

Using flow-cytometry in measuring platelet activity in type 2 diabetes and predicting macrovascular complications

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Abstract

Platelets are hyperactive in patients with type2 diabetes (T2DM), they adhere to vascular endothelium and play a key role in macrovascular complications. Platelets activity can be measured by flow-cytometry (cluster of differentiation (CD) 41, CD 42, CD 62, CD 63), which allows detection of surface antigens in a sensitive and specific manner. This study aimed to describe platelets activity in T2DM in association with cardiovascular and cerebrovascular complications in relation to duration of diabetes (DM). This was a case-control study with 130 participants (65 diabetic cases and 65 normal controls). All cases were subjected to history and clinical examination, base-line laboratory investigations and surface expression of platelets receptors e.g. CD 41% and mean fluorescent intensity (MFI), CD 42% and MFI, CD 62% and MFI, CD 63% and MFI were determined by flow-cytometry. There was a statistically significant higher expression of CD 62%, CD 62 MFI, CD 63% and CD 63 MFI ($p<0.001$ for all) in diabetic cases compared to controls. There were significantly higher CD 62 %, CD 62 MFI, CD 63% and CD 63 MFI in cases with cardiovascular complications ($p=0.001$, $p<0.001$, $p=0.05$ and $p=0.007$, respectively) and in cases with cerebrovascular complications compared to cases without complications ($p=0.05$, $p=0.008$, $p=0.035$, $p=0.017$, respectively). A significant positive correlation was found between glycated hemoglobin, body mass index and CD 62 %, CD 62 MFI and CD 63%. Using the receiver operating characteristic curve showed that CD 62 %, CD 62 MFI, CD 63 % and CD 63 MFI have a diagnostic ability to early predict DM (area under the curve (AUC)=0.998) as well as cardiovascular (AUC=0.855) and cerebrovascular (AUC=0.765) complications. In conclusion CD 62%, CD 62 MFI, CD 63% and CD 63 MFI markers have a diagnostic ability for early prediction of cardiovascular and cerebrovascular complications among diabetic patients.

Keywords: Platelet activity, Cardiovascular complication, Cerebrovascular complication.

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Introduction

Type 2 Diabetes Mellitus (T2DM) is a multisystem disease associated with both micro-vascular and macro-vascular complications.¹ The macro-vascular complications are manifested as accelerated atherosclerosis that results in premature coronary artery disease and increased risk of cerebrovascular diseases.² Patients with T2DM have a two- to four- fold increased risk of coronary artery disease and a three-fold increased risk of stroke compared with non-diabetic subjects.³ Chronic hyperglycemia is not the only cause of these complications but also associated with platelets hyper-activation, abnormal activation of coagulation proteins, abnormal endothelial function and hypo-fibrinolysis.⁴ Platelets are active participants in initiating and sustaining vascular inflammation as well as in pro-thrombotic complications.⁵

Platelets are hyperactive in diabetic patients as platelets in T2DM adhere to vascular endothelium and aggregate more readily than those in healthy people.⁶ Platelets are activated when they are in contact with damaged vascular endothelium. Once activated they secrete a wide spectrum of inflammatory mediators, as they initiate reactions by changes in the level of expression of surface glycoproteins results, which act as receptors for platelet agonist and for adhesive proteins involved in platelets aggregation.⁷

Platelets activity can be measured by flow-cytometry, which allows simultaneous detection of surface antigens in a sensitive and specific manner. It is therefore possible to examine aspects of the platelet membrane activity.

The available platelet markers are cluster of differentiation (CD) 41 and CD 42 both are used for platelet identification,⁸ while CD 62 and CD 63 are markers of platelet activation.⁹ The aim of the current study was to describe platelets activity in T2DM as a participant in vascular inflammation and describe the platelets activity in patients with T2DM in association with cardiovascular and cerebrovascular disease in relation to duration of diabetes.

Subjects and Methods

This was a case control study, which included 65 adult patients with T2DM, with age of 30-60 years old. Diabetic cases were recruited from the Diabetes Outpatient Clinic at Assiut University Hospital and patients admitted to the Department of Internal Medicine, Assiut University. The study also included 65 controls; they were apparently healthy workers in Assiut University Hospital.

Exclusion criteria: Patients that have type1 diabetes mellitus (T1DM), hypertension, renal impairment, hepatic impairment, thrombocytopenia, life threatening disease (e.g. malignancy) were excluded from this study.

Patients were subjected to clinical examination and complete medical history. Body mass index (BMI) was calculated as kg/m². Basic laboratory investigations included: complete blood count (CBC), was assessed on a hematology analyzer (ADVIA 2021i, Siemens, Germany), according to the manufacturer's instructions. Prothrombin time (PT) and prothrombin concentration (PC) and activated partial thromboplastin time (aPTT) were estimated on a coagulation analyzer (Sysmex CA-1500, Siemens, Germany), according to the manufacturer's instructions. Renal function, liver function, lipid profile, and glycated hemoglobin (HbA1C) were evaluated on a clinical chemistry analyzer (ADVIA 1800, Siemens, Germany), according to the manufacturer's instructions. Electrocardiogram (ECG) and echocardiography were processed by one of the authors. For controls, random blood sugar was assessed using a clinical chemistry machine (Advia 1800, Siemens, Germany), according to the manufacturer's instructions.

Surface expression of platelet markers

A venous blood sample (2 ml) was collected from each patient into a citrated tube for platelets determination. The Flow Cytometer (CytoFlex, Beckman Coulter, USA) was used to identify platelets by expression of CD 41 and CD 42 using commercial kits (Lot 200078 and 200069, respectively, Bechman coulter

company, Marseille, France,) and to evaluate their activation status by measuring the expression of CD 62P and CD 63 using commercial kits (Lot 200052 and 200050, respectively, Bechman coulter company, Marseille, France,) according to the manufacturer's instructions.

Assay Procedure

Citrated blood samples were centrifuged at 95 xg for 10 min at 25°C. Platelets rich plasma (PRP) was removed into separate tubes. Platelets were diluted by phosphate buffered saline to 50,000/ μ l. Four tubes were prepared for each sample. The first tube was the negative control, the second tube contained 50 μ l of sample incubated with 5 μ l of fluorescein isothiocyanate (FITC)-conjugated anti CD 41 and 5 μ l phycoerythrin (PE)-conjugated anti CD 42, the third tube contained 50 μ l of sample incubated with 5 μ l of FITC-conjugated anti CD 41 and 5 μ l PE-conjugated anti CD 62p and the fourth tube contained 50 μ l of sample incubated with 5 μ l of FITC-conjugated anti CD 41 and 5 μ l PE-conjugated anti CD 63. The tubes were incubated for 20 min at room temperature in the dark. Tubes were resuspended in 400 μ l phosphate buffer saline and then flow cytometry readings were done using a Flow Cytometer (CytoFlex, Beckman Coulter, USA), according to the manufacturer's instructions.

Analysis

For flow cytometry analysis, at first, platelets were identified according to their forward and side scatters characteristics (Figure 1A). Then the expression of CD 41 and CD 42 were determined to confirm the platelet gate (Figure 1B). Then the expressions of CD 62P and CD 63 in each tube were detected as co-expression with CD 41. The levels of CD 62P and CD 63 were expressed as percentages of total platelets. The mean fluorescent intensity (MFI) for each marker was determined (Figures 1C and 1D, respectively).

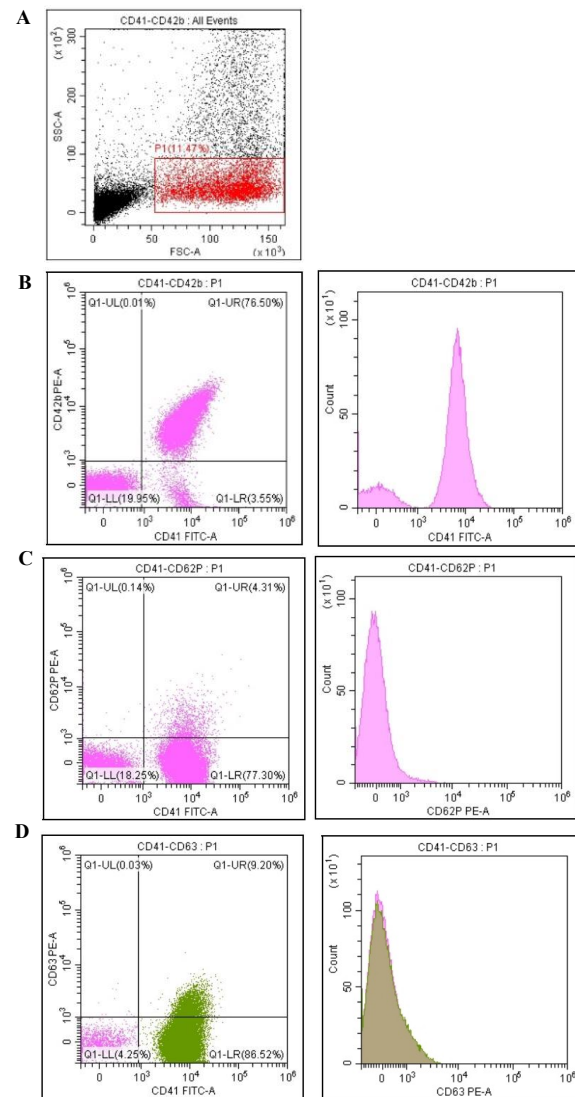


Figure 1. A: Platelets gate (P1) according to forward and side scatters. B: Dot plot for expression of CD 41 and CD 42 % (left), histogram showing CD 41 MFI (right). C: Dot plot for expression of CD 41 and CD 62 % (left), histogram showing CD 62 MFI (right). D: Dot plot for expression of CD 41 and CD 63 % (left), histogram showing CD 63 MFI (right).

Statistical Analysis

Data were analyzed using the Statistical Package for Social Science (SPSS), version 26.0 for Windows. Quantitative data were tested for normality by Shapiro-Wilk test, data are expressed as mean $\pm$ SD or median and range according to normality of data. Qualitative data are expressed as frequencies and percentages. Independent Sample T-test/ Mann Whitney U test was used to compare mean/median difference between two independent groups. The One Way ANOVA/ Kruskal Wallis test was used to compare mean/median differences between more than 2 groups. The pairwise comparison with Bonferroni correction was done to compare each two groups. The Chi square test was used to compare proportions between groups. The Spearman correlation was used to explore the correlation between CD markers and other variables. The receiver operating characteristic (ROC) curve analysis was done to identify the diagnostic ability of CD markers (CD 62 %, CD 62 MFI, CD 63 % and CD 63 MFI) in prediction of diabetes, and in prediction of cardiovascular and cerebrovascular complications in patients with diabetes. The area under curve (AUC), sensitivity, specificity, positive predictive value, and negative predictive value were calculated. Significant variables in bivariate analysis were entered in univariate linear regression, significant variables in univariate linear regression were entered in multivariate linear regression analysis adjusted with age to identify factors associated with increasing CD markers among type 2 diabetic patients. The level of significance was considered at p value of < 0.05 .

Results

Characteristics of the studied population

The studied cohort included 130 participants, 65 diabetic cases and 65 normal controls. The mean age among the cases was 53.02 ± 5.64 years and among controls 51.31 ± 5.44 years ($p=0.08$). Regarding the gender, 67.7% of the cases were males and 52.3% of the control were males ($p=0.073$).

The median duration of the diabetic disease was 10 years and ranged from 1 month to 25 years. Patients with duration of diabetes <5 years were 10.8%, 63.0% patients with duration of diabetes 5-10 years, and 26.2% of patients with duration of diabetes >10 years. The majority of cases had complications ($n=54$, 83.1%), cardiovascular complications were observed only in 44 (67.7%) cases and 10 (15.3%) cases had both cardiovascular and cerebrovascular complications.

Regarding laboratory investigations, the mean platelets count was 295.75 ± 109.6 ($10^3/\mu\text{l}$), the mean platelet volume (MPV) 9.88 ± 1.12 (femtolitre, fl), the mean blood urea 6.06 ± 1.55 (mmol/L), the mean serum creatinine 85.28 ± 21.27 ($\mu\text{mol/l}$), the mean HbA1c % 9.57 ± 2.18 mmol/mol, the mean PT 12.17 ± 1.23 sec, the mean PC 93.13 ± 12.58 %, the mean aPPT 30.74 ± 2.28 sec, the mean cholesterol level 162.86 ± 46.28 mg/dl, the mean triglyceride level was 190.84 ± 94.41 mg/dl, the mean high density lipoprotein (HDL) level 34.87 ± 9.27 mg/dl, and the mean low density lipoprotein (LDL) level 102.84 ± 36.83 mg/dl. The mean ejection fraction (EF) of diabetic patients was $50.89\% \pm 11.46\%$ which ranged from 16.0% to 73.0%. Of the studies diabetic patients, 40 % patients were with EF of $< 50.0\%$ and 60 % with EF $\geq 50.0\%$, Table (1).

As for relation between age, gender and disease complications; complicated cases had statistically significant higher mean age compared to non-complicated (53.74 ± 5.15 vs 49.45 ± 6.80 , respectively; $p=0.02$). Moreover, the majority of complicated cases were males (74.1%) vs. non-complicated cases (36.4%; $p=0.015$).

Among the studied diabetic cohort there was statistically significant increase in the mean HbA1c as the duration of diabetes increased (9.01 ± 1.76 in <5 years, 10.48 ± 2.42 in 5-10 years and 10.58 ± 2.95 in >10 years duration), $p=0.026$.

Moreover, there was statistically significant increase in the mean BMI as the duration of diabetes increased (27.75 ± 3.67 in <5 years, 28.52 ± 4.76 in 5-10 years and 31.66 ± 3.64 in >10 years duration), $p=0.003$. Also, BMI of complicated cases vs. non-complicated was (30.84 ± 3.62 vs 27.62 ± 2.82), $p=0.002$.

Table 1. Clinical and laboratory data of the 65 studied patients with T2DM.

Variables	Parameter
Clinical data	
<u>Duration of disease (years)</u>	
Median (range)	10.0 years (1 month-25 years)
<5 years	7 (10.8%)
5-10 years	41 (63.0%)
>10 years	17 (26.2%)
BMI: Mean $\pm$ SD (range)	28.17 $\pm$ 3.18 (20.40-36.10)
<u>Complications</u>	
Complicated	54 (83.1%)
Not complicated	11 (16.9%)
Laboratory data	
	Mean $\pm$ SD (range)
Platelets ($10^3/\mu\text{l}$)	295.75 $\pm$ 109.6 (140-603)
MPV (fl)	9.88 $\pm$ 1.12 (7.1-12.6)
Urea (mmol/L)	6.06 $\pm$ 1.55 (2.3-10.0)
Creatinine ($\mu\text{mol/l}$)	85.28 $\pm$ 21.27 (35.00-126.00)
HbA1c %	9.57 $\pm$ 2.18 (6.30-17.00)
PT (sec)	12.17 $\pm$ 1.23 (10.0-17.2)
PC (%)	93.13 $\pm$ 12.58 (50.5-121.0)
aPPT (sec)	30.74 $\pm$ 2.28 (24.7-36.0)
Cholesterol (mg/dl)	162.86 $\pm$ 46.28 (98.3-320.0)
Triglyceride (mg/dl)	190.84 $\pm$ 94.41 (44.0-471.0)
HDL (mg/dl)	34.87 $\pm$ 9.27 (20.5-69.0)
LDL (mg/dl)	102.84 $\pm$ 36.83 ((37.40-261.00)
Ejection fraction (EF)	50.89% $\pm$ 11.46% (16.0-73.0)
<50%	26 (40.0%)
$\geq 50\%$	39 (60.0)

aPTT: activated partial thromboplastin time, HbA1c: glycated hemoglobin, HDL: high density lipoprotein, LDL: low density lipoprotein, MPV: mean platelet volume, PT: prothrombin Time, PC: Prothrombin concentration. Data were expressed as mean $\pm$ SD/ median (range) or frequency and percentage (%).

Measurement of platelet markers expression

There was no difference between diabetic cases and controls regarding CD 41 %, CD 41 MFI, CD 42 % and CD 42 MFI expression (Figure 2, A, B). However, there was a statistically significantly higher median of CD 62% in the diabetic cases compared to controls (16.00 vs. 5.57, respectively) (Figure 2, C), CD 62 MFI in the

diabetic cases compared to controls (550 vs. 223, respectively) (Figure 2, C), CD 63% in the diabetic cases compared to controls (1.20 vs. 0.57, respectively) (Figure 2, D) and CD 63 MFI in the diabetic cases compared to controls (112.0 vs. 97.0, respectively) (Figure 2, D), ($p < 0.001$ for all), as shown in Table 2.

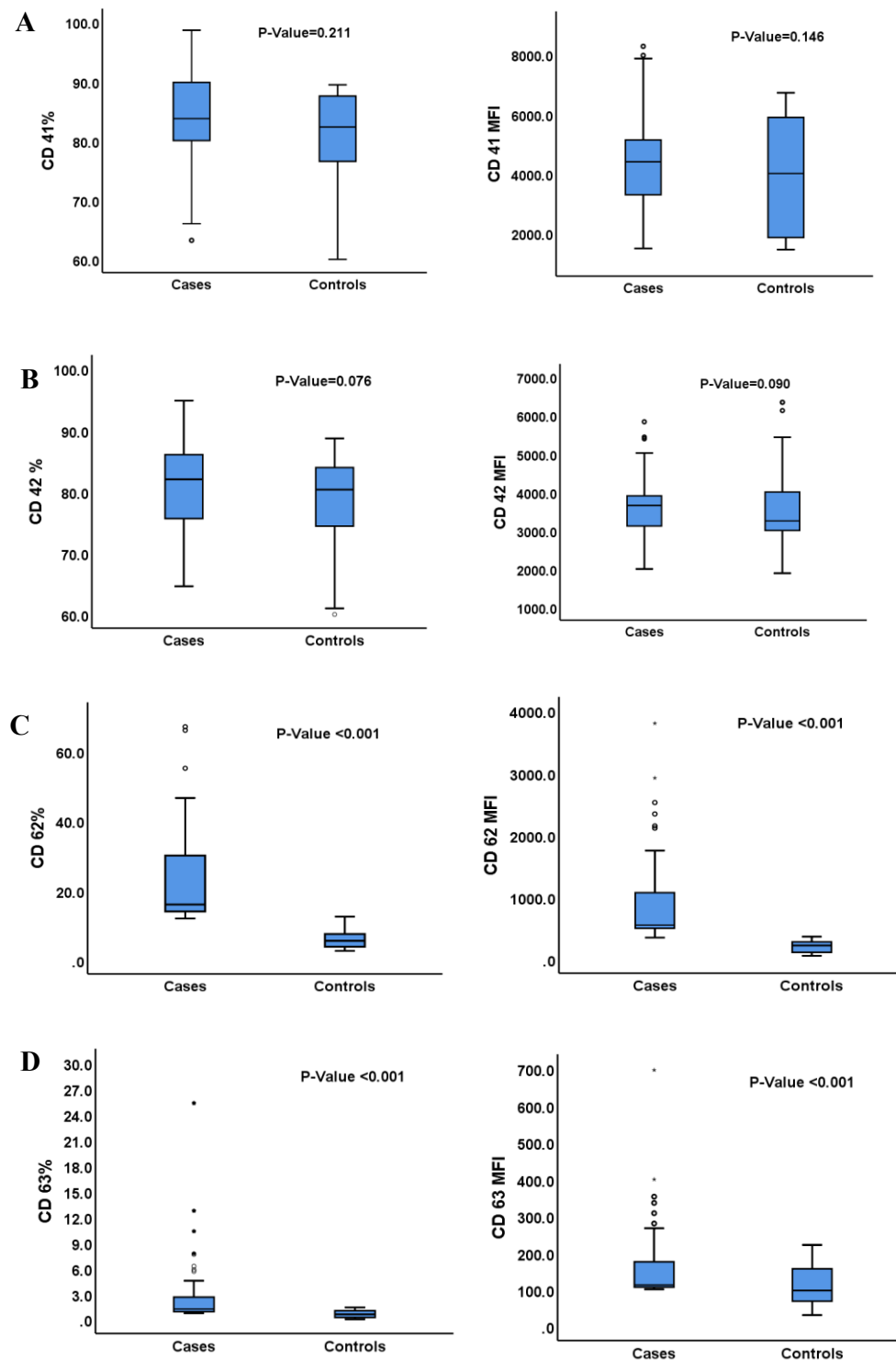


Figure 2. A: Boxplot for expression of CD 41 % among cases and controls (left), Boxplot for expression of CD 41 MFI among cases and controls (right). B: Boxplot for expression of CD 42 % among cases and controls (left), Boxplot for expression of CD 42 MFI among cases and controls (right). C: Boxplot for expression of CD 62 % among cases and controls (left), Boxplot for expression of CD 62 MFI among cases and controls (right). D: Boxplot for expression of CD 63 % among cases and controls (left), Boxplot for expression of CD 63 MFI among cases and controls (right).

Table 2. Surface expression of platelets by using different CD markers between cases and controls.

Variables [Median (range)]	Cases (n= 65)	Controls (n =65)	p-value*
CD 41%	83.70 (63.20-98.60)	82.30 (60.00-89.40)	NS
CD 41 MFI	4393.00 (1482.00-8272.00)	4000.00 (1450.0-6714.00)	NS
CD 42 %	82.00 (64.60-94.80)	80.30 (60.0-88.65)	NS
CD 42MFI	3650.00 (2000.0 -5828.00)	3250.0 (1886.0-6334.0)	NS
CD 62%	16.00 (12.00-66.90)	5.57 (2.69-12.55)	<0.001
CD 62 MFI	550.00 (440.0-3797.0)	223.00 (58.0-365.0)	<0.001
CD 63%	1.20 (0.70-25.30)	0.57 (0.01-1.38)	<0.001
CD 63 MFI	112.00 (100.0-696.0)	97.00 (30.0-221.0)	<0.001

* Mann Whitney U test was used to compare median between groups. $p > 0.05$ is not significant (NS).

CD: cluster differentiation, MFI: Mean fluorescence intensity.

Interestingly there was no difference between non-complicated, complicated diabetic cases and controls regarding CD 41 %, CD 41 MFI, CD 42 % and CD 42 MFI.

However, there were statistically significant higher median of CD 62%, CD 62 MFI, CD 63% and CD 63 MFI in the diabetic complicated and non-complicated cases compared to controls, ($p < 0.001$ for all) as described in Figures 3 A, B and Table 3.

For studying the expression of platelets markers in relation to the duration of diabetes, there was a statistically significantly increase in the median of CD 62 MFI with prolongation of duration of diabetes (509.0 in <5 years, 661.0 in 5-10 years and 980.0 in > 10 years), $p=0.005$. And on pairwise comparison with Bonferroni correction, the significance was between duration < 5ys and > 10 years, ($p=0.001$), (Table 3).

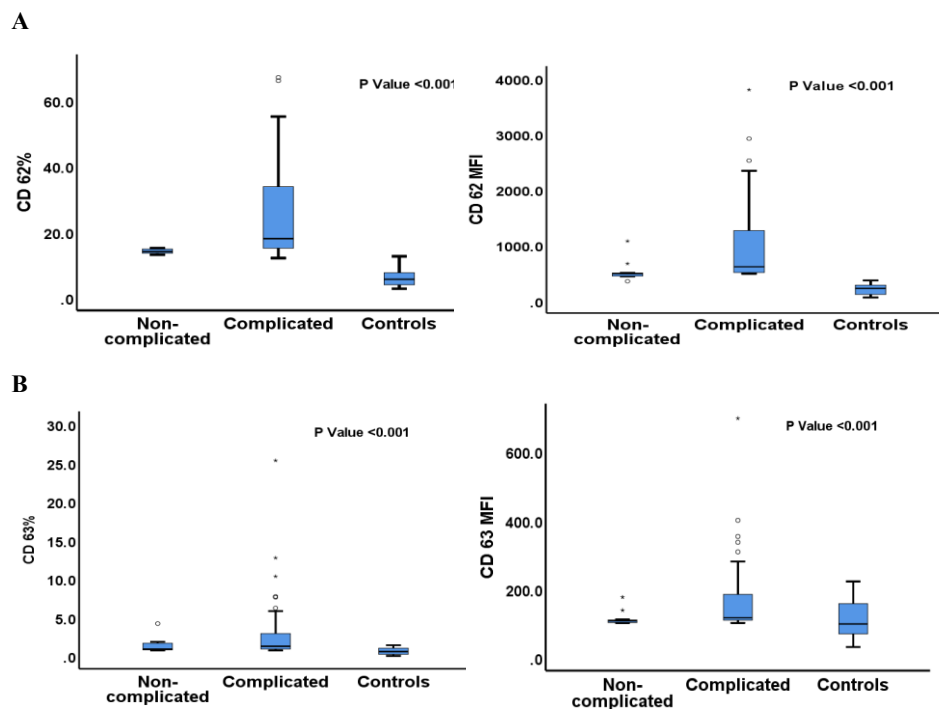


Figure 3. A: Boxplot for distribution of CD 62 % among non-complicated, complicated cases and controls (left), Boxplot for distribution of CD 62 MFI among non-complicated, complicated cases and controls (right). B: Boxplot for distribution of CD 63 % among complicated, non-complicated cases and controls (left), Boxplot for distribution of CD 63 MFI among complicated, non-complicated cases and controls (right).

Table 3. Distribution of platelets CD markers in non-complicated, complicated diabetic cases and controls.

Variables	Cases (n=65)		Controls (n =65)	<i>p</i> value*
	Non-complicated cases (n=11)	Complicated cases (n=54)		
CD 41%	83.7 (79.0-94.0)	83.7 (63.2-98.6)	82.30 (60.00-89.40)	NS
CD 41 MFI	4575.0 (3032.0-7142.0)	4391.5 (1482.0-8272.0)	4000.00 (1450.0-6714.0)	NS
CD 42 %	82.1 (68.4-91.9)	81.9 (64.6-94.8)	80.30 (60.0-88.65)	NS
CD 42MFI	3640.0 (2733.0-4590.0)	3695.0 (2000.0-5828.0)	3250.0 (1886.0-6334.0)	NS
CD 62%	14.0 (13.0-15.0)	17.9 (12.0-66.9)	5.57 (2.69-12.55)	<0.001
<i>p</i> Value**	Non-comp vs comp <i>p</i> =0.001	Comp vs Ctrl <i>p</i> <0.001	Non-Comp vs Ctrl <0.001	
CD 62 MFI	485.0 (350.0-1073.0)	611.5 (487.0-3797.0)	223.00 (58.0-365.0)	<0.001
<i>p</i> value**	Non-comp vs comp <i>p</i> =0.001	Comp vs Ctrl <i>p</i> <0.001	Non-Comp vs Ctrl <0.001	
CD 63%	0.88 (0.70-4.19)	1.25 (0.70-25.30)	0.57 (0.01-1.38)	<0.001
<i>p</i> value**	Non-comp vs comp <i>p</i> =0.051(NS)	Comp vs Ctrl <i>p</i> <0.001	Non-Comp vs Ctrl=0.012	
CD 63 MFI	106.0 (100.0-175.0)	115.0 (100.0-696.0)	97.00 (30.0-221.0)	<0.001
<i>p</i> value**	Non-comp vs comp <i>p</i> =0.055 (NS)	Comp vs Ctrl <i>p</i> <0.001	Non-Comp vs Ctrl=0.05	

Comp; complicated, Ctrl; Control, CD: cluster differentiation, MFI: Mean fluorescence intensity, non-comp; non complicated, vs; versus. *Kruskal Wallis test compares median between groups. **pairwise comparison with Bonferroni correction. *p* > 0.05 is not significant (NS).

Correlations between platelet activity markers, clinical and laboratory findings

As shown in Table 4, there was a statistically significantly positive mild correlation between CD 62 MFI and the duration of diabetic disease ($r=0.291$, $p=0.019$), CD62 % and HbA1c ($r=0.238$, $p=0.050$) Figure 4 A, CD62 MFI and HbA1c ($r=0.361$, $p=0.003$), CD63 % and HbA1c ($r=0.260$, $p=0.037$) Figure 4 B, CD63 % and LDL ($r=0.335$, $p=0.006$), and CD63 MFI and LDL ($r=0.252$, $p=0.043$) Figure 4 C.

Moreover, there was a statistically significantly negative mild correlation between CD 62 % and EF ($r=-0.372$, $p=0.002$) and CD62 MFI and EF ($r=-0.272$, $p=0.029$) Figure 4 D.

The diagnostic ability of platelet surface markers

This study used the Receiver Operating Characteristic (ROC) curve to determine the optimal cut off, accuracy, sensitivity, specificity and area under the curve (AUC) of different surface markers expressed with platelet increased activity.

Regarding CD 62%, at a cut of point of >12.55, it has 99.5% accuracy, 98.5% sensitivity, 100% specificity, 100% positive predictive value and 98.5% negative predictive value for prediction of diabetes, AUC=0.998 and $p<0.001$. While CD 62 MFI, at a cut of point of >365, it has 99.5% accuracy, 98.5% sensitivity, 100% specificity, 100% positive predictive value and 98.5% negative predictive value for prediction of diabetes, AUC=0.998 and $p<0.001$.

The platelet surface marker CD 63%, at a cut of point of >0.75 , it has 82.5% accuracy, 95.4% sensitivity, 69.2% specificity, 75.6% positive predictive value and 93.8% negative predictive value of for prediction of diabetes, $AUC=0.843$ and $p<0.001$. While CD 63 MFI, at a cut of point of >97 , it has 80.0% accuracy, 100% sensitivity, 60.0% specificity, 71.4% positive predictive value and 100% negative predictive value for prediction of diabetes, $AUC=0.713$ and $p<0.001$, Figure 4 A.

The diagnostic ability of platelet surface markers (CD 62 %, CD 62 MFI, CD 63% and CD 63 MFI) for prediction of cardiovascular complications in patients with type2 diabetes showed that CD 62%; at a cut of point of >15 , it has 82.5% accuracy, 64.8% sensitivity, 100% specificity, 100% positive predictive value and 36.7% negative predictive value for prediction of cardiovascular complications, $AUC=0.815$ and $p<0.001$. CD 62 MFI; at a cut of point of >500 , it has 81.5% accuracy, 81.5% sensitivity, 81.8% specificity, 95.7% positive predictive value and 47.4% negative predictive value for prediction of cardiovascular complications, $AUC=0.855$ and $p<0.001$.

On the other side, CD 63%, at a cut of point of >0.88 , it has 69.0% accuracy, 83.3% sensitivity, 54.5% specificity, 90.0% positive predictive value and 40.0% negative predictive value for prediction of cardiovascular complications, $AUC=0.685$ and $p=0.054$. CD 63 MFI, at a cut of point of >107 , it has 76.5% accuracy, 79.6% sensitivity, 72.7% specificity, 93.5% positive predictive value and 42.1% negative predictive value for prediction of cardiovascular complications, $AUC=0.761$ and $p=0.001$, Figure 4 B.

Also, the diagnostic ability of platelet surface markers (CD 62 %, CD 62 MFI, CD 63 % and CD 63 MFI) for prediction of cerebrovascular complications in patients with diabetes were determined. CD 62%, at a cut of point of >29 , it has 70.0% accuracy, 60.0% sensitivity, 80.0%

specificity, 35.3% positive predictive value and 91.7% negative predictive value of in prediction of cerebrovascular complications, $AUC=0.695$ and $p=0.025$. CD 62 MFI, at a cut of point of >1019 , it has 74.0% accuracy, 70.0% sensitivity, 78.2% specificity, 36.8% positive predictive value and 93.5% negative predictive value for prediction of cerebrovascular complications, $AUC=0.765$ and $p=0.001$.

While CD 63%, at a cut of point of >2.67 , it has 72.0% accuracy, 60.0% sensitivity, 83.6% specificity, 40.0% positive predictive value and 92.0% negative predictive value for prediction of cerebrovascular complications, $AUC=0.710$ and $p=0.033$. CD 63 MFI, at a cut of point of >110 , it has 72.5% accuracy, 90.0% sensitivity, 54.5% specificity, 26.5% positive predictive value and 96.8% negative predictive value for prediction of cerebrovascular complications, $AUC=0.738$ and $p=0.001$, Figure 4 C.

Identification of possible predictors of T2DM and its macrovascular complications

The univariate and multivariate linear regression analysis of factors associated with increasing platelet activity surface markers (CD 62%, CD 62 MFI, CD 63% and CD 63 MFI); the coefficient of determination (R^2) for the linear regression models were 0.162, 0.253, 0.309, and 0.226 for CD 62 %, CD 62 MFI, CD 63 % and CD 63 MFI, respectively (Figure 5).

We noticed that the presence of cerebrovascular complication was significantly associated with increasing CD 62 MFI ($\beta=538.93$, $p=0.022$), CD 63 % ($\beta=4.08$, $p<0.001$), CD 63 MFI ($\beta=91.37$, $p=0.004$). Also, the presence of cardiovascular complication was significantly associated with increasing CD 62% ($\beta=8.73$, $p=0.050$).

Finally, increasing LDL by one mg/dl was significantly associated with increasing CD 63 % by 0.03 ($\beta=0.03$, $p=0.001$), and with increasing CD 63 MFI by 0.81 ($\beta=0.81$, $p=0.009$).

Table 4. Correlation between platelets markers (CD62 %, CD 62 MFI, CD 63 % and CD 63 MFI) and patients clinical and laboratory findings.

		CD62%	CD62 MFI	CD63%	CD63 MFI
Age	r	0.200	0.194	0.095	0.082
	p-value	NS	NS	NS	NS
Duration of diabetes	r	0.208	0.291	0.170	0.044
	p-value	NS	0.019	NS	NS
Platelets (10 ³ /μl)	r	0.113	0.125	0.090	0.033
	p-value	NS	NS	NS	NS
MPV	r	-0.030	-0.023	0.055	0.025
	p-value	NS	NS	NS	NS
Urea (mmol/L)	r	0.152	0.186	0.095	0.099
	p-value	NS	NS	NS	NS
Creatinine (μmol/l)	r	-0.122	-0.075	-0.093	-0.078
	p-value	NS	NS	NS	NS
HbA1c (%)	r	0.238	0.361	0.260	0.096
	p-value	NS	0.003	0.037	NS
PT (sec)	r	0.201	0.031	0.251	0.125
	p-value	NS	NS	NS	NS
PC (%)	r	-0.226	-0.147	-0.166	-0.140
	p-value	NS	NS	NS	NS
APPT (sec)	r	-0.068	-0.174	-0.209	-0.129
	p-value	NS	NS	NS	NS
Cholesterol (mg/dl)	r	-0.151	-0.032	-0.066	-0.006
	p-value	NS	NS	NS	NS
Triglyceride (mg/dl)	r	-0.163	-0.123	-0.137	-0.002
	p-value	NS	NS	NS	NS
HDL (mg/dl)	r	-0.165	-0.074	-0.103	-0.111
	p-value	NS	NS	NS	NS
LDL (mg/dl)	r	0.125	0.183	0.335	0.252
	p-value	NS	NS	NS	NS
EF	r	-0.372	-0.272	-0.182	-0.142
	p-value	0.002	0.029	NS	NS
BMI	r	-0.081	-0.232	-0.159	0.002
	p-value	NS	NS	NS	NS

r (Spearman correlation). CD: cluster differentiation, MFI: Mean fluorescence intensity aPTT; activated partial thromboplastin time, HDL; high density lipoprotein, HbA1c; glycated hemoglobin, LDL; low density lipoprotein, MPV; mean platelet volume, PT; prothrombin time, PC; prothrombin concentration, EF: ejection fraction, BMI: body mass index.

p > 0.05 is not significant (NS).

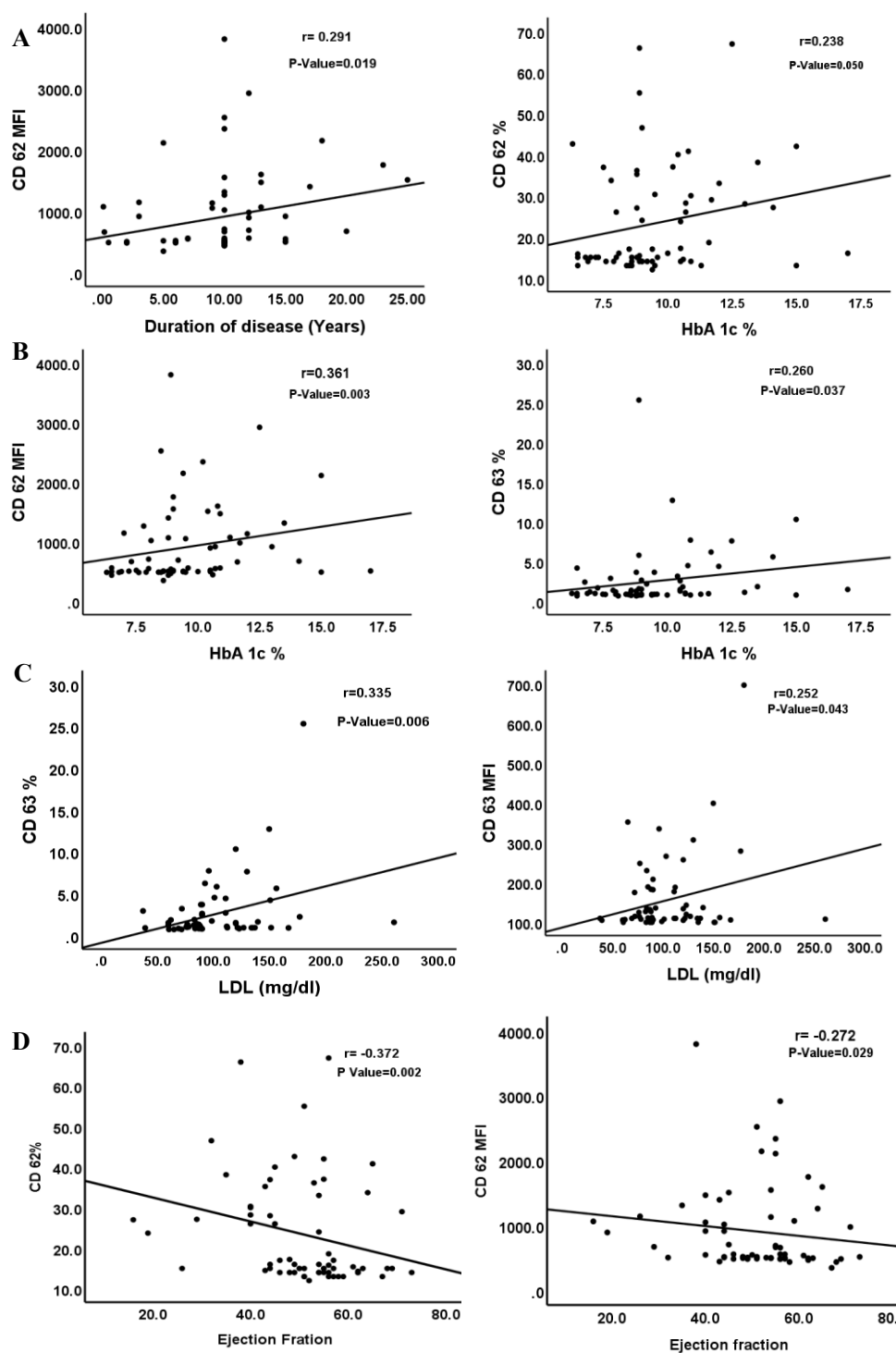


Figure 4. A: Scatter diagram for correlation between CD 62 MFI and duration of disease among diabetic patients (left). Scatter diagram for correlation between CD 62 % and HbA1c among diabetic patients (right). B: Scatter diagram for correlation between CD 62 MFI and HbA1c among diabetic patients (left). Scatter diagram for correlation between CD 63 % and HbA1c among diabetic patients (right). C: Scatter diagram for correlation between CD 63 % and LDL among diabetic patients (left). Scatter diagram for correlation between CD 63 MFI and LDL among diabetic patients (right). D: Scatter diagram for correlation between CD 62% and ejection fraction among diabetic patients (left). Scatter diagram for correlation between CD 62 MFI and ejection fraction among diabetic patients.

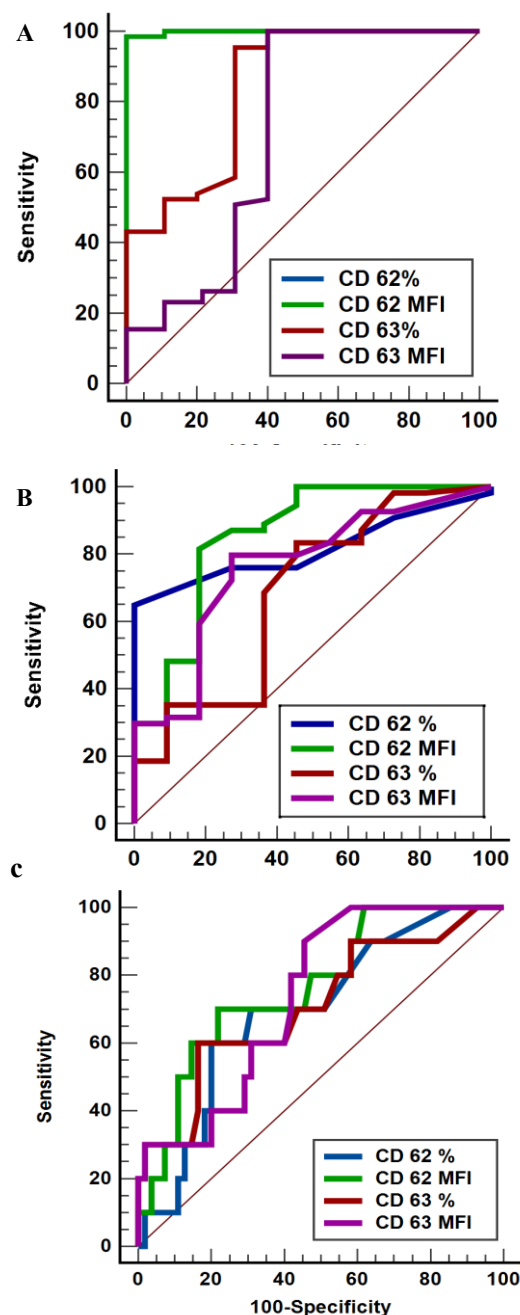


Figure 5. A: Receiver operating characteristic (ROC) curve for ability of platelet CD markers (CD62 %, CD 62 MFI, CD 63 %, CD 63 MFI) in prediction of diabetes. B: ROC curve for ability of platelet CD markers (CD 62 %, CD 62 MFI, CD 63 % and CD 63 MFI) in prediction of cardiovascular complications in patients with T2DM. C: ROC curve for ability of platelet CD markers (CD 62 %, CD 62 MFI, CD 63 % and CD 63 MFI) in prediction of cerebrovascular complications in patients with T2DM.

Discussion

Diabetes Mellitus (DM) has been shown to have a prothrombotic status.² Several factors contribute to the pro-thrombotic condition such as increasing coagulation, impaired fibrinolysis, endothelial dysfunction and platelets hyper-reactivity. Platelets play a crucial role in the pathogenesis of atherothrombosis in DM.¹⁰ The objective of the current study was to describe the platelets activity in patients with T2DM in association with ischemic cardiovascular disease in relation to duration of diabetes.

Uniquely, this work is one of the first studies conducted at Assiut University using four colors Flow-Cytometry for detection of platelet surface activation markers in T2DM patients, in a decent number of cases and controls (65 diabetic cases with and without complications and 65 controls). This study demonstrated up-regulation of the platelet expression markers P-selectin (CD 62P) and CD 63 in patients with T2DM, which agreed with findings of a previous study by Eibl et al., 2004.¹²

The results of the current study suggested that there is excess platelets activation in diabetic patients compared with “normal control subjects” as measured by expression of CD 62P and CD 63 with ($p < 0.001$). T2DM is already a known risk factor for platelets hyper-activation, that agreed with data reported by Israels et al., 2014, who established increased expression of both the platelets activation markers CD 62 and CD 63 in subjects with diabetes compared to healthy subjects.¹³ The study by Eibl et al., 2004, also identified increased baseline expression of CD 62P and CD 63 in participants with diabetes.¹² The study by Tschoepe et al., 2005, demonstrated that an increased fraction of activated (CD 62 and CD 63 positive) platelets in the circulation of type-1 and type-2 diabetic patients. Platelet activation in T2DM may have a different mechanism compared with T1DM and several factors like lipid abnormalities, non-enzymatic glycation of platelets membrane or increased intracellular thromboxane A2 formation are associated with altered platelet’s function.¹⁰

This study also established significant difference in the expression of platelet

activation markers CD 62%, CD 62 MFI, CD 63% and CD 63 MFI in diabetic patients with or without cardiovascular or cerebrovascular complications compared to controls ($p<0.001$). This is consistent with findings of the study by Soma et al., 2016, which obtained higher base level expression of the activation markers CD 62P and CD 63 among diabetics with or without cardiovascular complications in comparison to healthy controls.⁴

The present study observed that there was a statistically significant increase in the mean BMI as the duration of DM increased (27.75 ± 3.67 among <5 years, 28.52 ± 4.76 among 5-10 years and 31.66 ± 3.64 among >10 years), ($p=0.003$). Such finding matched the results obtained by the study by Sonmez et al., 2019.¹⁴ However, the study by Angiolillo et al., 2006, observed no correlation between platelets function profiles and BMI, as observed in our study.¹⁵

In the present study, there was statistically significant negative mild correlation between CD 62 % and EF ($r=-0.372$, $p=0.002$), CD 62 MFI and EF ($r=-0.272$, $p=0.029$). Interestingly there was an inverse proportion between left ventricular ejection fraction and (CD 62%, CD MFI). This comes in agreement with data of the study by Venturinelli et al., 2006, who cleared that P-selectin (CD 62P) level could be used as a marker of plaque destabilization in unstable angina and also level of P-selectin (CD 62P) in patients with multi-vessel disease could be higher than in those with single-vessel disease. So, P-selectin (CD 62P) may indirectly reflect clinical condition of patients with coronary artery disease, with potential diagnostic and therapeutic implications.¹⁶

In the current study, considering the correlation of laboratory characteristics with platelets activation markers, HbA1c had mild positive correlation with CD 63 ($r_s=0.260$, $p=0.037$), CD 62 ($r_s=0.238$, $p=0.050$), and CD 62 MFI ($r_s=0.361$, $p=0.003$). These observations agreed with findings of the study by Soma et al., 2016, who observed that increased platelets expression of CD 63, CD 62P in adults with T2DM was positively associated with HbA1c and improved glycemic control correlated with a decline in measured platelets activation markers. These findings suggest that platelets

hyperactivity can be reversed with improved metabolic control.⁴ On the contrary, the study by Neergaard et al., 2015, suggested that levels of HbA1c did not correlate with platelets aggregation and turnover in cardiovascular diseases patients with known T2DM. However, patients in these studies were on dual antiplatelet therapy and anti-diabetic drugs, therefore they were not entirely comparable with our study.¹⁷ Also, the study by Angiolillo et al., 2006, observed no correlation between HbA1C levels and platelet activity function test, this may be attributed to that their study was conducted in a tightly controlled diabetic population.¹⁸

The present study suggested that there was statistically significant increase in the mean HbA1c as the duration of DM increased (9.01 ± 1.76 among <5 years, 10.48 ± 2.42 among 5-10 years and 10.58 ± 2.95 among >10 years), ($p=0.026$). These findings agreed with those observed by the study of Verma et al., 2006, who showed that patients with poorly controlled DM showed a significant correlation between HbA1c level and duration of DM.¹⁹ Also, the current study confirmed that there was statistically significantly mild positive correlation between CD 63 % and LDL ($r=0.335$, $p=0.006$), and between CD 63 MFI and LDL ($r=0.252$, $p=0.043$), which also agreed with results reported by the study of Szarpak et al., 2021.²⁰

A previous study showed that DM is associated with persistent platelets activation and that P-selectin (CD 62p) levels predicted increased risk of acute cardiovascular complications, as reported by the study of Jenny et al., 2020.²¹ In accordance, the present study revealed that CD 62%, CD 62 MFI, CD 63% and CD 63 MFI can be used for early prediction of cardiovascular disease among diabetics ($p<0.001$). Furthermore, the most reliable markers for early prediction of cardiovascular complications were CD 62 MFI and CD 62% with (AUC =0.855 and 0.815, respectively). These data were also confirmed by findings of the study by Kim et al., 2013.²²

The present study revealed that CD 62%, CD 62 MFI, CD 63% and CD 63 MFI can be used for early prediction of cerebrovascular disease

among diabetics ($p < 0.001$). The most reliable markers for early prediction of cerebrovascular complications were CD 62 MFI and CD 63MFI with (AUC=0.765 and 0.7, respectively), These observations come in agreement with those reported by the study of Marquardt et al., 2002, who concluded that CD 63 could be used as a predictor for first or recurrent ischemic events.²³

In the present study, multivariate linear regression indicated that the presence of cerebrovascular complications was significantly associated with increasing CD 62 MFI ($\beta=538.93$, $p=0.022$), CD 63 % ($\beta=4.08$, $p<0.001$) and CD 63 MFI ($\beta=91.37$, $p=0.004$). A previous study conducted by Marquardt et al., 2002, showed that CD 62p and CD 63 are memory markers of platelets activation, and the increased expression of secretion-dependent antigens after stroke indicated that platelets had previously undergone activation. Therefore, CD 63 could be used as a predictor for first or recurrent ischemic events.²³

In the present study, increased LDL level by one mg/dl was associated with increased CD 63 % by 0.03 ($\beta=0.03$, $p=0.001$) and with increased CD 63 MFI by 0.81 ($\beta=0.81$, $p=0.009$). In the same line, the study by Gavina et al., 2023 reported that platelets and oxidized (ox)-LDL interactions play a key role in atherosclerosis development and progression due to (i) the presence of LDL-binding sites on platelets, (ii) generation of ox-LDL by platelets and (iii) targeting platelet by ox-LDL.²⁴

In conclusion, the up regulation of platelet activation markers in patients with T2DM may indicate that hyperlipidemia and hyperglycemia accompanying T2DM have a stimulatory effect on platelet activation which probably makes those patients more vulnerable to cardiovascular diseases and cerebrovascular disease than non-diabetics.

Author Contributions

FAE and MMA designed the study, HMM collected the data, ERB performed the experimental part, MMS, SMT, MHM, DAH supervised data analysis and results interpretation. All authors contributed to writing and editing the final manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

The study protocol was reviewed and approved by the Ethical Committee of the Faculty of Medicine, Assiut University (Approval dated December 2019). The study was registered as "Clinical Trials.gov ID NCT04027907".

Informed consent

Verbal consent was obtained from each study participant before being included in the study.

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