Suez Canal University Medical Journal Vol. 27 (9), 2024
Pages 58-71

Flow Cytometric Assessment of Activated Platelets in Patients with Type 2 Diabetes Mellitus and its Relation with Platelet Indices

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Abstract

Background: **Persistent hyperglycemia and disruption in the metabolism of proteins, lipids, and carbohydrates are characteristic of type 2 diabetes mellitus (T2DM). These metabolic disruptions cause hyperactivation of platelets resulting in a prothrombotic propensity.** ***Aim:*** **This work aims to assess platelet activation in type 2 diabetic patients, controlled and uncontrolled, and its relation with platelet indices.** ***Methods:*** **The study involved 75 participants split into 3 groups: Group 1: 25 Normal controls, Group 2: 25 controlled diabetics whose HbA1c < 7%; and Group 3: 25 uncontrolled diabetics whose HbA1c ≥7%. All had been subjected to glycemic markers, lipid profile, and platelet parameters including platelet indices and the flow cytometric assessment of CD62*p* expression.** ***Results:*** **The mean percentage of activated platelets, measured by CD62*p* expression, is significantly higher among diabetic patients, controlled and uncontrolled (28.3±12.4% and 59±18.5% respectively) in comparison to healthy controls (8.8±2.3%) with a p-value of <0.05. Additionally, it exhibits a strong positive correlation with FBS and HbA1c and has a substantial moderate positive correlation with PDW, MPV, P-LCR, and DM duration. Conclusion: Type 2 diabetic patients have greater numbers of activated platelets as seen by the positive correlation of CD62p and HbA1c in addition to platelet indices including MPV and PDW. These correlations suggest that proper management of patients with T2DM should include regular monitoring of platelet parameters.**

Keywords: T2DM, platelets, CD62*p*, flowcytometry.

Introduction

Diabetes Mellitus type 2 is a disorder of metabolism marked by persistent hyperglycemia and abnormalities in lipid, protein, and carbohydrate metabolism brought on by deficits in either insulin action, release, or both [(1)].

Patients with T2DM have been classified by the American Diabetes Association into two categories: controlled diabetics whose HbA1c level is < 7% and uncontrolled diabetics whose HbA1c level is ≥ 7% [(2)].

Up to 80% of diabetes-related deaths have been attributed to thrombosis and its associated complications. Since platelets are vital in how thrombi originate, develop, and are sustained, the primary causes of prothrombotic

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conditions in diabetics are platelet hyperactivity, increased coagulation, insufficient fibrinolysis, and endothelial dysfunction. Diabetes mellitus causes several platelet signaling pathways to become active, which causes long-term mortality and morbidity [3].

CD62*p* or P-selectin is a type of glycoprotein kept in vascular endothelial cells' Weibel-Palade bodies and platelets' α-granules. It promotes platelet adhesion to vascular endothelial cells and mediates neutrophils and monocytes rolling on their surface, so it is crucial in inflammation and thrombosis [4].

P-selectin levels in the blood have been found to be elevated in a variety of acute and chronic cardiovascular diseases, including coronary artery disease, peripheral arterial disease, hypertension, and acute myocardial infarction [5-6]. P-selectin is also involved in atherosclerosis [7], as well as hypercholesterolemia [8].

Many investigations have found that patients with diabetes mellitus have a greater plasma level of circulating P-selectin, as well as enhanced P-selectin expression on platelets [9].

Increased platelet activity can be detected by indices of platelet volume, including platelet-crit (PCT), mean platelet volume (MPV), and platelet distribution width (PDW). Hematological analyzers provide a broad range of platelet parameters that facilitate the early detection of prothrombotic conditions and changes in platelet shape. They could function as alerts to recognize and monitor the development of diabetic complications [10]. Therefore, we aimed to assess platelet activation in patients with T2DM, controlled and uncontrolled, and its relation with platelet indices.

Materials and Methods

Study Design: A comparative cross-sectional study.

Study Population and Setting. This study was conducted on patients who visited the internal medicine department's diabetic clinic at Suez Canal University Hospital (Ismailia, Egypt) between February and September of 2023 in cooperation with the clinical pathology department.

Inclusion criteria. Both males and females older than 18 years old. Those who have been given a T2DM diagnosis based on American Diabetes Association (ADA) guidelines [11], and on oral hypoglycemic medications throughout the last three months.

Exclusion criteria. Patients receiving insulin therapy currently, uncontrolled hypertension, organ failure, a history of blood component transfusions within the previous 14 days, or a documented life-threatening illness (AIDS, cancer, etc.). those using corticosteroids, nonsteroidal anti-inflammatory medicines, and anticoagulant medications, all of which may have an impact on MPV, or those with concurrent chronic conditions. As well as obese persons with BMI ≥30 kg/m^2 or patients have anemia (Hb <10 g/dl) and thrombocytopenia (<100 $x10^9/L$).

Data collection tool and technique

Laboratory methods. Blood samples were taken from each patient in 2 sessions as follows: In the first session total of 7 ml of blood "after 10 hours fasting" was collected: 4 ml of blood divided into 2 EDTA (Ethylenediaminetetraacetic acid) vacutainers; one for flow cytometric assessment of platelet markers, and the other for complete blood count and

glycated hemoglobin in an attempt to minimize platelet activation. 3.0 ml of blood in sterile plain vacutainers, for measuring serum cholesterol, triglycerides, high-density lipoprotein, and fasting blood sugar. In the second session, 3 ml of blood was collected in a sterile plain tube to measure post-prandial blood sugar after 2 hours of eating an ordinary meal without taking any drug. Whole blood was allowed to stand for 30 minutes at room temperature to facilitate clot formation, and then it was centrifuged for 10 minutes at 3500 rpm to make serum for PPBG, FBG, and lipid profile analysis.

Hematological and biochemical parameter measurement. CBC including platelet indices was performed on a fully automated analyzer (Sysmex XN1000 cell counter, Germany) using the 2 ml anticoagulated blood sample obtained in an EDTA vacutainer in our institute hematology laboratory. Biochemical laboratory tests (HbA1c, FBG, PPBG, & lipid profile) were performed on Cobas 6000, a completely automated auto-analyzer (Roche Diagnostics, Mannheim, Germany).

Flow cytometric assessment of platelet activation. The study utilized fluorescent-labeled antibodies for identifying and marking platelets. Anti-CD41 antibodies, which were labeled with phycoerythrin-cyanine7 conjugate (PC7), were used for the platelet identification. Anti-CD62p antibodies, labeled with Phycoerythrin (PE), were used for marking platelet activation. All these fluorescent-labeled antibodies were made by Beckman Coulter Inc., USA.

Procedure. Flow cytometric assessment of platelet markers was performed in 2 hours of blood collection and samples were handled carefully to reduce in vitro platelet activation during the sample preparation process. Two tubes were prepared for every blood sample collected. Each tube contained 2 µl of a whole blood sample from the EDTA vacutainer, The Unstained tube was run as a control, and the Stained tube contained 3µl of anti-CD41 antibodies labeled with PC7, and 5µl of anti-CD62p antibodies labeled with PE. After incubation in the dark at room temperature for fifteen min, 500 µl of PBS (phosphate-buffered saline) buffer was added. Then The samples were acquired on the 6-color NAVIOS EX 2 laser flow cytometer from Beckman Coulter Life Science Ireland, Inc., and then the data was analyzed by the Flowjo software. At a flow rate of fewer than 250 platelets per second, a total of 15 thousand platelets were analyzed. The platelets were gated using the following gating method [(1)] as shown in figure 1.

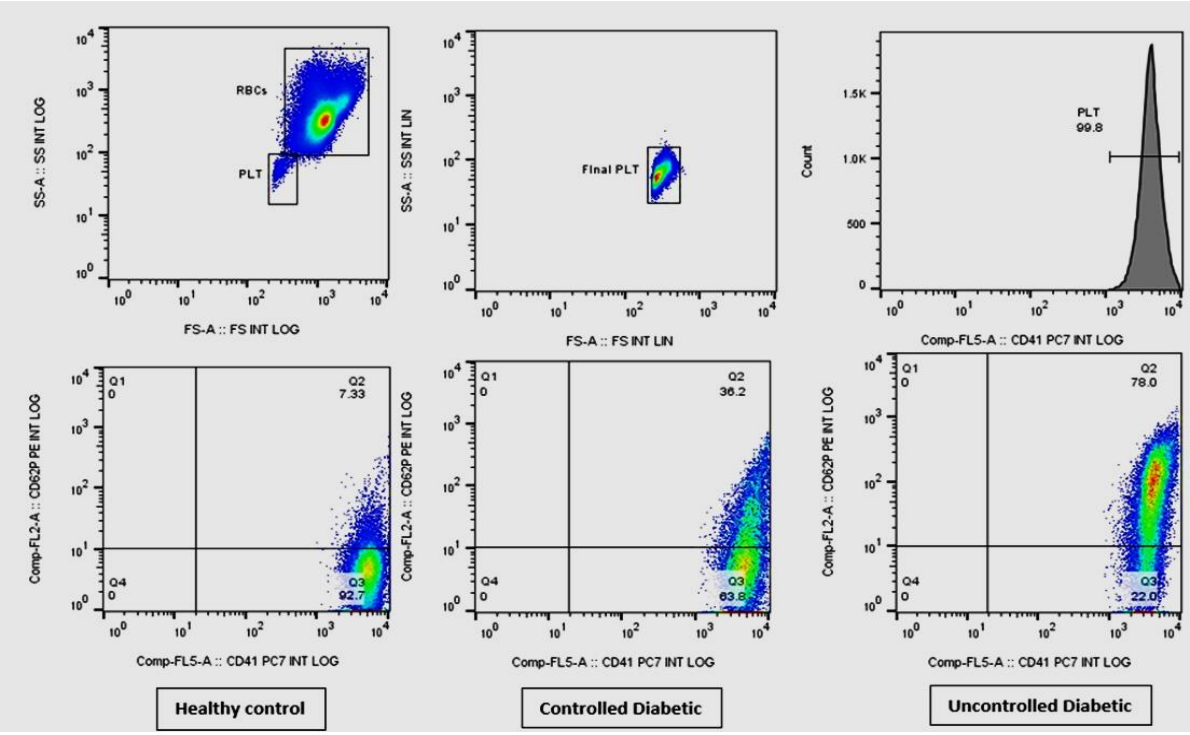

Figure 1. The expression of CD62*p* on Platelets (PLT) in the studied groups.

This figure shows an example of flow cytometric assessment of activated platelets in different study groups: Firstly, **it** shows the gating strategy of platelets, The gating on platelets was done based on forward scatter (FSC), side scatter (SSC), and after setting them on log scale. A gate was selected to exclude RBCs and locate platelets. The Flow was set to stop after acquiring 15,000 events in the selected gate. The purity of the gate was checked by assessing the expression of CD41 on the gated event. Co-expression of CD62p and CD41 was assessed to determine the percentage of the activated Platelets.

Ethical considerations.

The ethical approval was provided by the research ethical council of Faculty of Medicine Suez Canal University. Each participant provided their written consent after being fully informed throughout this research. The Suez Canal University Ethics Committee has given its stamp of approval to the study's methodology (number: 5269).

Statistical analysis.

SPSS or Statistical Package for Social Sciences was used to analyze the acquired data The data was examined for normal distribution using the Kolmogorov-Smirnov test, when dealing with normally distributed data, parametric tests were employed, while non-parametric tests were utilized for data that was abnormally distributed. Each continuous variable's mean and standard deviation were estimated. To evaluate the statistical variation among categorical variables, the Fisher exact test and the chi-square test were applied, as indicated. For comparisons of continuous variables between more than two groups, both parametric and non-parametric, One-Way ANOVA, and Kruskal-Wallis tests were utilized. Study

results were presented in tables and graphs. Spearman correlation coefficient test was used to assess the Correlation between CD62p expression and other different parameters.

Results

This study was conducted on 75 individuals divided into 3 groups as follows: 25 Healthy controls, 25 Controlled diabetics, i.e., HbA1c <7%, and 25 Uncontrolled diabetics, i.e., HbA1c > or =7%.

The controlled diabetic group mean age was (53.6±10.9) years, and the uncontrolled diabetic group was (53.2±9.3) years compared to the healthy control group which was (51.4±9.6) years. Regarding gender, the DM group either controlled or uncontrolled included 11 males and 14 females, whereas the control group included 12 males and 13 females. The three groups were matched for both age (P = 0.72) and gender (P = 0.95) (Table 1).

Table 1: Comparison between age and gender among studied groups.

Title		Healthy control n=25	Controlled diabetic n=25	Uncontrolled diabetic n=25	P value
Mean age + SD (years)		51.4 ± 9.6	53.6 ± 10.9	53.2 ± 9.3	0.72$
Gender Number (%)	Male	12 (48 %)	11 (44 %)	11 (44 %)	0.95#
	Female	13 (52 %)	14 (56 %)	14 (56 %)	

#Chi-Square Test $ Kruskal Wallis test *Statistically significant at 95% level of confidence.

The diabetes duration was significantly higher among uncontrolled diabetics (6.2±3.5) years, compared to controlled diabetics (4.3±4.2) years. But among the 3 groups under study, BMI, waist/hip ratio, or systolic and diastolic blood pressure were not statistically significantly different. (Table 2).

Table 2: Comparison between different groups regarding their clinical characteristics.

Title	Healthy control n=25		Controlled diabetic n=25		Uncontrolled diabetic n=25		P- value#
	Mean percent	SD	Mean percent	SD	Mean percent	SD	
Duration DM (years)	--------	---------	4.3	4.2	6.2	3.5	0.000*
BMI (Kg/m^2)	26.6	2.1	27.0	2.1	25.8	2.6	0.185
Waist/Hip ratio%	84.6	6.5	83.0	5.8	86.2	7.2	0.215
Systolic BP (mmHg)	119.6	4.8	118.6	6.0	119.0	6.5	0.820
Diastolic BP (mmHg)	78.4	4.3	77.8	5.0	78.0	5.0	0.900

#ANOVA test *Statistically significant at 95% level of confidence

Regarding the glycemic markers, the mean FBG level was (117.3±22.7 and 205.0±79.1 mg/dl) in diabetic groups controlled and uncontrolled respectively versus (88.9±8.2 mg/dl) in the healthy control group. The 2h P.P mean was (185.5±27.1, 279.4±91.8 mg/dl) in diabetic groups controlled and uncontrolled respectively versus (118.2±8.2 mg/dl) in the normal control group, and the HbA1c mean was (6.4±0.3% and 9.0±1.8%) in diabetic groups controlled and

uncontrolled respectively versus (5.2±0.5%) in the healthy control group. These parameters showed a statistically significant distinction, P value fewer than 0.05, among the studied groups.

Regarding lipid profile, the HDL mean level was (45.5±15.7, and 46.8±18.2 mg/dl) among diabetic groups controlled and uncontrolled respectively versus (56.8±11.7 mg/dl) in the control group. HDL had lower levels in the diabetic controlled and uncontrolled groups in comparison to healthy control group. Conversely, no statistically significant variation was observed in triglycerides (P = 0.093), cholesterol (P = 0.098), or LDL (P = 0.0122) among the 3 groups under study (Table 3).

Table 3: Comparison between different groups regarding blood sugar and lipid profile

Title	Healthy control n=25		Controlled diabetic n=25		Uncontrolled diabetic n=25		P-value#	Post- hoc test
	Mean percent	SD	Mean percent	SD	Mean percent	SD		
FBS (mg/dl)	88.9	8.2	117.3	22.7	205.0	79.1	0.000*	2,3=0.000* 1,3=0.000*
2h.P. P (mg/dl)	118.2	8.2	185.5	27.1	279.4	91.8	0.000*	1,2=0.000* 2,3=0.000* 1,3=0.000*
HbA1C (%)	5.2	0.5	6.4	0.3	9.0	1.8	0.000*	1,2=0.000* 2,3=0.000* 1,3=0.000*
Triglyceride (mg/dl)	93.7	28.1	94.7	26.6	96.5	23.7	0.93	--------
Cholesterol (mg/dl)	162.0	28.2	175.9	49.3	187.9	44.8	0.098	--------
HDL (mg/dl)	56.8	11.7	45.5	15.7	46.8	18.2	0.021*	1,2=0.000*
LDL (mg/dl)	86.7	29.8	102.1	39.6	109.9	48.5	0.122	--------

#ANOVA test *Statistically significant at 95% level of confidence

Regarding platelet parameters, the mean MPV values were 10.7±0.9 fL and 10.8±0.9 fL in diabetic cases controlled and uncontrolled respectively, while in non-diabetic cases the mean value was 9.7±0.9 fL. PDW Mean values were 12.4±1.8 and 13.0±1.9 fL in diabetic cases controlled and uncontrolled respectively compared to 10.8±1.3 fL in non-diabetics. P-LCR average values were 30.6±6.3 and 31.5±7.1% in diabetic patients controlled and uncontrolled respectively in comparison to non-diabetics where it was 23.4±5.8 %. When comparing diabetics (controlled or uncontrolled) to healthy controls, our study found that the PDW, MPV, and P-LCR p values were highly statistically significant. While PCT and platelet count revealed no significant difference between the three studied groups. The controlled and uncontrolled diabetic groups didn't show any significant discrepancy in between (Table 4, Figure 2).

Table 4: Comparison between different groups regarding platelet parameters

Title	Healthy control n=25		Controlled diabetic n=25		Uncontrolled diabetic n=25		P-value#	Post- hoc test
	Mean percent	SD	Mean percent	SD	Mean percent	SD		
Platelet count ($\times 10^3/\mu l$)	273.0	66.4	290.6	59.1	268.7	48.6	0.379	--------
PDW	10.8	1.3	12.4	1.8	13.0	1.9	0.000*	1,2=0.000* 1,3=0.000*
MPV (fL)	9.7	0.9	10.7	0.9	10.8	0.9	0.000*	1,2=0.000* 1,3=0.000*
P-LCR (%)	23.4	5.8	30.6	6.3	31.5	7.1	0.000*	1,2=0.000* 1,3=0.000*
PCT (%)	0.3	0.1	0.3	0.1	0.3	0.1	0.063	--------

#ANOVA test *Statistically significant at 95% level of confidence

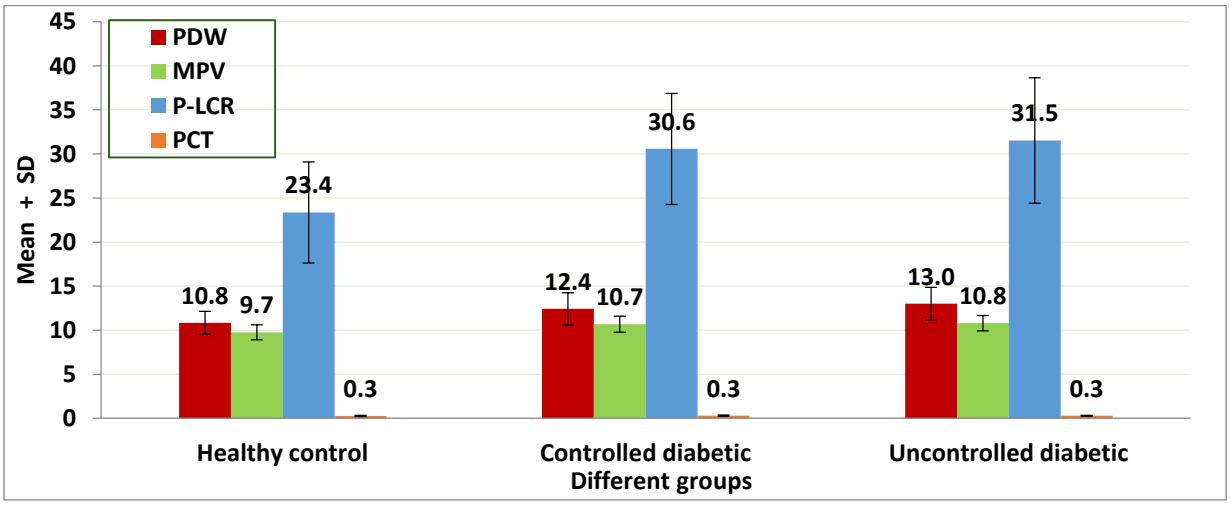

Figure 2: Comparison between different groups regarding their platelet parameters

The percentage of CD62p was significantly variable among the 3 groups. Uncontrolled diabetics showed the highest level compared to both controlled diabetics and healthy controls (59±18.5%, 28.3±12.4, and 8.8±2.3 respectively) with a p-value <0.05. Diabetics under control showed significantly increased levels of CD62p compared to controls (Table 5, Figure 3).

Table 5: Differences in CD62P percentage among the studied groups

Title	Healthy control n=25		Controlled diabetic n=25		Uncontrolled diabetic) n=25		P-value#	Post-hoc test
	Mean percent	SD	Mean percent	SD	Mean percent	SD		
CD62p (%)	8.8	2.3	28.3	12.4	59.0	18.5	0.000*	1,2=0.000* 2,3=0.000* 1,3=0.000*

#Kruskal walls test, F test between groups
*Statistically significant at 95% level of confidence

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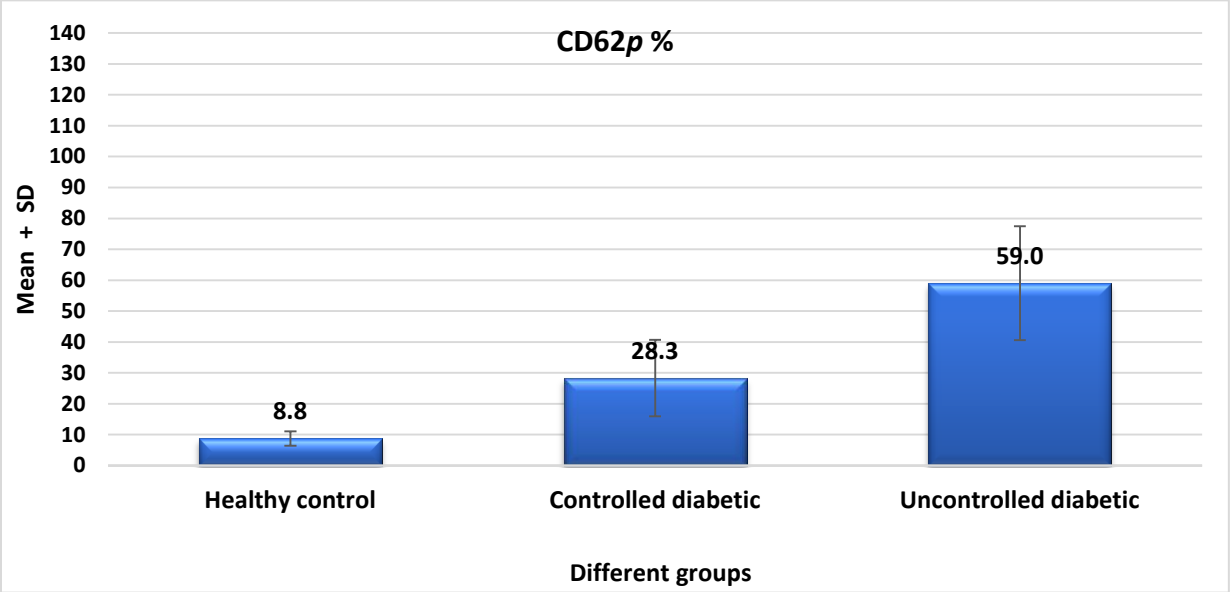

Figure 3: Differences in CD62*p* percentage among the studied groups

CD62*p* expression had a significant positive strong correlation with FBS (r=0.792), and HbA1C (r=0.856), and a positive moderate correlation with the DM duration (r=0.595), PDW (r=0.479), MPV (r=0.475), and P-LCR (r=0.469), but there was a significant negative moderate correlation between CD62p expression and HDL (r= -0.421), They all had a statistical significance with P < 0.05 (Table 6, Figure 4-9).

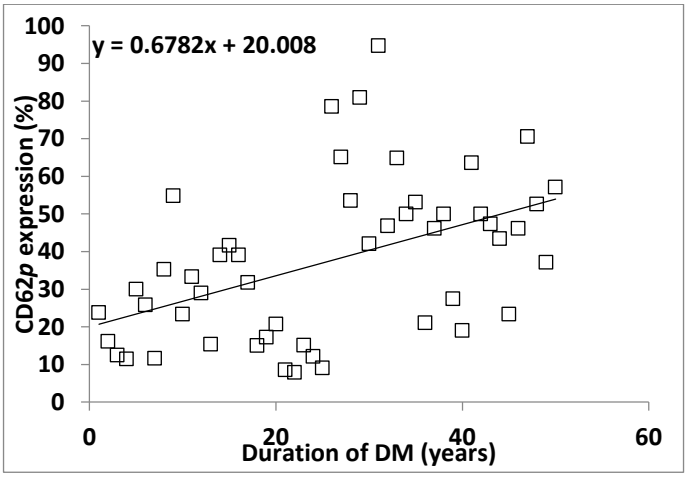

Figure 4. Correlation between duration of DM (years) and CD62*p* expression (%)

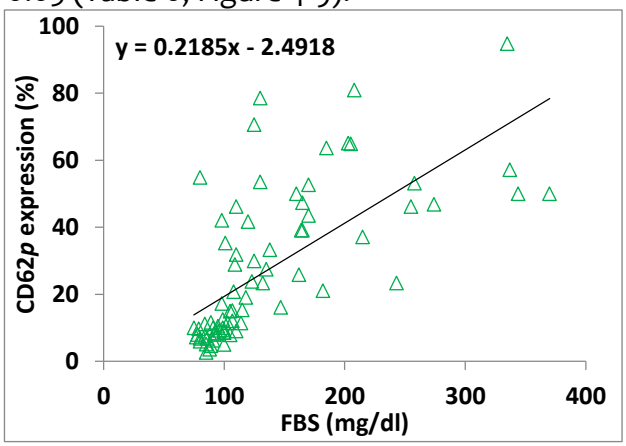

Figure 5. Correlation between FBS (mg/dl) and CD62*p* expression (%)

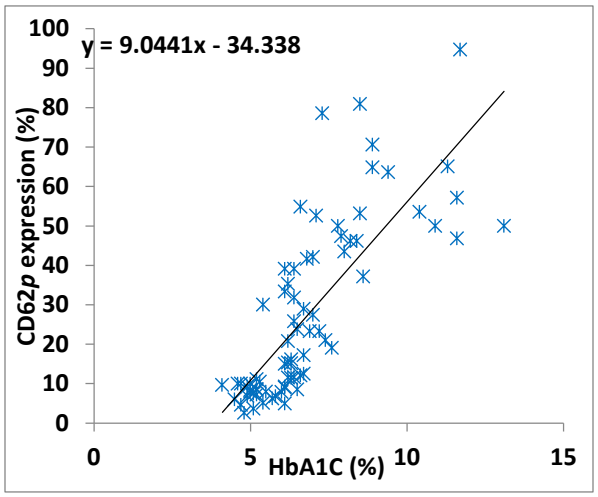

Figure 6. Correlation between HBA1c (%) and CD62*p* expression (%)

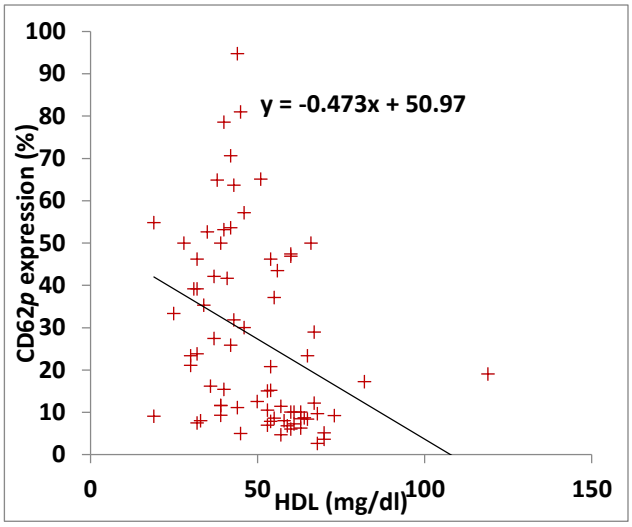

Figure 7. Correlation between HDL (mg/dl) and CD62*p* expression (%)

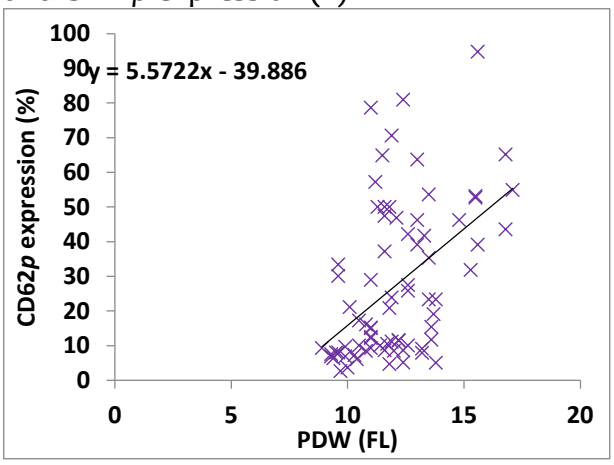

Figure 8. Correlation between PDW (fL) and CD62*p* expression (%)

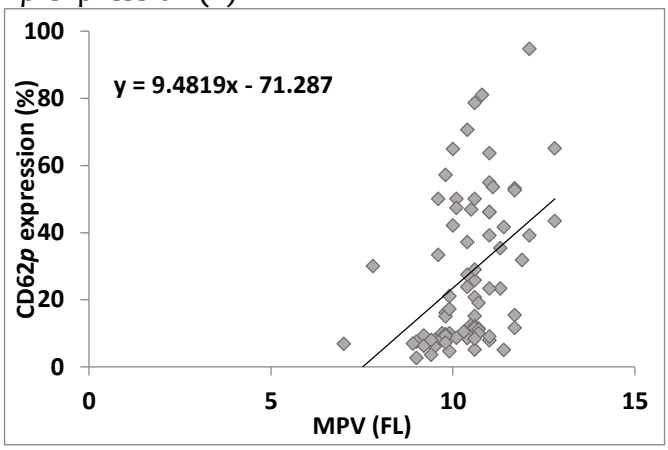

Figure 9. Correlation between MPV (fL) and CD62*p* expression (%)

Table 6: Correlation between CD62*p* expression and other different parameters		
Title	Rho	P-value
Duration DM (years)	0.595	0.000*
BMI (Kg/m^2)	-0.178	0.127
Waist/Hip ratio (%)	0.130	0.264
Systolic Blood pressure (mmHg)	0.077	0.514
Diastolic Blood pressure (mmHg)	0.057	0.630
FBS (mg/dl)	0.792	0.000*
HbA1C (%)	0.856	0.000*
Triglyceride (mg/dl)	0.034	0.77
Cholesterol (mg/dl)	0.135	0.247
HDL (mg/dl)	-0.421	0.000*
LDL (mg/dl)	0.134	0.251
Platelet count (×10^3/µl)	-0.030	0.799
PDW	0.474	0.000*

MPV (fL)	0.475	0.000*
P LCR (%)	0.469	0.000*
PCT (%)	0.171	0.142
#Spearman correlation	*Statistically significant at 95% level of confidence.	

Discussion

Diabetes Mellitus type 2 is a type of metabolic disorder that encompasses dyslipidemia, hypertension, and obesity. These metabolic disorders can cause platelet hyperactivation and lead to a prothrombotic tendency [12].

The current study shows that HDL levels are significantly lower among diabetics than controls. This is consistent with Abalı et al., 2014 [13] and Alhadas et al., 2016 [14], who agreed that diabetic patients had lower levels of HDL.

In this study, total cholesterol, triglyceride, and LDL are not significantly different between controls and diabetics. Conversely, other studies found that diabetics had considerably higher levels of triglycerides and cholesterol than controls[15,16].

Regarding platelet parameters, Diabetes, and control subjects' platelet counts did not differ significantly in the current study. However, Hekimsoy et al., 2004 [17], and Buch et al., 2017 [18] noted that diabetic patients' mean platelet counts were considerably lower than those of the control group.

Conversely, other studies revealed that diabetics had higher platelet counts than controls[19-21]. This rise in platelet count could be related to hyperglycemia, which acts on the Advanced Glycation End (RAGE) product receptors in the liver resulting in thrombopoietin synthesis. The effects of thrombopoietin are enhanced by increased levels of pro-inflammatory markers such as IL-6, that are involved in thrombopoiesis [22]. Other factors, like mean platelet survival and the platelet generation and turnover rate in type 2 diabetes, may account for these changes in platelet count [14].

Microvascular problems and preclinical atherosclerosis in type 2 diabetes may be related to platelet volume indices (PVI) that involve MPV, PDW, and P-LCR, which are markers of platelet activity [23]. Our study revealed that the levels of these indices in the controlled and uncontrolled diabetic groups show a statistically significant increase in comparison to healthy control group.

Platelet parameters were studied by Bhattacharjee et al., 2016 [24] and Jindal et al., 2011 [25], who also found that PVI were significantly higher among controlled and uncontrolled diabetic participants than in normal controls

The mean volume of platelets is a platelet index that signifies the platelets' average size which increases with the number of large platelets in circulation [26]. In comparison to smaller and less active platelets, larger platelets are more responsive, contain more dense granules, and have a higher thrombotic potential. [17-21].

Our results revealed a statistically significant elevation in MPV among diabetic patients controlled and uncontrolled compared to the healthy control group (10.7±0.9 and 10.8±0.9 vs. 9.7±0.9) respectively.

Ateş et al., 2009 [27] and Wahab et al., 2021 [28] found that group diabetics with complications had higher MPV than both the control group and group diabetics without complications. An increased MPV was detected in the diabetes group compared to the control group,

although, the difference wasn't statistically significant, according to a study by Alhadas et al., 2016 [14].

Conversely, Akinsegun et al., 2014 [29] found that while the mean platelet volume in non-diabetics (8.91±0.80 fL) was slightly higher than in diabetics (8.69±0.67 fL), there was no statistical significance, as evidenced by the p-value of 0.593.

PDW reflects platelet anisocytosis, and it is also influenced by active thrombosis and inflammatory reactions [26]. The current study declared that PDW shows a statistically significant higher level among the uncontrolled diabetics compared to controlled diabetics and healthy controls (13.0±1.9 vs. 12.4±1.8 and 10.8±1.3) respectively.

In agreement with our results, it was suggested that PDW was increased in diabetics experiencing complications than in the control group and those without complications [30,31].

Moreover, a statistically significant variation in P-LCR was seen among diabetic cases and healthy controls in our research. This is consistent with studies conducted by Jindal et al., 2011[25], Taderegew et al., 2021[32], Iqbal et al., 2022[33], and Mujahid et al., 2023[34], who found significantly elevated levels of P-LCR in diabetics compared to non-diabetics. Inversely, Buch et al., 2017[18] observed that PLCR levels weren't statistically significantly different between diabetic cases, with and without complications, and the normal control group.

Regarding PCT, we didn't discover any statistically significant distinction among the groups under study. The results of Taderegew et al., 2021[32], Iqbal et al., 2022[33], and Mujahid et al., 2023[34] coincide with this. However, Pujani et al.,2018[35] found that PCT levels were higher in diabetic cases controlled and uncontrolled.

CD62p glycoprotein, being expressed on circulating platelets' surface, signifies the activation of platelets in vivo [36]. It has a role in atherogenesis, inflammation, and thrombogenesis [37]. The primary finding of this study is a significant rise in the activated platelets percentage (indicated by CD62p expression) in diabetic patients (controlled and uncontrolled) when compared to the normal control group.

In this regard, our results align with the research conducted by Wahab et al., 2021[28] on markers of platelet activation in diabetics who have peripheral vascular disease. CD62p% showed a statistically significant rise in cases with diabetes more in uncontrolled than controlled when compared to the healthy control group. Kaur et al.,2018[38], Israels et al.,2014[37], and Eibl et al.,2004[39] found that the markers of platelet activation as CD63 and CD62*p* are more expressed in diabetics than healthy controls.

This study's findings are consistent with those of Véricel et al., 2004[40], who also found hyperactive platelets in diabetics under metabolic control who did not have cardiovascular problems.

The results of the present research reveal a strong positive correlation between HbA1C levels and CD62p expression. This is consistent with Wahab et al. in 2021[28] who also found a strong correlation between CD62p% and HbA1C levels in diabetic cases (P value = 0.015). This suggests that CD62p% can be used as a platelet marker for uncontrolled diabetes mellitus.

Our results confirmed the study of, Tayade et al., 2017[41] who reported a relationship between higher HbA1c levels and higher platelet activation, aggregation, turnover, and count.

In this study, CD62 expression has a significant negative correlation with HDL. In agreement with our results, Tan et al., 2005[42] an inverse relationship between CD62 and HDL levels, and having low HDL is associated with higher risk of having unfavorable cardiovascular events.

We also observed a statistically significant positive correlation between CD62*p* expression and platelet indices as MPV, PDW, and P-LCR among the groups under study.

Conclusion

The levels of activated platelets demonstrated by increased CD62p expression are higher in patients with T2DM than the control group with a positive correlation of CD62p and HbA1c as well as the indices of platelets including MPV and PDW. These correlations suggest that regular platelet parameter screening should be considered for type 2 diabetics' proper management. Glycemic control, food habits, and diabetes duration should all be closely monitored.

Data availability

The study's supporting data are not generally accessible, but they are available from the study's corresponding author upon justifiable request.

Conflicts of Interest

The authors have no competing interests associated with this study.

Authors' Contributions

There was equal participation from all authors in this research.

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