased risk of vascular disease<sup>6,22</sup>. In our study, p-selectin levels were similar between the patients and controls, but there was a significant correlation between p-selectin and MPV in the patient group. This result supports the inflammation-related activation of MPV and p-selectin. However, statistical analysis did not reveal any correlation between MPV, p-selectin, and the DR stages.

Bavbek et al.<sup>13</sup> studied MPV and p-selectin levels in type 2 diabetics with and without DR and found no significant correlation between these variables in patients with and without DR. Koskela et al.<sup>22</sup> compared vitreous-plasma concentrations of adhesion molecules and cytokines in DR with nondiabetic controls. P-selectin was not different between the two groups in vitreous and plasma, but the other molecules were higher in DR<sup>22</sup>. Although these results can partly be ascribed to the small sample sizes of the study groups, serum p-selectin level may not be an indicator of the DR stage.

Koskela et al.<sup>22</sup> showed that IL-6 level was significantly higher in the vitreous fluid of the patients with PDR compared to healthy controls, but there was no difference in the serum IL-6 levels between the two groups. Previous studies also reported that IL-6 produced by the retinal pigment epithelium against the inflammatory stimuli increased the disease activity in the vitreous fluid of the patients with PDR<sup>3,23</sup>. In our results, serum IL-6 was not different in the diabetics with or without DR, and no association was found with the DR stages. Thus, IL-6 activation induced by DM was limited in the retina locally, and it was not detected in the peripheral blood. Serum IL-1 $\alpha$  level was higher in the diabetics compared to controls, and this can explain the inflammatory nature of DM. However, IL-1 $\alpha$  did not correlate with the DR stages.

The important limitations of this study are the differences in the average age of the two groups and unequal numbers of

the cases classified according to DR stages. Additionally, the drug use that could affect the platelet parameters and the cytokines was not evaluated, and it is also thought of as an important limiting factor.

## CONCLUSION

The platelet indices and related cytokines cannot be used to assess the DR stages. Further studies including more patients in DR stages might exhibit significant results. It should also be emphasized that in the studies about platelet parameters, all factors that exert an influence on platelet activation should be taken into consideration.

## ETHICS COMMITTEE APPROVAL

The study was approved by the Local Ethics Committee of the University of Health Sciences Ankara Gulhane Training Hospital (06.07.2007/1491-454-07).

## INFORMED CONSENT

Written informed consent was obtained from all participants.

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## AUTHORS' CONTRIBUTIONS

**İK:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. **ATK:** Data curation, Methodology, Project administration, Visualization. **MEO:** Formal Analysis, Validation. **ET:** Software. **EK:** Supervision, Writing – original draft. **BD:** Resources, Writing – review & editing.

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