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Microparticle phenotypes are associated with driver mutations and distinct thrombotic risks in essential thrombocythemia

Essential thrombocythemia (ET) is a Philadelphia-negative myeloproliferative neoplasm with high platelet counts and an increased risk of thrombosis. Acquired somatic mutations delineate four ET subtypes: *JAK2-V617F*, *CALR*-mutated, *MPL*-mutated, and "Triple-Negative" (TN).[1] *CALR* mutations appear to drive a distinct phenotype, consisting of a higher proportion of males, younger age, higher platelet count, lower hemoglobin level and white cell count and lower thrombotic risk than *JAK2-V617F* carriers.[1-3] TN patients share the same profile, but lack the higher platelet count.[4]

Thrombogenesis in ET results from complex interactions between blood cells and plasma factors.[5] Microparticles (MPs) are thought to play a role in many thrombotic processes.[6] They are sub-micron plasma membrane vesicles, released into the circulation by blood and endothelial cells following activation or apoptosis.[6] They can be labeled with annexin V if phosphatidylserine (PS) is exposed during their formation.[6] So called platelet-derived microparticles (PMPs) are the most abundant MP subtype in the blood, and could originate mainly from megakaryocytes.[7]

Prior to the discovery of *CALR* mutations, an increase in PMPs was reported in ET patients as compared to healthy controls[8] and hypercoagulability was ascribed, at least in part, to MPs.[9,10] However, to our knowledge, the patterns of MPs according to mutational status have not yet been established. We hypothesized that circulating microparticles might account for the different thrombotic risks among ET subtypes. To test this hypothesis, we investigated cellular and procoagulant phenotypes of circulating MPs according to the mutational status in ET patients at diagnosis, prior to any cytoreductive therapy.

Following the informed consent of participants, 74 consecutive patients with newly diagnosed ET (WHO 2008 criteria), were enrolled in our study from November 2010 to June 2013. Approval was given by the local ethics committee (CPP France-North n°2009-A0062-55). At enrollment, none of the patients received cytoreductive therapy, whereas two-thirds of them had already been submitted to a low-dose aspirin treatment (Table 1). The 2 patients with rare *MPL* mutations were not considered for analysis.

MPs enumeration, phenotyping as well as procoagulant activities were analyzed in platelet-free plasma (PFP). PFP was isolated from citrated blood after two consecutive centrifugations at 2500g for 15 min at room temperature, aliquoted and stored at -80°C until testing.

JAK2-V617F mutation was detected by TaqMan® allelic discrimination, and *CALR* mutations by fragment analysis plus direct sequencing (Sanger).

Pre-analytical and flow cytometry procedures were performed according to the current ISTH guidelines.[11] MPs were detected with flow cytometry (FC500, Beckman-Coulter™) in a gate delineated with dedicated beads. Hereafter 'MPs' will refer to annexin V$^+$ events in the selected gate, and the cell of origin will be indicated as well. Phenotyping was performed by co-labeling with annexin V and lineage-specific antibodies (*Online Supplementary Material*): red cell-derived MP (RMP),

Table 1. Hematological characteristics of the patients with essential thrombocythemia at diagnosis prior to cytoreductive therapy, according to mutational status.

Parameters	All patients	A *JAK2- V617F*+	B *CALR*+	C Triple negative	*P* Global comparison	A *vs.* B	A *vs.*C	B *vs.* C
Number of patients (%)	72	45 (62)	15 (21)	12 (17)	–	–	–	–
Sex M/F (n, %)	24/48 (34/66)	15/30 (33/67)	7/8 (47/53)	2/10 (17/83)	ns	ns	ns	ns
Age (years)	58 [48-70]	61 [49-73]	58 [51-67]	55 [46-62]	ns	ns	ns	ns
Aspirin-treated patients (n, %)	49 (68)	31 (69)	10 (67)	8 (67)	ns	ns	ns	ns
Platelet count (x10^9/L)	709 [575-882]	664 [572-852]	861 [677-916]	610 [531-771]	<0.05	<0.05	ns	<0.05
Hemoglobin (g/dL)	13.8 [13-14.6]	14.3 [14-15.6]	13.8 [13.2-14.5]	13.7 [13-14.7]	ns	<0.05	ns	ns
Leukocyte count (x10^9/L)	8.4 [7.4-9.9]	8.7 [8.1-10.8]	8.0 [7.4-9.1]	7.9 [7.1-9.8]	ns	ns	ns	ns
Thrombosis before diagnosis (n, %)	11 (15)	9 (20)	0 (0)	2 (17)	ns	ns	ns	ns
High IPSET-thrombosis score, (n, %)	37 (51)	37 (82)	0 (0)	0 (0)	<0.0001	<0.0001	<0.0001	ns

Data are presented as number and percentage (%) or median with interquartile range [IQR]. ns indicates not significant. P values ≤ 0.05 were considered significant. IPSET-thrombosis stands for International Prognostic Score of Thrombosis in ET.

monocyte-derived MP (MoMP), granulocyte-derived MP (GMP), endothelial cell-derived MP (EMP), so-called platelet-derived MP (CD41⁺MP, hereafter referred to as PMP). PMPs were further characterized as coexpressing CD62P (P-selectin⁺ PMP) or not. 'Total MPs' refer to all annexin V⁺ events in the selected gate, whatever their cellular origin.

We also determined procoagulant activity of MPs with two assays: (i) phospholipid-dependent procoagulant activity of MPs using the Zymuphen™ MP-activity assay (Hyphen BioMed), and (ii) thrombin generation using Calibrated Automated Thrombography (CAT). The experimental conditions for both assays are specified in the *Online Supplementary Material*.

Statistical analysis was performed using SPSS software. Quantitative data were compared between subtypes with the Kruskal-Wallis and Mann-Whitney tests (for multiple and single comparisons, respectively) and qualitative variables with the χ^2 test. Correlation tests between MP counts and procoagulant activities were analyzed by Spearman's correlation test. *P* values <0.05 were considered significant. The performances and cutoff of MP enumeration were evaluated as predictive criteria for thrombotic risk using receiver operator characteristic (ROC) analysis.

Patients' characteristics at diagnosis (45 *JAK2*-mutated, 15 *CALR*-mutated, and 12 TN) were consistent with previous reports (Table 1). In particular, platelet counts were higher in *CALR*-mutated patients, no thrombotic events were recorded for *CALR*-mutated patients, and none of the *CALR*-mutated and TN patients were placed in the high-risk category of the IPSET-thrombosis score[12] (Table 1).

There was no correlation between PMP count and age, platelet count, leukocytosis, and hemoglobin, and no difference according to sex.

The MP patterns (Figure 1) of *CALR*-mutated and TN patients were similar and shared the same differences with those of the *JAK2*-mutated patients. Total MP counts were higher in *JAK2*- than in *CALR*-mutated and TN patients. We found no differences between the three molecular subtypes regarding MoMP, GMP and EMP counts. RMP counts were higher in *JAK2*- than in *CALR*-mutated and TN patients. Such a difference is reminis-

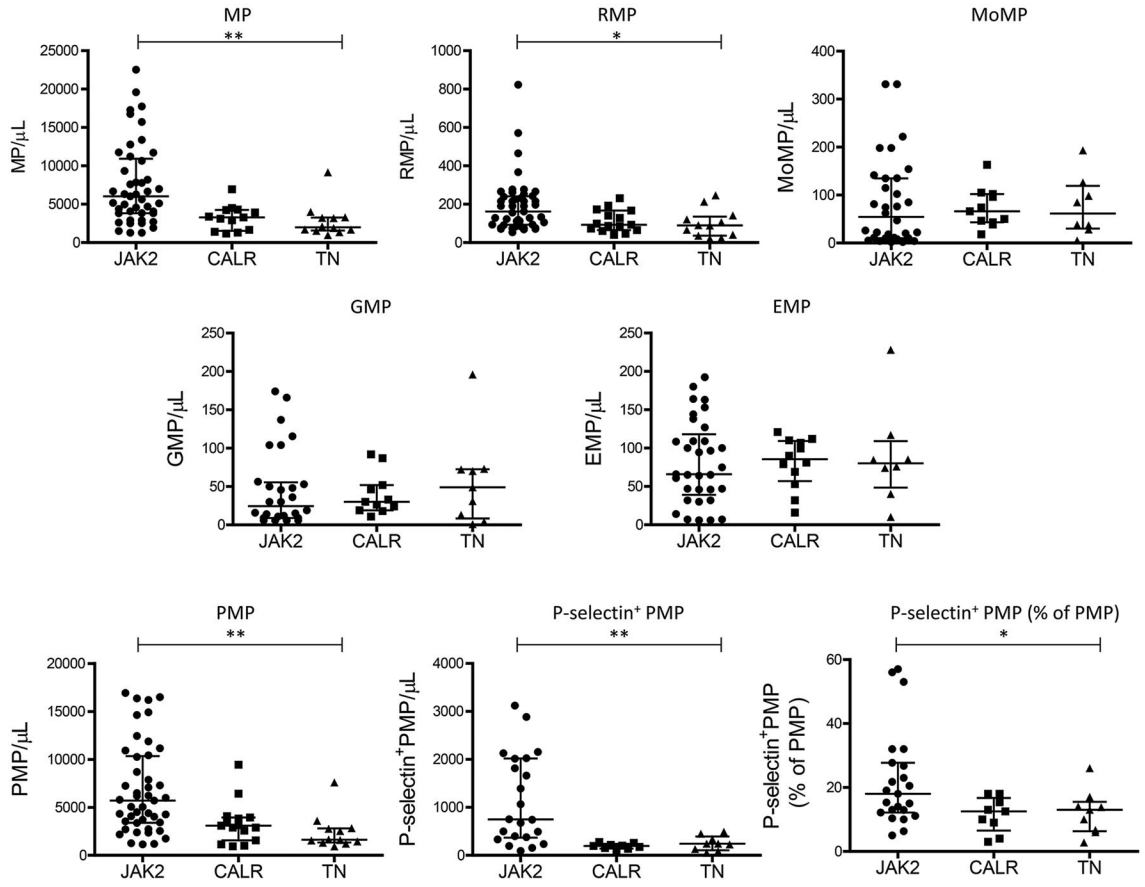

Figure 1. Circulating microparticle counts with respect to the mutational status of ET patients. JAK2: *JAK2-V617F* mutated patients; CALR: *CALR*-mutated patients; TN: so-called "triple negative" patients, for which neither the two former mutations, nor a mutation in the *MPL* gene, were found. *indicates *P*<0.01, ** indicates *P*<0.001. In addition to individual data, median values and interquartile ranges are plotted. MPs indicates all annexin V⁺ events in the gate delineated with dedicated beads (Megamix® beads, BioCytex, Stago, Marseille, France), whatever their cellular origin. Phenotyping was performed by co-labeling with annexin V and lineage-specific antibodies (as detailed in the *Online Supplementary Material*): RMP: red cell-derived microparticles, MoMP: monocyte-derived microparticles, GMP: granulocyte-derived microparticles, EMP: endothelial cell-derived microparticles, PMP: CD41⁺ MP, so-called platelet-derived microparticles, P-selectin⁺ PMP: PMPs coexpressing P-selectin, P-selectin+ PMP % of PMP: percentage of PMPs coexpressing P-selectin among PMPs. MoMPs, GMPs and EMPs were enumerated in a subset of 45 ET patients. Labeling procedure is detailed in the *Online Supplementary Material*.

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cent of the proximity of *JAK2*-mutated ET with polycythemia vera.[2] Despite higher platelet counts, PMP counts were significantly lower in *CALR*-mutated than in *JAK2*-mutated patients. Of note, PMP counts were not related to platelet counts, suggesting that their production is not merely a reflection of thrombocytosis.

To our knowledge, this is the first description of the MP patterns in untreated ET patients with up-to-date molecular annotation. A study carried out before the *CALR* era found no differences in MP patterns according to *JAK2* mutational status in 21 ET patients, with one third already receiving a cytoreductive drug.[8] Of note, we observed a consistent decrease in PMPs following the start of a cytoreductive treatment (*data not shown*).

The higher PMP counts we found in *JAK2*-mutated patients could be explained by an abnormal platelet sensitivity to activating stimuli due to *JAK2-V617F* mutation,[13,14] in agreement with a previous report showing increased platelet P-selectin expression on platelets of *JAK2*-mutated patients.[13] To investigate the activated PMP subset, we enumerated P-selectin$^+$ PMPs (CD62P$^+$, CD41$^+$, annexin V$^+$),[7] as membrane expression of CD62P is a well-established marker of platelet activation. We found that P-selectin$^+$ PMPs, expressed either as counts or percentages, were higher in *JAK2*- than in *CALR*-mutated and TN patients (Figure 1). Nevertheless, this subset was low in most of the ET patients and megakaryocytes are likely the main source of PMPs.[7] Thus another explanation is that *JAK2-V617F* could change megakaryocyte biology beyond driving myeloid proliferation, leading to an increased vesiculation in the bone marrow, with the release of numerous MPs into the bloodstream. This hypothesis is in line with a report on a *JAK2-V617F* ET mouse model where *JAK2-V617F*-positive megakaryocytes produced higher amounts of proplatelets than control megakaryocytes.[14]

In functional studies we found a relation between MP counts and procoagulant activities, assessed either as procoagulant phospholipids of captured MPs or with a more global assay (CAT), taking into account all MPs, whatever their size and membrane composition (Spearman's test, r=0.53, *P*=0.0002 and r=0.47, *P*=0.01, respectively). These results indicate that MPs are likely to enhance thrombin generation and thus contribute to the thrombotic risk. Procoagulant activity is linked to PS exposure, but polyphosphates associated with MPs could also play a role. MP-associated procoagulant activities were higher in *JAK2*-mutated than in *CALR*-mutated and TN patients (25.7 [17.9-33.5], 11.5 [5.8-29.6], 12.8 [6.9-17.5] phosphatidylserine equivalents (nM), respectively; *P*=0.01). This difference probably depends more on the MP counts than on differing PS exposures, as ratios for PS-equivalent/total MPs count were not different between the mutational subtypes.

To further explore whether MP counts could be related to the thrombotic risk, we compared MP counts according to the IPSET-thrombosis score[12] and performed a ROC analysis. Circulating MP counts were higher in the group with high thrombotic risk than in the groups with low- or intermediate-risk (6320/μL [4080-11700], 4030/μL [3000-6750], 3100/μL [1660-3920], respectively, *P*<0.001). A cutoff value of 4600 MPs/μL was calculated with ROC analysis and discriminated the high thrombotic risk ET patients with a specificity of 81% (30/37) and a sensitivity of 77% (Figure 2). Of note, ROC performances were similar considering either total MP or PMP counts (PMP cutoff value: 4000/μL). These results need to be confirmed with a standardized methodology thanks to the ongoing efforts of international societies.[15]

In our study, we based the detection of MPs on annexin V binding. Preanalytical conditions, especially a freeze-thaw cycle, may induce PS exposure, thus possibly triggering artifacts on procoagulant assays and MP enumeration after annexin V staining.[15] We minimized the risk by removing residual platelets, the main source of PS exposure after freeze-thawing.[11]

In conclusion, we report for the first time that in molecularly annotated ET patients at diagnosis, *JAK2-V617F* patients have more circulating PMPs and higher MP-associated procoagulant activities than *CALR*-mutated and TN ET patients. This could be partly explained by platelet activation, as assessed by P-selectin expression on PMPs in the *JAK2*-mutated patients. Abnormal *JAK2-V617F* megakaryocytes are presumably also involved in higher PMP generation. Since we found a relation between MP counts and thrombin generation, circulating

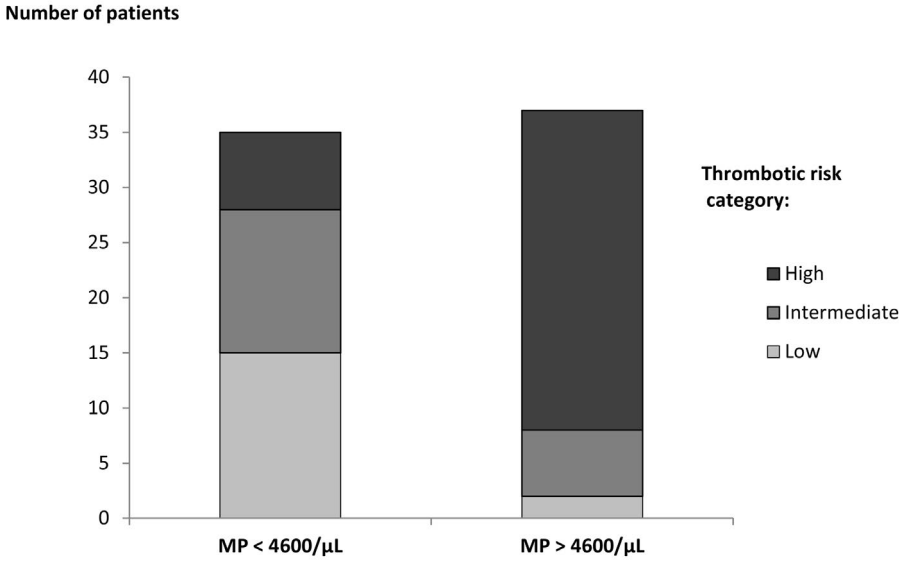

Figure 2. Relation between MP counts and prothrombotic state in ET patients. Distribution of ET patients according to circulating MPs cutoff value and thrombotic risk assessed with IPSET-thrombosis score.[12] ET patients were distributed between low, intermediate and high thrombotic risk groups. The cutoff value of 4600 MP/μL was calculated by statistical analysis (ROC curve analysis). This value discriminates between high and intermediate or low thrombotic risk patients with a specificity of 81% and a sensitivity of 77%.

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MPs could play their part in thrombotic complications through hypercoagulability. Indeed, we found a relation between MP counts and thrombotic risk. Consequently, in *JAK2*-mutated patients, the higher count of circulating PMPs might contribute to a higher incidence of thrombosis than in the other ET patients. Similarities in the patterns of *CALR*-mutated and TN patients suggest that the low thrombotic risk in *CALR*-mutated patients is mainly related to the absence of *JAK2-V617F* rather than to a "protective" role of *CALR* mutations, for reasons that remain to be elucidated. Finally, our results suggest that microparticles might account, at least in part, for the distinct thrombotic risks according to mutational status in essential thrombocythemia.

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