

Utility of Platelet derived microparticles in predicting complications of Diabetes mellitus

ABSTRACT

Background : The long term complications related to DM are the main cause of mortality and morbidity in DM. However, many of the Diabetes related complications are detected later at end stage . Thus, there is a need for early diagnosis of the complications and biomarker that predicts the outcome of the disease and its complications. PMPs are the recent particles of interest that play a major role in pathophysiology of the disease and can be used to detect complications earlier.

Aims and Objectives :

Aim – To study the role of PMPs in DM

Objectives- To compare the levels of PMPs in adults who are Diabetics and in complicated diabetes

Introduction : Diabetes Mellitus(DM) is a global pandemic. The long term microvascular and macrovascular complications related to DM are the main cause of mortality and morbidity in DM. However, many of the Diabetes related complications can be prevented or delayed with early detection. Thus, there is a need for early diagnosis of the complications and biomarker that predicts the outcome of the disease and its complications. . Recently, platelet derived microparticles have been discovered to be involved in the onset and progression of Diabetes and various Diabetic complications. Platelet derived microparticles (PMPs) are small sized membrane bound vesicles released from platelets during platelet activation. They now have emerged as important markers associated with endothelial injury, inflammation and prothrombotic state seen in DM. Due to substantial burden of the disease and associated problems, there is a need to explore the possibility of new markers that will help in predicting the onset and progression of diabetes. Also, PMPs can be helpful in assessing the vascular events and early assessment and management of various complications related to Diabetes. Hence, in this present study the levels of PMPs in Diabetics and non-

diabetics were compared and found to be higher in Diabetics. Similarly, higher levels of PMPs were found in patients with diabetic complications. Thus, PMPs can be further studied to establish its role as a biomarker in Diabetes that can help in early onset and detection of various DM related complications.

Material and Methods-

A prospective case control study was done in Department of Pathology and Department of Medicine. 30 Diabetic patients were taken as cases while 30 healthy adults were taken as controls. Complete hematological profile was done for both the groups. Immunophenotyping using antibodies like Cd 45, CD41, Cd61 and Annexin V+ was used to distinguish PMPs from platelets. The annexin V + particles were identified first and separately gated which were then observed in the CD41 vs CD 61 plots. The particles that were annexin V +, CD41+, CD 61+ were taken as PMPs.

Results –There was significant difference between Hemoglobin, total leucocyte count, platelet count and Annexin V between cases and controls as $p < 0.05$. PMPs ranged from 2 ± 169 with a mean $\pm$ SD 53.7 ± 57.49 in controls and from 36 ± 2256 with a mean $\pm$ SD 574.50 ± 647.98 in diabetic cases while PMPs in the complicated diabetes group was from 848.42 ± 810.51 (78-2264). Thus, higher levels of PMPs were seen in Diabetes and Diabetic complications and the difference was statistically significant.

Conclusion – Thus , this study shows that there is increase in the levels of PMPs in DM and its complications and thus can play a role in the thrombotic state of the disease as well as can give rise to various DM related complications.

Abbreviations : DM, Diabetes mellitus, PMPs, Platelet derived microparticles, GFR, glomerular filtration rate, ADA, American Diabetes Association, WHO, World Health Organization, Hb, hemoglobin, Hct, haematocrit, RBC, red blood cell count, MCH, mean corpuscular volume, MCH, mean corpuscular haemoglobin, MCHC, mean corpuscular haemoglobin Concentration, TLC, total leucocyte count, PC, and platelet count.

Key words : Platelets, microparticles, diabetes mellitus, complicated diabetes, thrombosis, platelet microparticles.

Introduction- Diabetes Mellitus (DM) is one of the most common group of metabolic disorders affecting multiple systems of the body. It is associated with various long term macro and microvascular complications which add to the morbidity and mortality seen with the disease.(1) The microvascular complications include Diabetic retinopathy, diabetic nephropathy, and diabetic neuropathy while the macrovascular complications include peripheral arterial disease, stroke, and coronary artery disease. Diabetic nephropathy is usually detected by microalbuminuria, elevated GFR levels, Diabetic neuropathy is detected when patient presents with sensory neuropathy while diabetic retinopathy presents with retinal hemorrhages, exudates and detachment. At the time of detection of complications, they present with end stage manifestations that are often irreversible. Most of these complications are detected quite late as patients remain asymptomatic for a long time. Hence, there is a need for biomarkers for predicting the early onset of complications associated with DM so that aggressive intervention can be done.

Currently, routine ophthalmologic screening and microalbuminuria are the only methods which can be used for detection of complications.(2) Hence there is a need for early indicators of complications. Platelets, their indices and micro particles are of active interest as their potential use as biomarkers. Diabetes is a prothrombotic state which causes a number of structural, functional and metabolic changes.(3) Hyperglycemia contributes to increased platelet reactivity and hyper aggregation of platelets which leads to release of microparticles which are nano sized fragments (100-1000nm) that are released upon platelet activation or stress.(4,5). DM and its complications share a common pathophysiologic environment of inflammation, hypercoagulability and endothelial dysfunction. (4,6) As per many studies, due to this deranged environment, there is an increased release of PMPs which could be used as biomarkers of vascular dysfunction and potentially explored for their role in various complications. It has been found that there is an increase in levels of PMPs in DM as

compared to healthy adults while even more significant raised levels of PMPs are found in patients with various Diabetic complications. (7) Hence, there is a possibility that PMPs can be used a potential biomarker for early detection and onset of various DM related complications and needs to be explored. Therefore, this study aims to explore the utility of PMPs as potential biomarkers for DM and its complications.

Aim and Objectives : To explore the role of PMPs as potential biomarkers for DM and its complications.

Material and Methods

This was a case control study that was conducted in a tertiary care hospital from November 2019 to April 2021. 30 adult patients aged between 18- 60 years diagnosed clinically as DM according to American Diabetes Association (ADA) and World Health Organization(WHO) were enrolled in the study. (8) 30 healthy adults with no diabetes, no coronary artery disease, no cerebrovascular disease, not on any antiplatelet drugs were taken as controls. Among the 30 cases, 16 cases had diabetes with no complications while 14 cases had diabetes associated with complications like hypertension(4 cases), diabetic nephropathy (4 cases), cataract(3 cases), coronary artery disease (2 cases) and 1 case of diabetic retinopathy.

The exclusion criteria included: Pregnant females, patient with history of cancer/chemotherapeutic agents, history of autoimmune disease other than type 1 Diabetes Mellitus, current or recent infections and or inflammatory processes, patients on antiplatelet drugs like aspirin, clopidogrel. Detailed history was taken and routine physical examination was done for all subjects. Informed consent was taken from all subjects before blood collection. Approval from Institutional Ethics Committee for Human Research (IEC-HR) was obtained.

Sample collection for PMPs estimation

Five ml venous blood sample was collected under aseptic precautions from patients for the following investigations- 2.5 ml in EDTA vial was collected for complete hemogram done on 5 part Automated Haematology analyzer (Mindray BC-6800). Parameters noted were hemoglobin (Hb), haematocrit (Hct), red blood cell count (RBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin Concentration (MCHC), total leucocyte count (TLC), and platelet count (PC). Another, 2.5 ml of blood was collected in another 3.2 % Trisodium citrate (TCA) vial for flowcytometry based immunophenotyping for detection of PMPs.

Isolation & processing of PMPs

The sample was processed within 4 hours for making Platelet poor plasma for isolation of PMPs. The sample obtained was stained using the standardized 'Stain-lyse-wash' method. One tube was taken for each sample. The tube was stained with a cocktail of antibodies-containing CD 45, CD41, CD61, and Annexin V according to the panel.

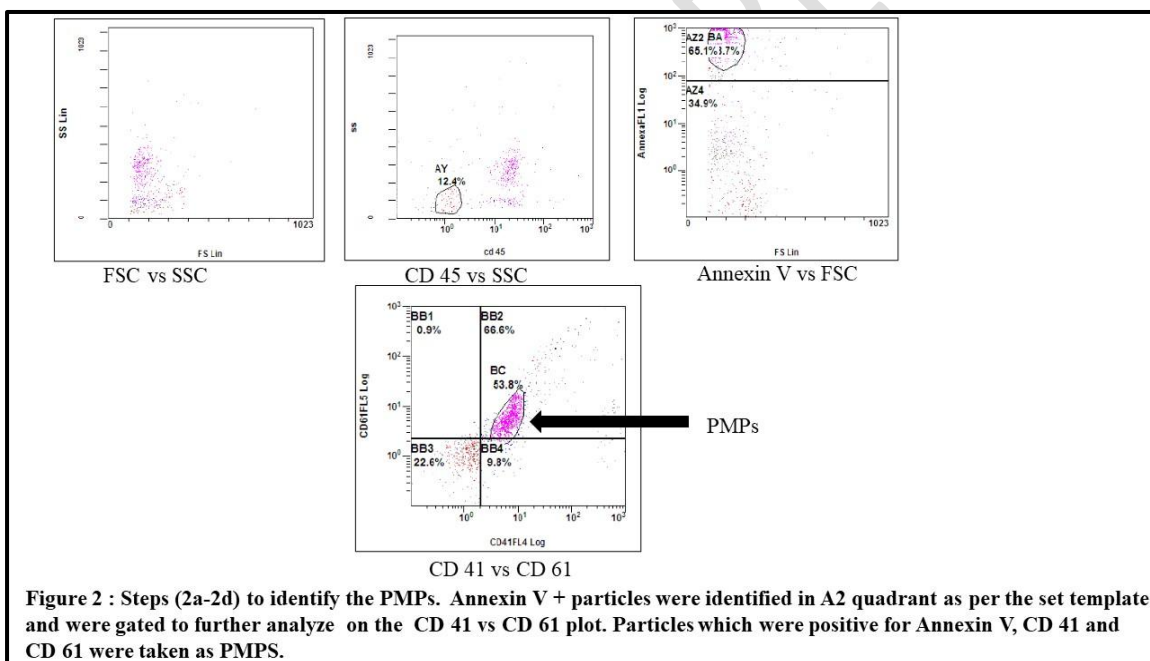
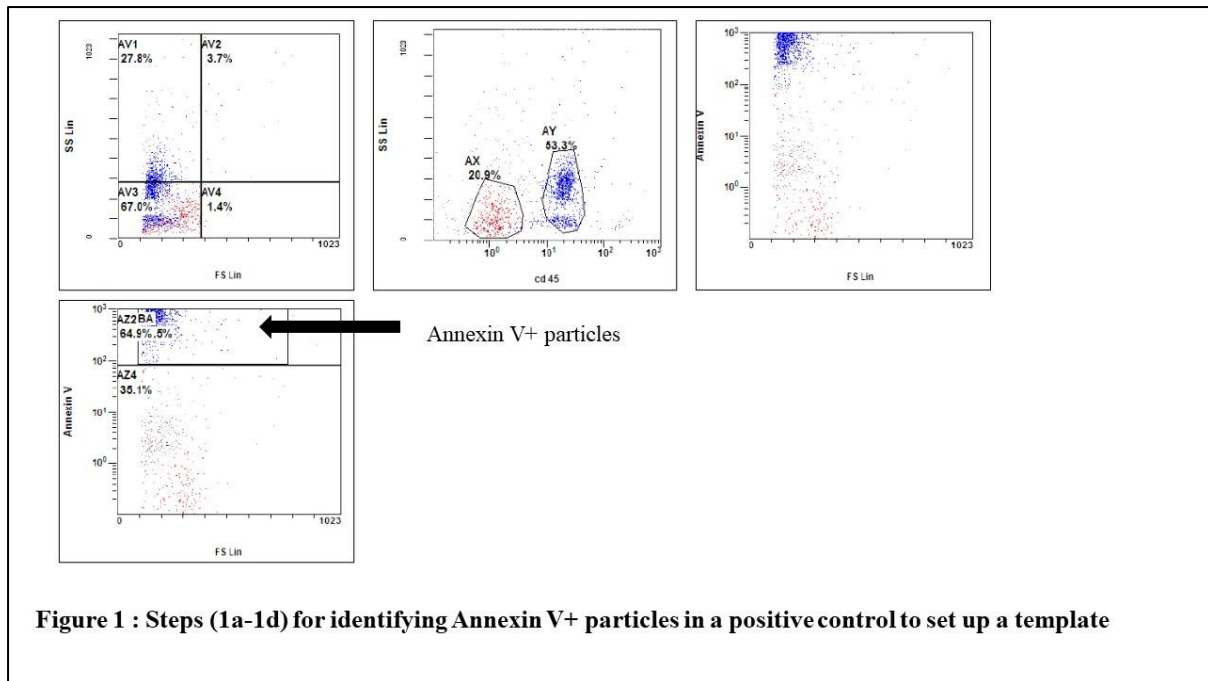
Measurement of PMPs by immunophenotyping

Immunophenotyping was done on Beckman Coulter (FC 500), which is a 5 color flow cytometer. 5 μ L of a cocktail containing antibodies CD45-ECD (J33), CD41(P2)- PC.5, CD 61(SZ21) - PC.7 were added to the tube. The tube was incubated for 20 minutes in a dark room at room temperature. The mixture was then vortexed well and centrifuged for 8 min at 3600rpm. The supernatant was decanted leaving behind the cell pellet. The cell pellet was then washed with ice-cold sheath fluid and centrifuged at 3600 rpm for 10 mins. The supernatant was again decanted and resuspended in 100 μ L of 1X Annexin V binding buffer (1 X binding buffer preparation for annexin V – 10 x binding buffer was diluted 10 fold with distilled water and the diluted buffer was placed on ice. A quantity sufficient for the expected number of assays was prepared). The tube was kept on ice. 1 μ L of **Annexin V-FITC**

solution was added to 100 μ L of cell suspension prepared previously. The tube was kept on ice and again incubated for 15 minutes in the dark. After this, 400 μ l of ice-cold 1X binding buffer was added to the tube and was mixed gently. Following this, the sample was ready to be acquired in flow cytometry.

Acquisition of events: The sample was acquired to a maximum of 300 seconds or a total of 10,000 events whichever was first.

Gating strategy: Viability gating was done on SSC vs CD 45 to exclude the debris. Platelet population was confirmed using CD 41 and CD 61. Total Annexin V + particles were identified on Annexin V vs FSC plot. The positive annexin V + was identified using the previously set template as per the positive control (Figure 1). The absolute number, as well as the percentage of Annexin V+ particles, was noted. *PMPs were identified using Annexin V and platelet lineage-specific markers like CD 41 and CD 61* (Figure 2). The particles which were CD 41+ CD61+ but Annexin V- were identified as platelets . The Annexin V + were analyzed further and the particles which were positive for all 3 – CD41, CD 61, and Annexin V were considered as PMPs.



Statistical Analysis:

The collected data was transformed into variables, coded, and entered in Microsoft Excel. Data were analysed and statistically evaluated using the SPSS-PC-19 version. Quantitative

data were expressed in mean $\pm$ standard deviation or median with interquartile range and the difference between two comparable groups were tested by student's t-test (unpaired) while qualitative data were expressed in percentage and statistical differences between the proportions were tested by chi-square test or Fisher's exact test. Pearson correlation was used to see the correlation between two quantitative variables and Lin's concordance test was to see the concordance between two variables.

RESULTS

30 diabetic cases and 30 healthy adults were recruited in the study. The cases included 15/30 females (50%) and 15/30 males (50 %) while the control group included 22/30 males (73.3 %) and 8/30 females (26.6 %). Among the diabetic cases, 16/30 had diabetes with no complications while 14/30 had diabetes with complications.

Hematological profile of subjects

A complete evaluation of hematologic profile of all the patients was done and compared between diabetic cases and controls. These parameters were further compared between the diabetic cases without complications and diabetic cases with complications. (Table I, II)

Table I :Comparison of Hematological parameters between Diabetic cases(n = 30) and healthy controls (n = 30)

	Group		p value
	Cases (n =30) (Mean $\pm$ SD)	Controls (n =30) (Mean $\pm$ SD)	
Hb (g/dL)	9.91 $\pm$ 2.23 (5.1-14.9)	12.9 $\pm$ 1.88 (7.7 -15.7)	0.000**(p = 0.01)
HCT (%)	32.1 $\pm$ 5.18(25- 48.3	37.2 $\pm$ 5.61(23.6 %-	0.001*

	%)	47%)	
MCV (fL)	87.91± 10.91(70.2-128.9)	96.04± 6.21(86.6 – 109)	0.001*
MCH (pg)	27.83± 3.95(20.2-41.9)	31.24 ±1.90(28.1-35.7)	0.001*
MCHC (g/dL)	31.65± 1.31(28- 34)	32.42±0.91(30.7- 34.1)	0.011
TLC (× 10 ⁹ /L)	9.74± 3.77(3.77-17.6)	6.61± 1.49(3.7 -9.84)	0.000**(p = 0.01)
RBC (× 10 ¹² /L)	3.85 ±1.157(2.17- 6.6)	3.97 ± 0.64(2.63-5.22)	0.626
Platelets (× 10 ⁹ /L)	226.43 ±124.4(35-530)	185.7 ± 51.85 (109-325)	0.106

Table II : Hematological profile between uncomplicated diabetic cases (n = 16) and complicated diabetes (n = 14)

	Cases (N= 16) Uncomplicated DM Mean ± SD	Cases (N = 14) DM with complications Mean ± SD	p value
Hb (g/dl)	9.806±2.352(6.5-14.9)	10.028 ±2.170(5.1-12.3)	0.790
Hct (%)	32.766 ±5.666(25.6-48.3%)	31.335 ±4.655(25-41 %)	0.454
MCV (fL)	90.656 ± 12.941(70.2- 128.9)	84.778 ±7.246(71-101.1)	0.132

MCH (pg)	28.587± 4.686(20.2 – 41.9)	26.964 ±2.846(21.6- 32.7)	0.256
MCHC (g/dL)	31.475 ±1.322(28.8 - 33.2)	31.864 ± 1.315(28- 34)	0.427
TLC (x 10 ⁹ /L)	8.602 ± 3.426(3.77 – 15.12)	11.042 ± 3.860(5.01 – 17.6)	0.080
RBC (x 10 ¹² / L)	3.875 ± 1.246(2.17 – 6.6)	3.840 ± 1.092(2.2 – 5.6)	0.937
Plt count (x 10 ⁹ / L)	249.062 ±155.889(35-530)	200.571 ± 71.664(108- 371)	0.276

*Denotes significant difference between the two groups

Test : Independent t test

Immunophenotyping

Immunophenotyping was done using peripheral blood samples for evaluating the total Annexin V+ particles and PMPs between cases and controls. The Annexin V + particles and percentage of Annexin V+ particles were evaluated among the diabetic cases and healthy controls. Similar evaluation was done for diabetic cases without complications and diabetic cases with complications. After estimating the Annexin V+ particles, the PMPs were identified in cases & controls as per the set protocol. (Figure 3, 4) . The total number of PMPs and the percentage of PMPs were analyzed in healthy controls and compared with diabetic cases. Also, the levels of PMP+ particles and percentage of PMPs were assessed between the uncomplicated diabetic cases and complicated diabetic cases.(Table III, IV)

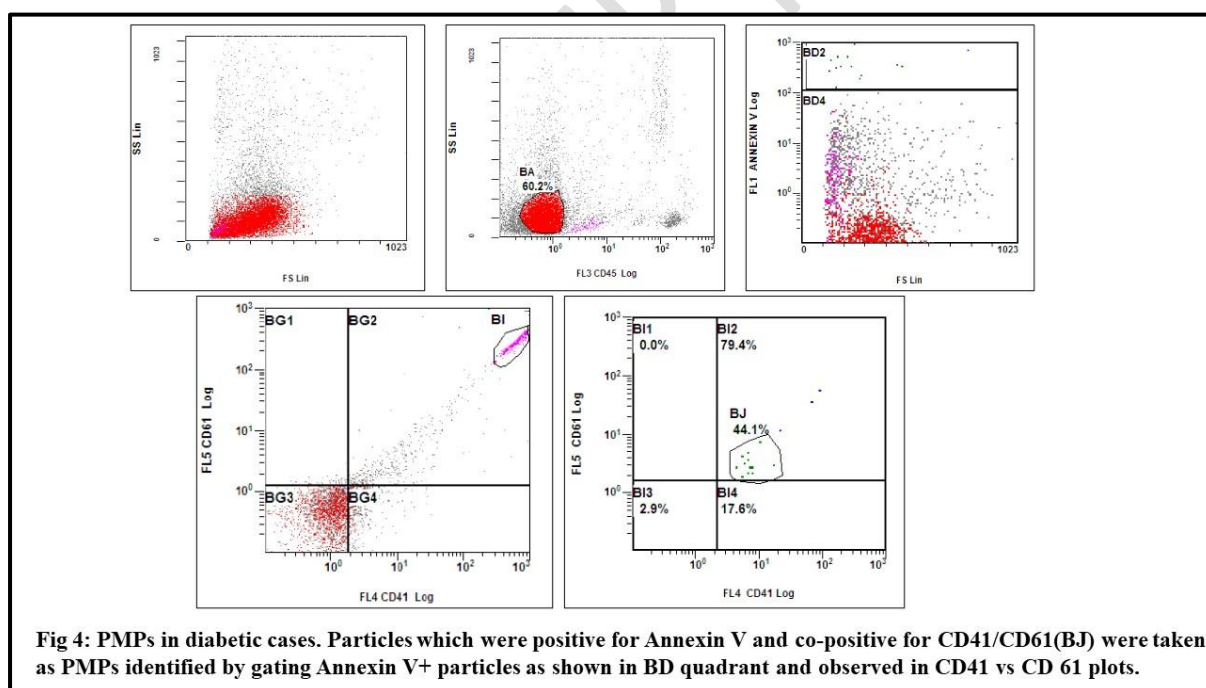
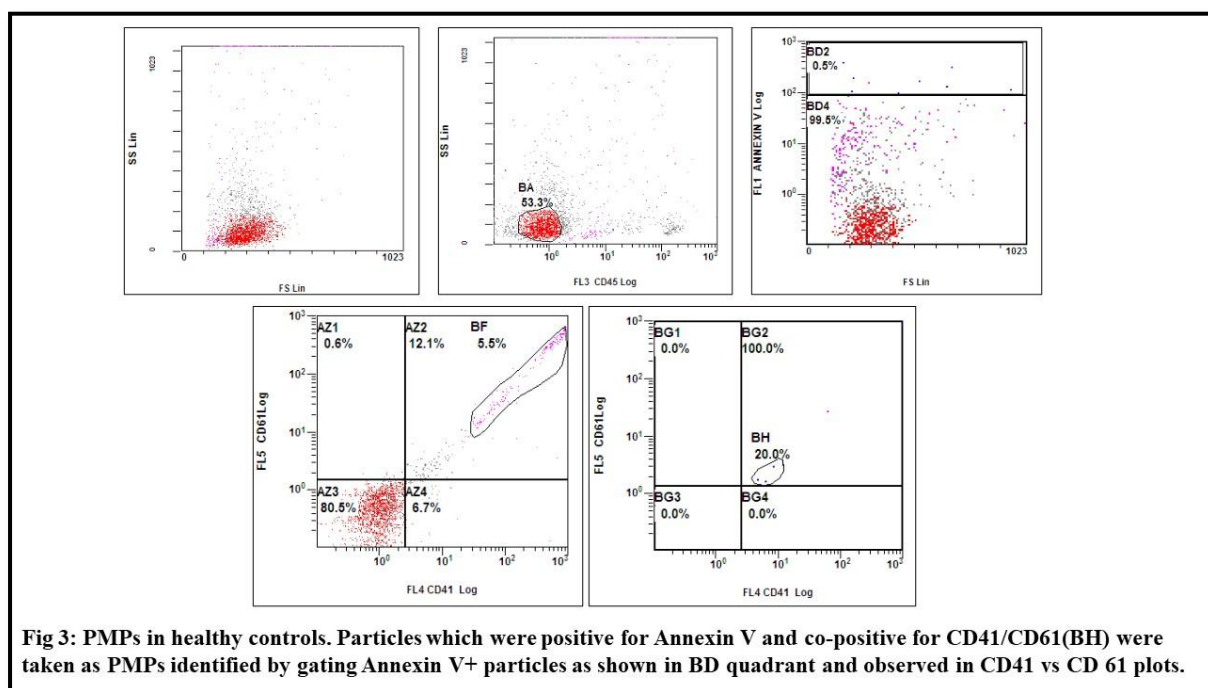


Table III: Comparison of Annexin V+ particles between diabetic cases(n = 30) and healthy controls (n = 30)

	Healthy controls	Diabetic cases	P value
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Annexin V+	135.4±199.7 (7 - 1060)	697.43±744.4 (65-2600)	<0.001 *
Annexin V + %	2.45 ±3.35 (0.03 - 12.58)	8.61 ±7.5 (0.12-24)	<0.001*
PMPs	53.7 ±57.49 (2-169)	574.5 ±647.98 (49-2264)	<0.001*
PMPs %	1.86±2.96 (0.01-12.24)	6.80±5.24 (0.06-36.6)	<0.001*

*Denotes statistical significance between two groups

Test : Independent t test

Table IV: Annexin V+ values between diabetic without complications (n = 16) and complicated diabetes (n = 14)

	Diabetic cases without complications	Diabetic cases with complications	P value
Annexin V+	463.37.4±453.34 (65 -1589)	964.92±924.46 (89-2600)	0.081
Annexin V + %	7.39 ±5.52 (0.03 - 12.58)	8.59 ± 5.5(0.12-24)	0.55
PMPs	334.81 ±333.34(49 - 1316)	848.42± 810.51 (78-2264)	0.041*
PMPs %	6. 21± 5.59 (1.17-15.9)	7.48± 4.95 (1.07-14.72)	0.52

*Denotes statistical significance between two groups

Test : Independent t test

There was a significant difference in the absolute value of Annexin V+ particles and PMPs between diabetic cases and healthy controls. Similar results were seen on comparing the percentage of Annexin V+ particles and PMPs between cases and controls as p value<0.001. However, on comparing between the uncomplicated diabetic cases and complicated diabetic cases, statistically significant difference was found between only the absolute values of PMPs and none of the other parameters.

DISCUSSION

DM is a multifactorial, systemic chronic disease that is mainly characterized by insulin resistance and hyperglycaemia. Hyperglycaemia and various other ongoing complex pathogenesis cause the patients of diabetes to develop prothrombotic changes. These prothrombotic changes lead to various vascular complications seen in DM. However, most of these complications develop quite late and the patient may remain asymptomatic for a long time. Hyperglycaemia in DM contributes to increased platelet reactivity and increased release of platelet contents and microparticles. This poses a major risk to the quality of life of DM patients and the extra demand for health services. Early detection of complications can alter the outcome of disease and prognosis of many diabetics. Hence, we need to have biomarkers that can help us to predict and assess the onset and progression of complications in diabetic patients. (2

PMPs are 0.1-1 μm fragments that are shed from the plasma membrane of platelets undergoing activation, stress, or apoptosis and are the most abundant microparticles found in blood. They have a phospholipid-based structure and express functional receptors from platelet membranes. PMPs accounting for 70-90 % of those circulating in the blood and they express the procoagulant phosphatidylserine on their surface and most likely complement the functions of platelets in haemostasis, thrombosis, and inflammation and can also act as promoters of tissue regeneration.(10) In the deranged microenvironment observed in Diabetes, PMPs, apart from being biomarkers of vascular dysfunction, also contribute significantly to the pathogenesis and progression of the disease.(11)

Early hyperglycaemia through a metabolic memory triggers endothelial activation as the initial vascular abnormality. In this context, early hyperglycaemia-induced PMP release can be considered as novel markers of endothelial activation/dysfunction.(9) This mechanism is also associated with the ability of PMPs to promote coagulation by externalization of anionic phospholipids (phosphatidylserine) and the subsequent assembly of coagulation complexes (TF: FVII a, FVIIa, IXa) and thereby thrombin formation.(7) Further pro-inflammatory state seen in DM can increase PMP release which may directly promote and exaggerate the inflammatory responses. Thus, PMPs in DM may act as markers for thrombosis, endothelial dysfunction and inflammation associated with various Diabetic complications.(7, 9)

The mean values of Hb between diabetic cases and controls showed that most diabetics had lower Hb as compared to controls. In our study, among the 30 diabetic cases, 16 had mild to moderate degrees of anaemia. This is consistent with other studies which state that diabetics have a higher chance of developing anaemia and anaemia can also potentially contribute to the pathogenesis of diabetic complications. Both uncomplicated and complicated diabetic cases show presence of anaemia which is consistent with previous studies.(12, 13) There were 12/16 (75 %) cases of uncomplicated diabetes with anaemia while 8/14(57%) cases of

complicated diabetes developed anaemia. *DM is often accompanied by mild to moderate anaemia called anaemia of inflammation or anaemia of chronic disease* caused due to hyperactive mononuclear phagocyte system triggered by various mechanisms that lead to early removal of RBCs.(12) According to many studies, anaemia is more commonly seen in Diabetics with renal impairment due to impaired production of erythropoietin. It is also found that normochromic, normocytic anaemia can occur before evidence of renal impairment is present in Diabetic nephropathy patients.(13)

The mean of MCV, MCH, and MCHC in both cases and controls was found to be in the normocytic normochromic range. On comparing between diabetic cases and healthy controls, MCV, MCH among the diabetic cases were found to be on the lower side as compared to controls and showed wide variation. The lower MCV in cases could be due to *associated anaemia of chronic disease or iron deficiency anaemia or both*. Our findings contrast with a study done by Alamri et al which stated that RBC count, MCV, MCH, and MCHC are raised in Diabetics due to hyperglycaemia.(14) There was a significant difference between the MCV, MCH, and MCHC values between diabetic cases and controls. In our study, MCV and MCH were also lower in complicated diabetes than uncomplicated diabetes. This contrasts with a study done by Alamri and Jaman et al which stated that RBC indices are raised in diabetics and more in complicated diabetes MCHC in our study lay within the normal range in both the groups.(15) Thus, it can be deduced *that most diabetics have normocytic normochromic anaemia to microcytic hypochromic anaemia consistent with anaemia of chronic disease seen in chronic conditions like DM*. The lower RBC indices seen in our study as compared to the West could be possibly due to coexistent iron deficiency anaemia, malnutrition which is in high prevalence in our population as well because of anaemia of chronic disease associated with DM, and several other comorbidities.

TLC among the diabetic cases was found to be on the higher side and even higher in complicated diabetes cases. 9 diabetics showed a higher TLC($> 11 \times 10^9/L$). Our findings are consistent with other studies which suggest *in diabetes there is an activation of the immune system and inflammation which leads to higher TLC.* (16)

There was *no statistically significant difference observed in any of the haematological parameters on comparing cases of uncomplicated DM and cases of DM with complications.* This could be due to the lesser number of complicated diabetic cases in our study and lesser follow-up time. Thus, findings of our study suggest that only CBC cannot be used for distinguishing complicated diabetic cases from uncomplicated diabetes cases.

There are several markers for determining the platelets on flow cytometry, but there is no precise marker for PMPs. Annexin V, which is a marker of apoptosis is usually used in conjugation with other lineage markers of platelets like CD41, CD 61, and CD 42 to determine the presence of PMPs in a blood sample. (17).

In our study, *Annexin V+* particles and the percentage of Annexin V+ particles in both diabetic cases and controls was also noted and analyzed using the flowcytometry plots. Annexin V + are considered being markers for apoptosis and they are widely used to identify microparticles. Annexin V+ microparticles are released during stress or apoptosis of cell in deranged environment and it has been found that Annexin V + microparticles are raised in chronic conditions like DM, coronary artery disease. (18, 19)

In this study, there was a statistically significant difference in the absolute value and percentage of Annexin V+ particles between both groups. This is in contrast to study done by Biilgir et al who assessed the serum annexin V levels between healthy adults and diabetics. (20)As the results of Annexin V+ particles in different studies is controversial, further studies

are needed to establish the levels of Annexin V+ particles and their role in various chronic diseases like DM.

Similarly, the absolute value of PMPs and percentage of PMPs in both diabetic cases and controls showed significant statistical difference. There is no established reference range for PMPs but *the levels of PMPs were found to be much higher in the diabetic cases*. This is consistent with various other previous studies which showed that diabetics have a higher range of PMPs as compared to normal controls. (7, 9, 21) DM also being a prothrombotic and proinflammatory state leads to increased release of PMPs(6). Hyperglycemia seen in DM activates platelets and is a potent stimulator of microparticles (PMPs) formation. The hyperactive platelets in DM lead to increased PMP release which may lead to capillary microembolization by the formation of microaggregates seen in diabetic complications. The platelets in DM also develop various functional and metabolic changes leading to an increase in the release of PMPs. PMPs can thus initiate endothelial injury, vascular abnormality, and display procoagulant and proinflammatory activity. (6) These changes lead to various vascular and microvascular complications which can be detected early by assessing the PMPs prior to their clinical presentation. It was found by Nomura et al that the number of PMPs was significantly raised in diabetes and also in diabetics with low-density lipoproteins suggesting that PMP levels also can contribute to the progression of atherosclerosis in DM. Patients with Chronic kidney disease exhibited significant platelet activation and endothelial dysfunction (early signal for renal function deterioration), leading to the release of more PMPs.(6, 7) Many investigators found that PMPs might be associated with hyperglycaemia-induced organ damage. Study done by Zhang et al observed that diabetic subjects. Similar to previous studies, in our study also it was found that both absolute number as well as PMPs % were increased in patients with DM than in normal controls.(25,26,27,28) Similar results were seen on comparing the absolute value of PMPs

among uncomplicated diabetes and complicated diabetes.(24,25,26) This implies that PMPs can be helpful to identify diabetics with complications.

Despite the increased association between PMPs in Diabetes and its complications in many studies, there is no standardized protocol for identifying PMPs and no known specific antibodies known for detection for PMPs. Also, there are very few studies that elaborate on the potential role of PMPs in Diabetic complications This leads to an inter-study variation of results and inaccurate analysis. Thus, further studies are needed to devise a standardized protocol for determining the range of PMPs in DM for accurate analysis. Due to the substantial burden of the DM and associated complications, there is a need to explore the possibility of utilizing PMPs as biomarkers that will help in predicting the onset and progression of diabetes. Also, they can help assess the vascular events and early assessment and management of various complications related to Diabetes.

Conclusion : The present study demonstrated that PMPs and Annexin V levels are increased in diabetics as compared to healthy controls. Further, PMPs are also significantly increased in diabetics with complications and can be further explored for their discriminating power in picking up the complications related to DM. On the other hand in our study, CBC did not have a discriminating role in differentiating healthy populations from diabetic subjects. This could be due to lesser subjects in our study and lesser follow-up time. Hence, these results need to be validated by a larger sample size. Further, the exact role of PMPs in differentiating DM from DM with complications should be explored further to establish its role as a biomarker in picking up complications.

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