

ORIGINAL ARTICLE

Plasma-Derived Microparticles in Polycythaemia Vera

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Abstract

Introduction: Microparticles are membrane bound vesicles, measuring less than 1.0 μm , which are released during cellular activation or during apoptosis. Studies have shown that these circulating microparticles play a role in coagulation, cell signaling and cellular interactions. Increased levels of circulating microparticles have been observed in a number of conditions where there is vascular dysfunction, thrombosis and inflammation. The objective of this study was to determine the various plasma-derived microparticles in patients with polycythaemia vera (PV) in Universiti Kebangsaan Malaysia Medical Centre and to compare them with normal control. **Methods:** A total of 15 patients with PV and 15 healthy volunteers were included in this cross-sectional descriptive study. Plasma samples from both patients and healthy volunteers were prepared and further processed for isolation of microparticles. Flow cytometry analyses were then carried out in all samples to determine the cellular origin of the microparticles. Full blood count parameters for both groups were also collected. Data collected were analyzed using SPSS version 12.0. **Results:** Patients with PV had a significantly higher percentage of platelet derived microparticles compared to healthy controls ($P < 0.05$). The control group had a higher level of endothelial derived microparticles but the differences were not statistically significant ($P > 0.05$). **Conclusion:** The median percentage of positive events for platelet derived microparticles was higher in patients with PV compared to normal healthy controls.

Keywords: Microparticles, polycythaemia vera, haemostasis, flowcytometry

INTRODUCTION

Microparticles were first described by Wolf in 1967 as “platelet dust”, composed of vesicles measuring less than 0.1 micrometer (μm) in diameter. Two main cellular processes implicated in the formation of microparticles are cellular activation and apoptosis by chemical or physical stress. Both of these processes lead to cytoskeletal reorganisation, membrane blebbing and generation of microparticles.¹⁻³ The majority of *in vivo* microparticles are platelet microparticles. Microparticles derived from erythrocytes, granulocytes, monocytes, lymphocytes and endothelial cells are present in lower quantities compared to those derived from platelets.⁴ Previous studies have demonstrated that differences exist in these subpopulations of microparticles detected in healthy individuals as compared to those with various diseases. Microparticles are mainly composed of lipids

and proteins; their composition depends on the cellular origin as well as the processes which led to their formation.¹ In the resting state, various phospholipid components are distributed asymmetrically within the bilayer of phospholipid which surrounds the microparticles. The disruption of this asymmetrical balance subsequently results in the exposure of negatively charged phospholipids such as phosphatidylserine and phosphatidylethanolamine on the surface of microparticles. This results in a prothrombotic state as phosphatidylserine efficiently binds coagulation factors and promote the formation of activity of tenase and prothrombinase complexes.^{5,6} Microparticles formed during cell activation or apoptosis have surfaces which are rich in negatively charged phospholipids that can promote procoagulant activity. Phospholipids on the surface of microparticles derived from platelets and endothelial cells provide binding

sites for coagulation factors Va, VIII, IXa and IIa.⁵⁻⁹

Another factor which contributes to the procoagulant feature of microparticles is the expression of tissue factor (TF), which is the primary initiator of *in vivo* coagulation. TF is expressed by microparticles derived from leukocytes, platelets and endothelial cells.⁸ Monocyte derived microparticles can bind to activated platelets via a mechanism involving P-selectin glycoprotein ligand-1 (PSGL-1) found on microparticles and P-selectin, found on platelets. As such, the binding of monocyte microparticles to activated platelets and endothelial cells within a developing thrombus will further promote TF accumulation and localised thrombin generation. Thus, circulating microparticles can serve as potential biomarkers for individuals with increased risk of thrombosis.⁷⁻⁹

A study by Villmow *et al.* (2002) reported an increase level of platelet derived microparticles in patients with polycythaemia vera (PV) and essential thrombocythaemia (ET). They also observed an increase in platelet activation markers in both groups.¹⁰ The increase in platelet microparticles in these patients could explain partly the increase risk of thromboembolic events in both ET and PV as these platelet microparticles promote thrombin generation. Generally, there are two main mechanisms of hypercoagulable state associated with PV. The first mechanism involves the abnormal components of the haematopoietic system (i.e. platelets, erythrocytes and leucocytes), derived from the clonal proliferation of the haematopoietic progenitor cells which have a prothrombotic phenotype. The second mechanism is due to the inflammatory response from the host towards cytokines and the inflammatory mediators released by malignant cells.^{11,12} It is interesting to note that inflammation and thrombosis are linked by common activation pathways and feedback regulation system, thus establishing a relationship between these two processes.

In this study, we determined the level of various tissue derived microparticles in patients with PV in Universiti Kebangsaan Malaysia Medical Centre (UKMMC), using flow cytometry and we also compared them to the normal control.

MATERIALS AND METHODS

Study setting

A cross sectional descriptive study among

patients with PV in UKMMC was carried out from 28th March 2012 to 31st December 2013. A total of fifteen patients with PV (12 females and 3 males) and fifteen healthy volunteers (5 females and 10 males) were included in this study after given informed consent. This study was approved by the Institutional Review Board and Ethics Committee of Universiti Kebangsaan Malaysia (UKM 1.5.3.5/244/SPP3).

Blood collection and isolation of microparticles

Eight mls of peripheral blood samples were obtained with a 21-gauge needle and after discarding the first three mls, the samples were transferred into a 5ml tube containing 3.2% citrate (BD, Plymouth UK). Plasma was prepared within one hour after blood collection by centrifugation for 20 minutes at 1550 x g at room temperature. Aliquots of plasma were then stored at -75 °C until use. For isolation of microparticles, 250 µL of plasma was thawed in a water bath (temperature of 37 °C), and then centrifuged for 30 minutes at 17,500 x g at 20 °C. Subsequently, 225 µL of supernatant (microparticle free plasma) was removed. The remaining 25 µL containing microparticle pellet was resuspended in 225 uL of phosphate buffered saline (PBS). Samples were then centrifuged for 30 minutes at 17,500 x g at 20 °C; thereafter 225 µL of supernatant was removed and the microparticle pellet was resuspended in 125 µL of PBS.

Selection of surface markers for microparticle subgroups

Annexin-V-Allophycocyanin (APC) was used as a general marker for microparticles. Anti-CD61 FITC (fluorescein isothiocyanate) was used for the identification of platelet derived microparticles, whereas anti-CD144 PE (phycoerythrin) was used for identifying endothelial derived microparticles. 5 µL of the microparticle samples were diluted with 35 uL PBS containing 2.5 mM CaCl₂ (pH 7.4). The samples were then incubated for 30 minutes at room temperature in the dark with 5 µL annexin-V-APC and 5 µL of each of the monoclonal antibodies. After incubation, 1000 µL of PBS containing 2.5 mM CaCl₂ (pH 7.4) were added prior to flow cytometric analysis. All antibodies were purchased from BD Biosciences and flow cytometric analysis was performed using a Becton Dickinson FACS CALIBUR flow cytometry.

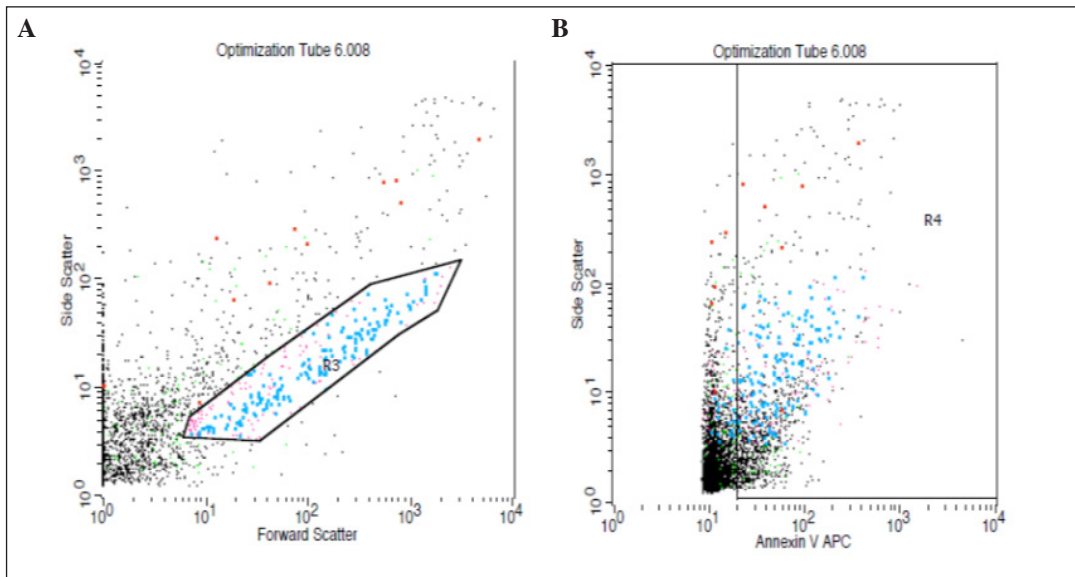


FIG. 1: Flow cytometric microparticle analysis in a patient with PV. Forward Scatter (FSc) and Side Scatter (SSc) were set in logarithmic mode and were used to illustrate the microparticle population. Plot A showed the gated microparticle population (R3). Events defined as microparticles were selected for their annexin-V binding, determined by positivity for annexin V- allophycocyanin (APC) fluorescence on the x-axis (Plot B).

Detection and Acquisition of Microparticles using Flow Cytometry

To identify annexin-V-positive events, a threshold was set in a microparticle sample prepared without calcium. Events defined as

microparticles were then selected for their annexin-V binding, determined by positivity for annexin-V-APC fluorescence on the x-axis (Fig. 1 and Fig. 2). Samples were acquired on a medium flow rate and a total of 10,000 events

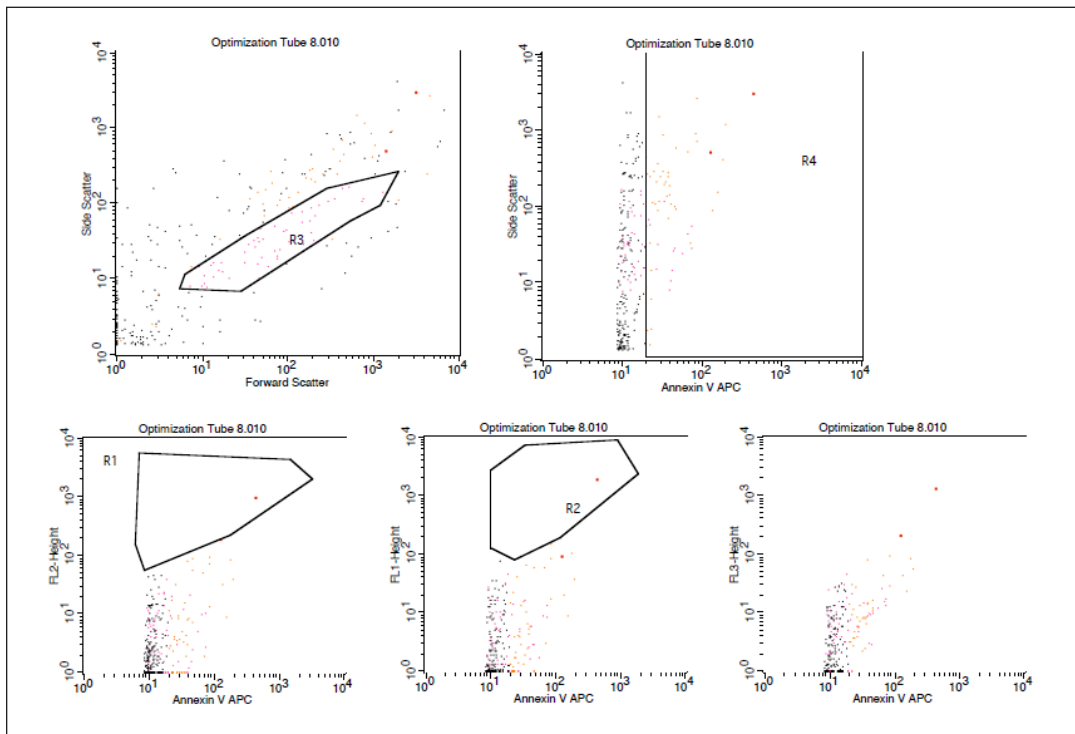


FIG. 2: Flow cytometric analysis of an unstained sample in the absence of calcium buffer. In the absence of calcium, there will be no annexin-V binding to the microparticles.

TABLE 1: Comparison of age, white cell count (WCC), haemoglobin (Hb), haematocrit (HCT), red cell count (RCC) and platelet (PLT) levels for patient group and control group

| | Group | N | Median | IQR (25-75) | Mean Rank | Mann-Whitney U | *P value |
|---------------------------|---------|----|--------|-------------|-----------|----------------|----------|
| Age | Control | 15 | 32 | 30-35 | 8.27 | 124 | 0.001 |
| | Case | 15 | 60 | 48-66 | 22.73 | | |
| WCC (x10 ⁹ /L) | Control | 15 | 7.1 | 5.9-8.3 | 10.23 | 33.50 | 0.001 |
| | Case | 15 | 9.9 | 7.9-14.6 | 20.77 | | |
| Hb (g/dl) | Control | 15 | 13 | 11.8-13.1 | 11.00 | 45.00 | 0.01 |
| | Case | 15 | 14.8 | 13.5-15.7 | 20.00 | | |
| HCT (%) | Control | 15 | 39.5 | 36.6-41.1 | 12.17 | 62.50 | 0.04 |
| | Case | 15 | 45.7 | 38.8-47.4 | 18.83 | | |
| RCC (x10/L) | Control | 15 | 4.55 | 4.38-4.88 | 12.37 | 65.50 | 0.05 |
| | Case | 15 | 5.4 | 4.3-6.98 | 18.63 | | |
| PLT (x10/L) | Control | 15 | 260 | 246-301 | 12.20 | 63.00 | 0.04 |
| | Case | 15 | 402 | 256-655 | 18.80 | | |

* P<0.05 is considered as significance

were acquired for each sample. Flow cytometric analyses were carried out on Cellquest Software.

RESULTS

Sample population

A total of fifteen patients with PV (12 females and 3 males) and fifteen healthy controls (5 females and 10 males) participated in this study. The median age for PV patients was 60 years old as compared to the healthy controls, which was 32 years old.

Haemoglobin (Hb), haematocrit (HCT), red cell count (RCC), white cell count (WCC) and platelet (PLT) counts for PV patients and controls.

The median haemoglobin, red cell count, white cell count and platelet levels were significantly higher in PV patients than in the controls ($p < 0.05$). Table 1 below showed the median

comparison for the parameters mentioned above.

Total percentage of the subpopulations of microparticles

The median percentage of positive events for platelet derived microparticles was significantly higher in PV patients than in controls [1.3% (0.82-9.69) versus 0.65% (0.4-0.93); $p=0.01$]. However, for endothelial derived microparticles, the control group had a higher median percentage of positive events than PV patients (3.15% versus 1.97%), but the differences observed were not statistically significant ($p=0.43$). Table 2 which showed the median levels for these results. No linear correlation was observed between the platelet count and the percentage of positive events for platelet derived microparticles in both groups [(patients' group, $R=-0.29, p>0.05$), (control group $R=0.15, p>0.05$)(Fig. 3).

TABLE 2: Comparison of median values for percentage of endothelial (Endo) and platelet (PLT) derived microparticles (MPs) in patients with Polycythaemia Vera and in control group.

| | Group | N | Median | IQR (25-75) | Mean Rank | Sum of Ranks | Mann-Whitney U | *P value |
|----------------------|---------|----|--------|-------------|-----------|--------------|----------------|----------|
| PLT derived MPs (%) | Control | 15 | 0.65 | 0.4-0.93 | 11.43 | 171.50 | 51.50 | 0.01 |
| | Case | 15 | 1.3 | 0.82-9.69 | 19.57 | 293.50 | | |
| Endo derived MPs (%) | Control | 15 | 3.15 | 1.51-4.2 | 16.77 | 251.50 | 93.50 | 0.43 |
| | Case | 15 | 1.97 | 1.23-3.28 | 14.23 | 213.50 | | |

* P<0.05 is considered as significance

DISCUSSION

The clinical course of PV is often complicated by thrombotic events, which could be the first presentation of the disorder itself. In PV, thrombosis can be of arterial, venous or microcirculatory in origin. Leukocytosis, advanced age, previous history of thrombosis and V617F mutation are risk factors for thrombosis in PV patients. The neutrophils and platelets in PV patients circulate in an activated state and activated cells could lead to membrane vesiculation and microparticle release. As these microparticles express phosphatidylserine, they are a potential prothrombotic marker in this disease.

In this study, the median percentage of positive events for platelet derived microparticles was significantly higher in PV patients than in controls (1.3% [0.82-9.69] versus 0.65% [0.4-0.93]). The platelet derived microparticles were defined as events which were positive for both Annexin-V-APC and CD61 FITC. This result was

consistent with findings by Trappenburg *et al.* (2009) who were the first group that conducted an extensive analysis of microparticles in patients with myeloproliferative neoplasm.⁴ They found that patients with essential thrombocythaemia (ET) had a significantly higher number of platelet as well as endothelial derived microparticles. Similarly, Villmow *et al.* (2002) also found a significantly higher level of platelet microparticles in patients with PV.¹⁰ They also observed that patients with myeloproliferative neoplasm with a previous history of thromboembolism had a higher level of microparticles than patients with myeloproliferative neoplasm without a prior history of thromboembolism.

Increased number of platelet microparticles have also been observed in a large number of thrombotic disorders, such as acute coronary syndromes, diabetic patients with atherothrombotic diseases and peripheral arterial disease.¹³ This could be attributed to the highly procoagulant surface of platelet microparticles

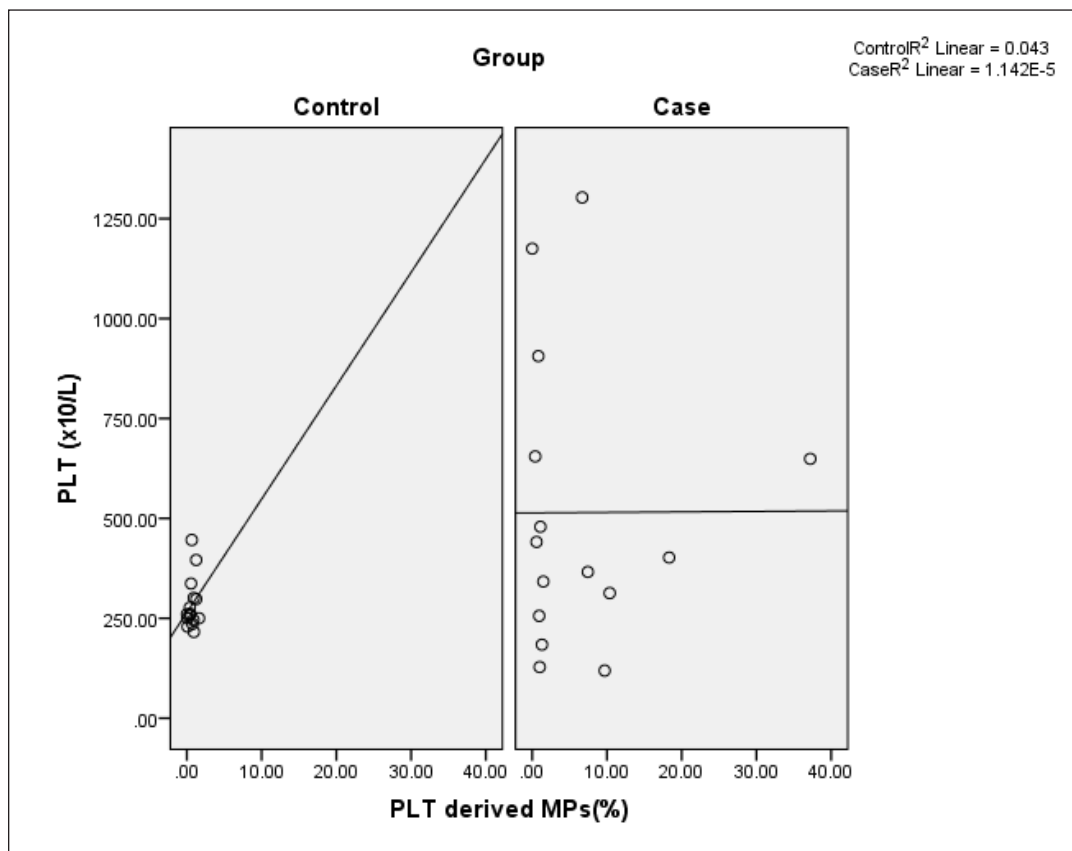


FIG. 3: No correlation was found between percentage of positive events for platelet derived microparticles and the platelet count in both patients with polycythaemia vera and the control group. R (patients' group) = - 0.29 (P>0.05), R (control group) = 0.15 (P>0.05).

as it may be fifty to one hundredfold more procoagulant than the surface of activated platelets per se.⁹ Platelet microparticles will bind to subendothelial matrix, thus directly promoting fibrin formation and ultimately leading to thrombosis. Furthermore, platelet microparticles also participate in the regulation of leucocyte interactions and amplify leucocyte mediated tissue injury in thrombotic and inflammatory disorders.^{8,9}

No correlation was observed between the platelet count and the percentage of positive events for platelet derived microparticles in both groups. This could be due to the small sample size. More samples would need to be analysed in the future for a better correlation study between these two parameters. Similarly, Trappenburg *et al.* (2009) also did not find a correlation between platelet count with the microparticle level in ET patients as well as in the healthy controls. This finding has led the authors to suggest that microparticle formation was a regulated process and not constitutive.⁴

Apart from being involved in coagulation, platelet derived microparticles have also been reported to play a part in angiogenesis and wound healing. They serve as delivery carriers of growth factors to damaged cells and tissues. In one study, platelet microparticles are found to enhance the proliferation as well as migration of stem cells *in vitro* and affect the ability to deposit mineralised matrix, thus potentially playing a part in tissue regeneration.¹⁴

Microparticles have also been linked to cancer-induced thrombosis. The release of microparticles expressing tissue factor from both cancer cells and host cells may result in a hypercoagulable state. In terms of tumour progression, platelet microparticles can induce angiogenesis and affect both the tumour microenvironment as well as cancer cell interactions. As microparticles also contain proteins, lipids and nucleic acid, circulating microparticles may serve as reservoir of prognostic and predictive biomarkers to monitor genetic tumour progression, angiogenesis, thrombosis and responses to targeted therapies.^{8,14}

The endothelium derived microparticles in PV patients were lower than the controls, but the differences were not statistically significant. In healthy individuals, platelet microparticles account for 70-90% of circulating microparticles, with the remainder being leucocyte and endothelial derived microparticles.⁴ However, in another study, endothelial derived microparticles

accounted for 43.8% of the total circulating microparticles.¹⁵ The low levels observed in this study could be due to variations in the method of sample preparation and sampling.^{16,17} Medications could possibly affect the levels of circulating endothelial derived microparticles in the plasma, as they may affect the cellular vesiculation and microparticle release. Hypertensive and type 2 diabetes mellitus patients who were treated with nifedipine and benidipine showed a reduction in the level of circulating endothelial derived microparticles. Eicosapentaenoic acid also showed a significant reduction in the concentrations of plasma endothelial derived microparticles in diabetics.¹⁸ It would be worthwhile to identify the patients' medications in the future to study the effects of drugs on circulating microparticles.

Activated endothelial cells will release microparticles, which can lead to endothelial dysfunction. These microparticles can bind to platelets and promote platelet aggregation in patients with coronary atherosclerosis. Patients with venous thromboembolism have increased levels of endothelial microparticles, endothelial microparticles-monocytes conjugates and E-selectin, thus indicating that the release of endothelial microparticles and its subsequent binding to monocytes are key events in thrombogenesis.⁸ Correlation between endothelial microparticle levels and other markers of endothelial dysfunction, such as thrombomodulin and soluble intercellular adhesion molecule (sICAM) have been reported by previous studies. They discovered that the level of both endothelial derived microparticles and sICAM were elevated in patients with diabetes mellitus.⁷ *In vitro* studies have also shown that endothelial microparticles express ultra large von Willebrand factor. As such, they are able to promote as well as stabilize platelet aggregates.^{8,13}

The median age for our PV patients in this study was almost similar to the essential thrombocythaemia patients in a study by Trappenburg *et al.* (2009), which was 58 years old, but the median age for their healthy controls were 46 years old, as compared to ours, which was 32 years old. The number of circulating endothelium-, platelet-, and monocyte-derived microparticles are reported to be increased in low-oestrogen menopausal women.¹⁹ In another study, the level of endothelial microparticles was lower in patients who were above the age of 80 years old, both in males and females, but

the procoagulant activity was preserved.²⁰ The authors compared the procoagulant activity of microparticles in both the elderly and young patients, under physiological as well as pathological conditions. They discovered that the number of endothelial microparticles were lower in the elderly patients, but the procoagulant activity was also preserved as compared to the younger patients. This was attributed to the fact that basal endothelial microparticle level was determined by the balance between endothelium repair and stress. Thus, the age associated reduction in endothelial microparticles could be attributed to the slowing of the turnover process and an indicator of a senescent endothelium and a lower cell metabolism activity.²⁰

Our study had some limitations. Firstly, our sample numbers are small in this study. Secondly, there is still no general consensus on the protocol for microparticle detection by flowcytometry. Pre-analytical variables such as blood collection, centrifugation speed, storage and freezing can significantly affect the number of microparticles detected. Short periods of ultracentrifugation can remove up to 80% of the total circulating microparticles.¹⁷ Ayers et al (2011) reported that the number of microparticles recovered from plasma inversely correlated with the centrifugation speed.¹⁷ Another potential source of variability is whether the plasma samples were analyzed fresh or upon storage. But if the analysis is limited to only using fresh samples, this will prevent collaborative studies among different laboratories and will be labor intensive. Snap freezing and storing the plasma at -70°C for two weeks could potentially increase the number of circulating microparticles by 90% as compared to analyzing the microparticles from fresh samples. This is attributed to further microparticle production by any residual platelets in the plasma or further breakdown of larger platelet microparticles into smaller platelet microparticles.²¹ These illustrate the need to standardize the flow cytometry protocol used for the detection as well as analysis of microparticles in order to allow for direct comparison of results between different studies.

In conclusion, we demonstrated that the median percentage of positive events for platelet derived microparticles was higher in PV patients. However, due to the small sample size, this result needs to be cautiously interpreted. More extensive studies should be carried out in the future as microparticles are involved not only in coagulation but also in the cross-talk between

the cellular elements of the coagulative and inflammatory systems, through the transfer of signaling molecules and receptors of their cell of origin to other cell types. Most importantly, circulating microparticle may serve as potential biomarkers for disease status or a possible therapeutic target.

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