High Level of Circulating Microparticles in Patients with *BCR/ABL* Negative Myeloproliferative Neoplasm – a Pilot Study

Vysoká hladina cirkulujících mikropartikulí u pacientů s *BCR/ABL* negativními myeloproliferativními chorobami – pilotní studie

Aswad M. H.[1,2], Kissova J.[1,2], Rihova L.[1,2], Zavrelova J.[1,2], Ovesna P.[3], Penka M.[1,2]

[1] Department of Clinical Hematology, Faculty of Medicine, University Hospital Brno
[2] Faculty of Medicine, Masaryk University
[3] Institute of Biostatistics and Analyses, Faculty of Medicine, Masaryk University, Brno

Summary

Background: Microparticles (MPs) are small (0.1–1 μm) cell-derived vesicles released during activation or apoptosis, with a surface-exposed phosphatidylserine along with antigens indicating the cellular origin. The level of MPs is known to be elevated in thromboembolic diseases and malignancies; it is believed that MPs are not only amplifying but can also initiate the thrombogenesis processes. *BCR/ABL* negative myeloproliferative neoplasms (MPNs) are clonal haematopoietic diseases, which include polycythemia vera, essential thrombocythemia and primary myelofibrosis. One of the main problems of MPN patients is high risk and incidence of thrombosis which affect the survival, quality of life and life expectancy. ***Patients and methods:*** The clinical significance of circulating MPs was assessed in a group of 179 patients with *BCR/ABL*-negative MPNs. Analysis of MPs was done using flow cytometry on 417 samples, and MPs procoagulation activity was performed using a functional assay called Zymuphen MP-activity (Hyphen Biomed, Neuville-sur-oise, France) on 274 samples. ***Results:*** Significantly higher absolute and relative count of platelet MPs was found in MPN patients when compared with healthy group, respectively ($p = 0.001$, $p = 0.043$). Erythrocyte MPs were also significantly higher in MPN patients than in the healthy group ($p < 0.001$). Procoagulation activity of MPs was as well significantly higher in patients compared to the control group ($p < 0.001$). Patients with primary myelofibrosis had decreased absolute and relative count of platelet MPs compared to polycythemia vera and essential thrombocythemia patients, respectively ($p = 0.008$, $p = 0.014$). Presence of *JAK2V617F* mutation was associated with higher absolute and relative count of platelet MPs, respectively ($p = 0.045$, $p = 0.029$). ***Conclusion:*** Although some literature data support the hypothesis of a direct relation between MPs and thrombotic events in MPN patients, further studies are needed to evaluate the clinical implication of MPs in the hypercoagulation state of MPN patients.

Key words

microparticles – myeloproliferative disorders – procoagulation activity – thrombosis – Janus Kinase 2

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Mohamed Hussam Aswad, M.D.
Department of Clinical Hematology
Faculty of Medicine
University Hospital Brno
Jihlavska 20
625 00 Brno
e-mail: dr.hussam89@gmail.com

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Souhrn

Východiska: Mikropartikule (MP) jsou malé vezikuly (0,1–1 μm) uvolněné v průběhu aktivace nebo apoptózy, na jejichž povrchu je exprimován fosfatidylserin spolu s antigeny indikujícími jejich buněčný původ. Je známo, že hladina MP je zvýšena u tromboembolických onemocnění a malignit; předpokládá se, že MP nejen zesilují, ale mohou také iniciovat proces trombogeneze. *BCR/ABL* negativní myeloproliferativní choroby (myeloproliferative neoplasms – MPN) jsou klonální onemocnění krvetvorby, která zahrnují pravou polycytemii, esenciální trombocytemii a primární myelofibrózu. Jednou z hlavních komplikací u pacientů s MPN je vysoké riziko a incidence trombózy, které ovlivňují přežití, kvalitu i délku života. ***Soubor pacientů a metody:*** Klinický význam cirkulujících MP byl hodnocen u skupiny 179 pacientů s *BCR/ABL* negativními MPN. Analýza MP byla provedena použitím průtokové cytometrie u 417 vzorků a prokoagulační aktivita MP byla provedena pomocí funkčního testu Zymuphen MP-activity (Hyphen Biomed, Neuville-sur-Oise, Francie) u 274 vzorků. ***Výsledky:*** Významně vyšší absolutní a relativní počet destičkových MP byl nalezen u pacientů s MPN ve srovnání s vyšetřením zdravých dárců (p = 0,001; p = 0,043). Erytrocytární MP byly také významně vyšší u pacientů s MPN než u zdravých jedinců (p < 0,001). Prokoagulační aktivita MP byla rovněž významně vyšší u pacientů než ve skupině zdravých dárců (p < 0,001). Pacienti s primární myelofibrózou měli významně nižší absolutní a relativní počet destičkových MP ve srovnání s pacienty s pravou polycytemií a esenciální trombocytemií (p = 0,008; p = 0,014). Přítomnost mutace *JAK2V617F* byla spojena s vyšším absolutním a relativním počtem destičkových MP (p = 0,045; p = 0,029). ***Závěr:*** Ačkoliv některá literární data podporují hypotézu o přímém vztahu mezi MP a trombotickými událostmi u pacientů s MPN, pro vyhodnocení klinického významu MP ve vztahu k hyperkoagulačnímu stavu těchto pacientů jsou nezbytné další klinické studie.

Klíčová slova

mikropartikule – myeloproliferativní choroby – prokoagulační aktivita – trombóza – Janus Kinase 2

Background

Microparticles (MPs), which were called "platelet dust" when originally discovered by Peter Wolf in 1967 [1], could be found in both serum and plasma [2]. Generally, MPs are produced from all cell types during activation or apoptosis and this applies in healthy individuals as well as patients [3]. Although the majority of MPs are platelets-derived, other cell types produce MPs such as erythrocytes, leukocytes, endothelial cells and tumour cells [4–6].

A standard definition of MPs was set in the International Society on Thrombosis and Haemostasis 51st annual meeting in 2005 when vascular biology subcommittee defined MPs as 0.1–1 micrometer cell-derived vesicles, with neither nucleus nor synthetic capacity, containing a membrane skeleton, varying amounts of surface-exposed phosphatidylserine along with antigens from the cells of origin [7]. The expression of the tissue factor and phosphatidylserine on MPs surface play a critical role in starting and amplifying of thrombosis due to their procoagulant properties. In addition, MPs are involved in the transport and transfer of bioactive molecules, cell activation and inflammation processes [8–10].

BCR/ABL negative myeloproliferative neoplasms (MPNs) are chronic haematological diseases characterised by proliferation of myeloid cells. The classical *BCR/ABL* negative MPNs include essential thrombocythemia (ET), polycythemia vera (PV) and primary myelofibrosis (PMF). The clinical course of *BCR/ABL* negative MPNs is frequently complicated by thrombotic events, which can initially be manifestation of the disease and may occur at venous, arterial or microcirculatory sites. Thrombosis represents a common complication in MP and significantly contributes to morbidity and mortality. The prevalence of thrombotic events ranges 10–29% in ET and 34–39% in PV patients [11]. In patients with myelofibrosis the rate of thrombotic events is 2.2% per patient/year, which is close to the rate observed in ET [12]. Approximately one third of patients with PV, up to 29% of patients with ET and 13% of patients with PMF have thrombotic events prior to or at diagnosis of MPN, and the cumulative rate of thrombosis after an MPN diagnosis is estimated to be approximately 3% per patient/year in PV and ET and around 2% per patient/year in PMF [13].

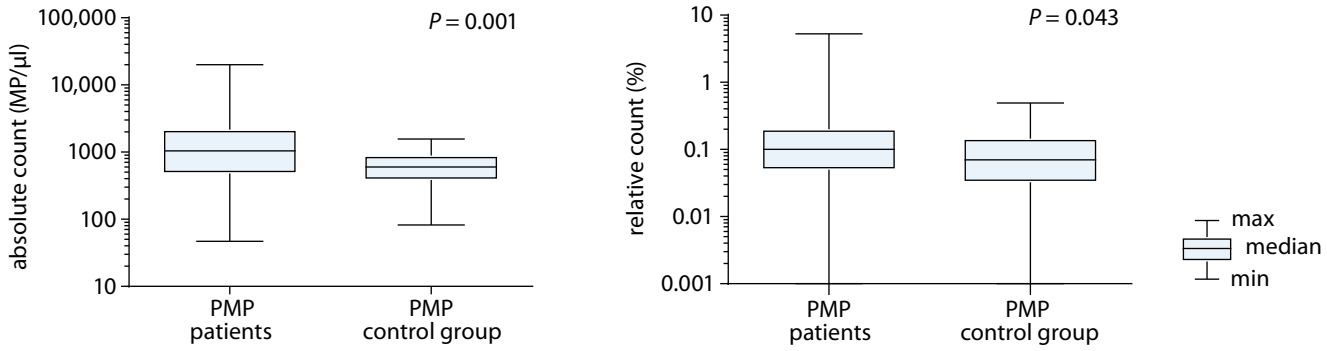
Well-described risk factors for thrombotic complications include older age and history of prior thrombosis. Cardiovascular risk factors, such as smoking, hypertension and diabetes have been associated with higher risk of cerebral or cardiac events in MPN and are included in the International Prognostic Score of Thrombosis for ET (IPSET-thrombosis) [14]. Patients with both MPN and underlying thrombophilia have a higher inclination to thrombosis. *JAK2V617F* mutation has been found to carry an increased risk of thrombotic complications, whereas *CALR* has a lower risk than *JAK2V617F* mutation. Studies have also focused on evaluation of the risk of thrombosis due to higher cell counts. Several studies have shown an association with leukocytosis or thrombocytosis and higher risk of thrombotic complications [15,16], *JAK2V617F* mutation status also seems to impact the risk of thrombosis [17,18]. Management of MPN focuses on the prediction risk of thrombosis and initiating prophylaxis to prevent complications in patients at high risk of thrombosis. Based on these facts the global interest is shifted nowadays towards identifying and proving of an effective treatment as well as identifying prognostic factors in order to minimise the risk and the complications [16,19].

The aim of this study is to evaluate the level of circulating MPs and their procoagulant activity in connection with the occurrence of thrombotic complications in patients with *BCR/ABL* negative MPNs.

Patients and methods

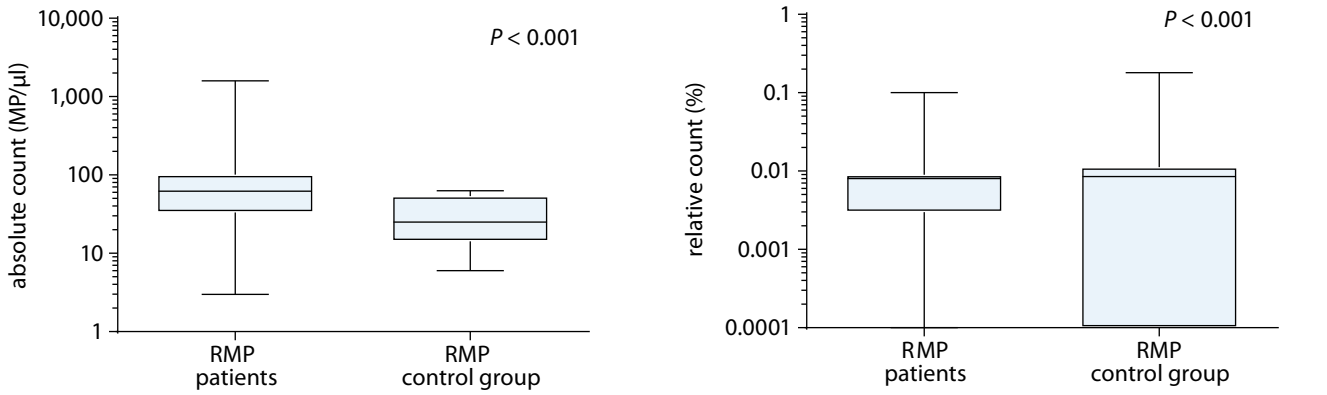
Patients

In this study we analysed the clinical and laboratory data from 179 patients who were diagnosed for *BCR/ABL* negative MPNs in the Department of Clinical Hematology University Hospital Brno. The study was approved by the local Ethical Committee. Written informed consents



MP – microparticles, PMP – platelet microparticles

Graph 1. Comparison of absolute and relative count of PMPs between patients with myeloproliferative neoplasms and healthy individuals (control group).



MP – microparticles, RPM – erythrocyte microparticles

Graph 2. Comparison of absolute and relative count of RMPs between myeloproliferative neoplasms patients and control group.

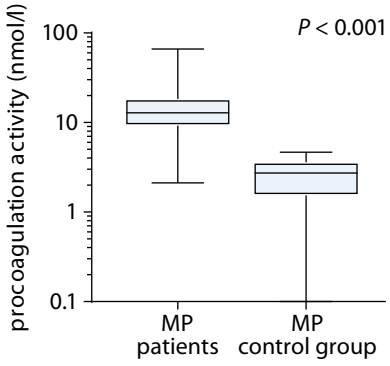
were obtained from all patients. Patients were diagnosed according to the criteria of the World Health Organization (WHO), only 5% according to Polycythemia Vera Study Group criteria.

The cohort of 179 patients with MPN (77 males, 102 females, median age at diagnosis 56 years) includes 99 patients with ET (55%), 51 patients with PV (29%) and 29 patients with PMF (16%). In this cohort the level of platelet and erythrocyte MPs was assessed using flow cytometry (FCM) and the procoagulant activity of MPs measured by using functional assay. A total of 142 patients were positive for *JAK2V617F* mutation (79.3%), 25 patients for *CALR* mutation (14%), 3 patients for *MPL* mutation (1.7%), and 9 patients were triple-negative (5%).

A total of 61 patients of our cohort had a history of thrombosis (34.1%); 29 patients (16.2%) had arterial thrombosis events, 19 patients (10.6%) had venous thrombosis events, 7 patients had microvascular thrombosis events (3.9%), 4 patients had both venous and arterial thrombosis events (2.2%) and 2 patients had arterial and microvascular thrombosis events (1.1%).

Pre-analytical conditions of MPs examination

After using a light tourniquet, blood samples were collected from patients' antecubital vein through a 21-gauge needle to reduce blood-cell activation or damage which affect the MPs level. The tubes used for collecting blood contains sodium citrate as anticoagulant and K3-ethylenediaminetetraacetic acid for molecular studies and blood count evaluation. The first few millilitres were discarded. Plasma was prepared within approximately 120 min after blood collection. Centrifugation process was used for the purpose of MPs examination to remove platelets and other large blood components from the plasma in order to avoid cellular activation leading to unintended production of MPs.



MP – microparticles

Graph 3. Comparison of procoagulation activity of MPs between myeloproliferative neoplasms patients and control group.

For FCM this was done by using only one phase-centrifugation process at 2,500 g for 15 min at room temperature to avoid using breaks when reproducing platelet-poor plasma which may affect the credibility of the results. Then 200 mL of the plasma supernatant was carefully moved into a polypropylene haemolysis tube with a micropipette to avoid disturbing or mixing the cellular layer with the plasma. The plasma supernatant was then immediately further processed for FCM examination.

For procoagulation activity of MPs, the supernatant of the platelet-poor plasma which was produced by centrifugation at 1,500 g for 15 min, was centrifuged again for 2 min at 13,000 g at room temperature, then plasma samples were stored frozen at –80 °C. On the day of analysis, an aliquot of plasma was thawed for 15 min at 37 °C before use. Thawed specimens were tested within 4 h stored at room temperature. A complete blood count was ordered for all patients at the time of MPs examination.

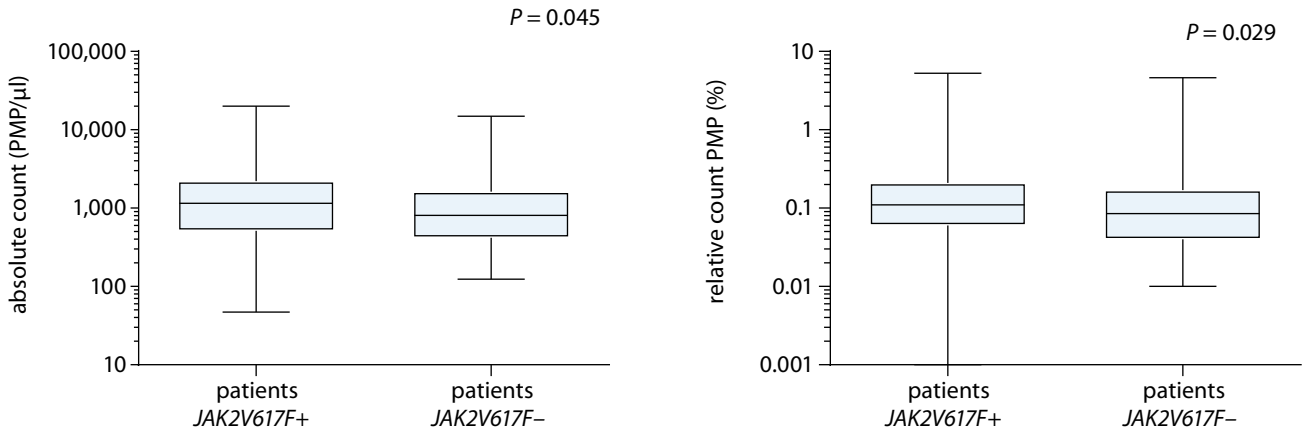
Analytical conditions for MPs examination

FCM assay was performed by using FC500 Cytomics (Beckman Coulter, Hialeah, FL, USA). After incubation of platelet-poor plasma with CD41-PC7 (Beckman Coulter), CD235a-PE and annexin V-FITC (both EXBIO) in Ca++ buffer, calibrated beads FlowCount (Beckman Coulter) were added. Sample was immediately analysed on protocol adjusted to the MPs size with Megamix beads (Biocytex). Platelet MPs (PMPs) were identified as positive annexin V and CD41 (Beckman Coulter) events, while erythrocyte MPs (RMPs) were identified as positive annexin V and CD235a (EXBIO) events. Relative (%) and absolute (MP/µl) counts of MPs were acquired using FlowCount (Beckman Coulter).

Measurement of MP procoagulant activity in plasma was performed using a functional assay, called Zymuphen MP-activity (Hyphen Biomed, Neuville-sur-oise, France). In this assay the diluted plasma samples supplemented with calcium, Factor Xa and thrombin inhibitors are introduced to ELISA plate coated with biotinylated annexin V and streptavidin. During the incubation period the phospholipids of MPs bind to the annexin V. After applying a washing step, Factor Xa and Va are added together with prothrombin. This prothrombinase complex in the presence of calcium will accumulate on the surface of the captured MPs as they expose phosphatidylserine, then the active complex FXa-FVa will convert prothrombin to thrombin. The concentration of MPs phospholipids is directly proportional to the amount of generated thrombin, which is measured via its specific activity on the thrombin substrate. The results were expressed as nanomolar phosphatidylserine equivalent.

Statistical methods

Each patient had at least one evaluation of MP, all MP examinations were analysed. Due to the skewed shape of the data, log transformation was applied. Statistics after back transformation are



PMP – platelet microparticles

Graph 4. Analysis of absolute and relative count of PMPs in relation with the positivity or negativity of *JAK2V617F* mutation of myeloproliferative neoplasms patients.

shown – geometric mean and 95% confidence interval (CI). Since MP examinations in one patient can be correlated, mixed effect models were applied – patient's examination was included as a random effect factor whereas evaluated variable as fixed effect factor. P-values of fixed effects are shown. No significant difference among examinations in time was proven. Analysis was performed in IBM SPSS Statistics 23 (IBM, USA).

Results

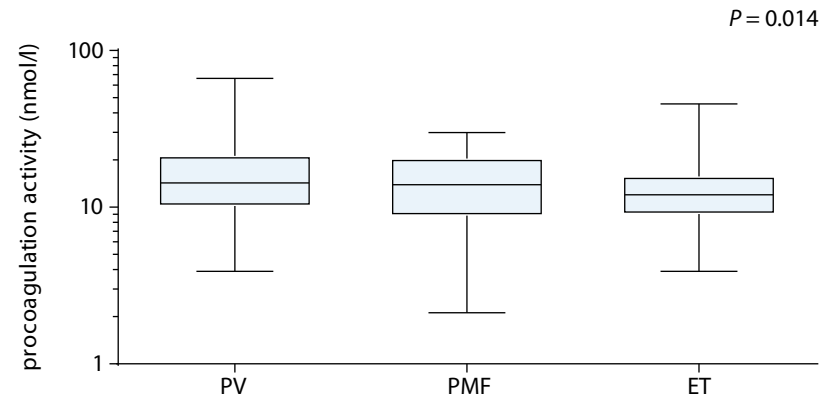
The total number of samples examined by FCM reached 417 samples with at least one sample for each patient from the 179-patient cohort, while the sample size of MPs concentration detected using procoagulation activity assay was 274 samples for 121 patients. As a control group, samples from 20 healthy donors not using drugs that affect the coagulation system (10 males, 10 females, median age at MPs examination 37.5 years) were used. The normal range of procoagulant activity of MPs was confirmed by the results obtained from the healthy group, with no result of the tested samples reached or was above 5 nmol/L (0–4.67).

The mean and 95% CI of the absolute and relative count of PMPs in MPNs patients was 1,046.6 MPs/µl (139.7–7,842) and 0.109% (0.012–0.951), respectively. For RMP the mean and 95% CI of the absolute and relative count was 57.3 MPs/µl (10.2–322.8) and 0.006% (0.001–0.043). Mean procoagulation activity was 12.8 nmol/L (4.8–34).

A weak positive correlation was found between the results of procoagulation activity of MPs and the relative count of PMPs in our MPN patients (p = 0.029), but no significant correlation was found with RMPs.

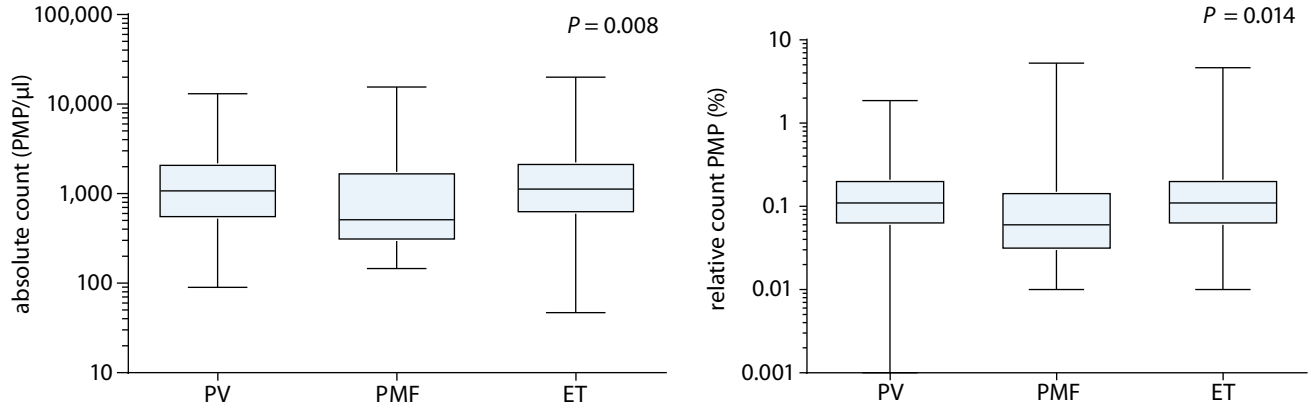
The difference between patients and healthy individuals was statistically significant for absolute and relative count of PMPs, respectively (1,047 vs. 519 MPs/µl, p = 0.001; 0.109 vs. 0.068%, p = 0.043) (Graph 1), for absolute and relative count of RMPs (57 vs. 24 MPs/µl; 0.006 vs. 0.003%, both p < 0.001) (Graph 2) and for procoagulation activity of MPs (12.9 vs. 1.9, p < 0.001) (Graph 3).

Patients with positive *JAK2V617F* mutation appeared to have higher absolute and relative count of PMPs compared with patients with negative *JAK2V617F*



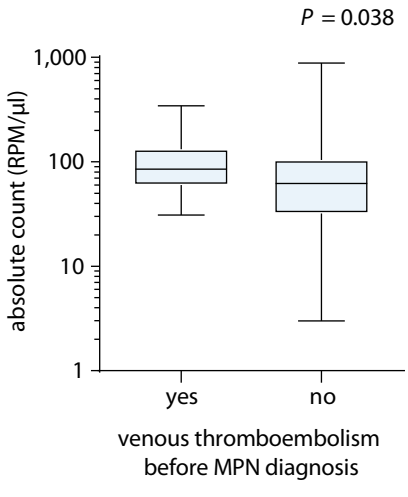
PV – polycythemia vera, PMF – primary myelofibrosis, ET – essential thrombocythemia

Graph 5. Comparison of procoagulation activity of MPs between patients according to the type of myeloproliferative neoplasms (PV, PMF, and ET).



PMP – platelet microparticles, PV – polycythemia vera, PMF – primary myelofibrosis, ET – essential thrombocythemia

Graph 6. Comparison of absolute and relative count of platelet microparticles between patients according to the type of myeloproliferative neoplasms (PV, PMF, and ET).



RPM – erythrocyte microparticles, MPN – myeloproliferative neoplasms

Graph 7. Comparison of absolute count of RPMs between myeloproliferative neoplasms patients with or without venous thromboembolism occurred before MPN diagnosis.

mutation, (1,150 vs. 807 MPs/µl, p = 0.045; 0.11 vs. 0.085%, p = 0.029) (Graph 4), but no statically significant difference was found when comparing the RMPs count in the two groups. When specifically studied, the relationship between the type of mutation and the level of PMPs, *JAK2V617F* positive patients were found to have higher absolute and relative count of PMPs than patients with *CALR* or *MPL* mutations (1,100 vs. 973 vs. 727 MPs/µl; 0.116 vs. 0.096 vs. 0.064%).

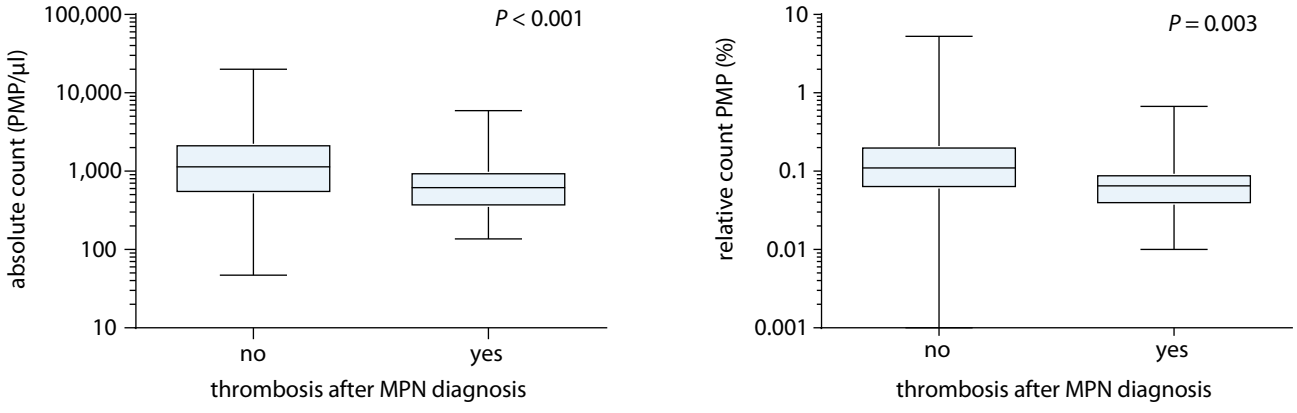
Statistically significant difference was found when assessing the MPs according to the type of MPNs. Patients with PV had pathological values of procoagulation activity of MPs more frequently than PMF and ET patients (p = 0.014) (Graph 5). Absolute and relative count of PMPs in patients with PMF was lower than in PV and ET patients (748 vs. 1,037 vs. 1,147 MPs/µl, p = 0.008; 0.077 vs. 0.11 vs. 0.119%, p = 0.014) (Graph 6), on the contrary and when the same evaluation was made for RMPs, patients with PMF were found to have higher absolute and relative count than in PV and ET patients, but it was not statistically significant.

No statistically significant difference was found when studying the correlation of the procoagulation activity of MPs in patients with or without a history of thrombosis in general. The data of patients with thrombosis were analysed according to the time of thrombosis into three groups – before MPN diagnosis, at and after MPN diagnosis. The absolute count of RMPs was significantly higher in patients who had a history of venous thrombosis before diagnosis than those who did not have (91 vs. 56 MPs/µl, p = 0.038) (Graph 7). Patients with thrombosis occurred after MPN diagnosis had significantly lower PMP as compared with patients without thrombosis, in absolute and relative values (p < 0.001; p = 0.003) (Graph 8). When comparing PMPs value in patients who had thrombosis and according to the time of occurrence it was found that patients with thrombosis before MPN diagnosis had significantly higher absolute count of PMPs than those who had thrombosis at or after MPN diagnosis (p = 0.022) (Graph 9). Detailed analysis was performed on the ET patients, specifically on those who have positive *JAK2V617F* mutation; it was found that absolute and relative values of PMPs were significantly lower in patients who had a history of thrombosis than those who did not have any thrombosis (p = 0.026; p = 0.014) (Graph 10).

The correlation in PMPs absolute and relative count with the results of simultaneously done complete blood count was studied, and statically significant correlations were found between PMPs absolute count with leukocytes, erythrocytes and platelets count along with haemoglobin and haematocrit values (p = 0.023, p = 0.027, p = 0.002, p < 0.001, p < 0.001), resp. for relative PMPs count (p = 0.024, p = 0.040, p = 0.018, p = 0.002, p = 0.018).

Discussion

Considered mechanism that triggers thrombosis in MPN include vascular dis-



PMP – platelet microparticles, MPN – myeloproliferative neoplasms

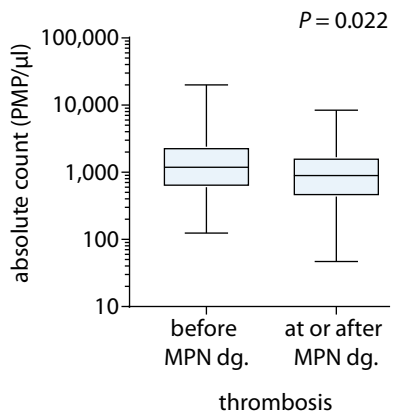
Graph 8. Comparison of absolute and relative count of PMPs between myeloproliferative neoplasms patients with or without thrombosis occurred during MPN treatment.

turbances, platelet activation, endothelial damage, and microparticle induced coagulation [13]. While the mechanisms leading to thrombosis are not completely understood, clinical and lab-based risk factors have been identified. Circulating MPs are considered to be biomarkers indicating the procoagulant state. Majority of circulating MPs are derived from platelets or megakaryocytes, but the other subtypes of MPs are important too. Erythrocyte-derived MPs, which are together with platelet-derived MPs exposing PS on the surface, are not only capable of propagation of the coagulation but can also solely start the production process of thrombin [20].

Numerous studies have shown that levels of circulating MPs are elevated in cancer patients, and that MPs contribute to thrombosis development. The elevated level of MPs or its procoagulant activity has been reported in MPN patients [21–24]. The aim of this study is to investigate a link between the parameters of circulating MPs and thrombotic complications in MPN patients. Like others [22,24], we observed a significantly higher procoagulant activity [22,24] and level of MPs [25,26] in comparison with control groups. These results can demonstrate the persistent prothrombotic state in patients with MPN.

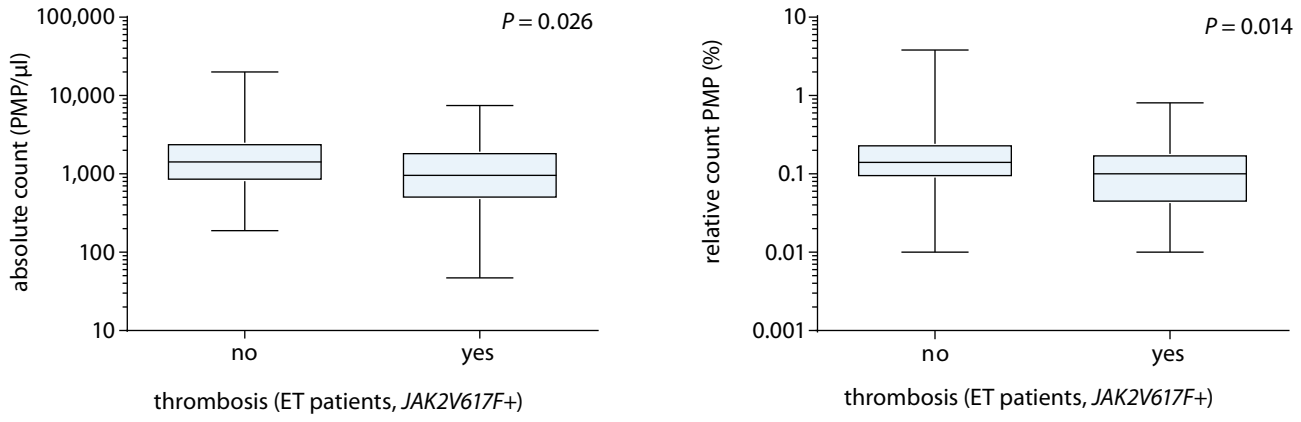
JAK2V617F mutation has been reported frequently in MPN patients [27]; recent studies show that *JAK2V617F* mutation or its allele burden is associated with the increased risk of thrombosis [17,18,28–31]. We found that *JAK2V617F* positive patients have significantly higher absolute and relative count of PMPs than those who are *JAK2V617F* negative, but no significant difference was found for RMP. Similar results were previously published by Zhang et al. [25], where the PMPs level in patients with *JAK2V617F* mutation was increased compared to patients without the mutation. Results can demonstrate the prothrombotic phenotype typical for this group of patients and can support the predicted involvement of MPs in pathogenesis of thrombotic complications in *JAK2V617F* positive patients. *CALR* mutation has been associated with lower risk of thrombosis compared to *JAK2V617F*, as demonstrated in one single-centre study of WHO-defined ET that found rates of major thrombosis of 13.5% for *CALR* vs. 30.1% in *JAK2V617F* (p = 0.01) [32]. Our comparison of MPs level between *JAK2V617F* positive and *CALR* positive patients did not confirm a significance due to the low number of *CALR* patients.

In our study we also analysed the differences of MP characteristics between the three subgroups of MPN. In previous study of our department [24] we found higher MP procoagulant activity in PV patients when compared to ET and PMF; the results of the current study are the same. Higher level of PMP was found in patients with PV and ET in comparison with PMF; the finding is different to the recent study of Zhang et al. [25], where the authors describe higher level of PMP and RMP in patients with PMF. The difference in outcome may be as a result of a lower number of MPN patients compared to our study, especially PMF patients. According to the published data, PV and ET are associated with higher risk of thrombosis as compared with



PMP – platelet microparticles,
MPN – myeloproliferative neoplasms

Graph 9. Comparison of absolute count of PMPs between myeloproliferative neoplasms patients with thrombosis occurred before and during or after MPN diagnosis.



PMP – platelet microparticles, ET – essential thrombocythemia

Graph 10. Comparison of absolute and relative count of PMPs between ET patients (*JAK2V617F* positive) with or without thrombosis.

PMF [13,30] and these circumstances can support the clinical implication of our results. Higher level of MPs in PV and ET patients may promote thrombogenesis and therefore lead to higher risk of thrombosis in this disease.

Unlike Zhang et al. [25] and Kissova et al. [24], but as reported by Duchemin et al. [22] we found no correlation between MPs and history of thrombosis in MPN patients. When we analysed the MP levels in patients with thrombosis according to the time of thrombosis development, we determined that patients with thrombosis during follow-up for MPN diagnosis had a lower level of PMPs than patients without thrombosis. The disadvantage of this study is the lack of MPs analysis in context with treatment. As previously documented in other studies [16,22,26], the patients with cytoreductive therapy were characterised by lower procoagulant activity of MPs than patients without cytoreduction. This analysis could clarify these results.

Well-defined risk factors for thrombotic complications in MPN patients include older age and history of prior thrombosis. Comparison of MPs levels in patients with thrombosis before diagnosis of MPN and patients after diagnosis of MPN showed higher levels of PMPs in the patients with venous thrombosis prior to MPN diagnosis. This finding supports the strategy of management of MPN patients according to the individual risk factors; the patients with a history of thrombosis belong to the high-risk group and should be appropriately treated. Further analysis of these results in context with therapy could provide more detailed information.

We examined the MP characteristics periodically regardless of the stage of disease or its treatments. And these circumstances could affect the results of the study even in the same patient. Therefore, further analyses of MPs in patients with MPN are necessary, especially in relation to therapy.

Conclusion

The present study observed a higher level of MPs in MPN patients, a high level of MPs was found in *JAK2V617F* positive patients and patients with a history of venous thrombosis prior to MPN diagnosis. The results of MP characteristics in patients with thrombosis require more detailed analyses in relation to the therapy.

Further studies are needed to clarify the clinical benefit of MPs as a potential biomarker of thrombosis as well as a diagnostic and prognostic factor in MPN patients.

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