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Blood cell activation as an indicator of prothrombogenesis in polycythemia vera: Case control study

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Abstract:

BACKGROUND: Polycythemia vera (PV) is a chronic myeloproliferative stem cell disease usually combined with *JAK2* mutation. Active *JAK2* mutation directly promote platelets and granulocytes activation and indirectly initiate endothelial activation. Such interactions can provoke endothelial injury with subsequent release of procoagulant factors. The aim of this study was to understand of this study is to understand the pathophysiology of increased thrombosis in PV in terms of the effect of *JAK2*^{V617F} gene mutation in relation to the intensity of the neutrophil CD11b and platelet CD62P.

SUBJECTS AND METHODS: A group of 53 patients was examined comprised 30 PV patients and 23 with secondary polycythemia. In parallel, 30 aged- and sex-matched, nonsmokers healthy volunteers served as a control group. Hemoglobin level, packed cell volume, white and red blood cells count, mean corpuscular volume, and platelet count were estimated for all the 83 participants. Flow cytometry for the detection of neutrophil CD11b and platelet CD62P were done for all the participants; whereas quantitative polymerase chain reaction technique for the assessment of *JAK2* mutation rate was done for the 30 PV patients only.

RESULTS: We were able to detect significantly higher neutrophil CD11b and platelet CD62P expressions in PV patients. Within PV, *JAK2* mutation rate was significantly higher in those with a history of thrombosis. A positive relationship was demonstrated between the *JAK2* mutation rate and each of CD11b and CD62P.

CONCLUSION: There is an overexpression of neutrophil CD11b and platelets CD62P in patients with PV which can be considered as a marker of procoagulant activity in blood cells. Active *JAK2* mutation directly promote platelets and granulocytes activation and indirectly initiate endothelial activation and in turn endothelium into a proadhesive and procoagulant surface.

Keywords:

CD11b, CD62P, flow cytometry, *JAK2*, polycythemia vera

Introduction

Polycythemia vera (PV) is a hematologic malignancy demonstrates uncontrolled hematopoiesis that leads to hyperviscosity along with a tendency to thrombosis.[1,2] In 2005, *JAK2*^{V617F} mutation was discovered as the principal cause of PV[3] where 96% of them were positive for *JAK2*^{V617F} and only 2%–3% have mutations in exon 12 of *JAK2*.[4-6]

Increased hematocrit (Hct), red blood cell (RBC), platelets, white blood cells (WBCs), and elevated *JAK2*^{V617F} allele burden were demonstrated by many researchers in patients with PV.[7,8] Among the nonconventional hazardous for thrombosis in PV is blood cell activation. Leukocytes and platelets activation plus endothelial cells were suggested to promote a prothrombotic state.[9] Plasma elastase,

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elastase activity, leukocyte alkaline phosphatase, CD11b, and plasma myeloperoxidase are representative of leukocyte activation markers and found to be elevated in patients with PV.[10]

Enhanced platelet activation by leukocytes and platelets aggregation denoted increased levels of CD11b/CD42b, CD11b/CD62P (markers for leukocyte-platelet aggregation) plus higher percentage of circulating activated platelets (CD62P) have been found in patients with PV.[11] They thought to be a central component of leukocyte-driven thrombosis.[12]

In human diseases, an interrelationship between *JAK2*^{V617F} and platelet activation is experimentally intimated were megakaryocyte-specific knock-in of *JAK2*^{V617F} produces hyperreactive platelets to thrombin and collagen.[13]

The objective of this study is to understand the role of blood cell activation in thrombogenesis in PV patients taking into account the effect of *JAK2*^{V617F} gene mutation in relation to the intensity of neutrophil CD11b and platelet CD62P.

Subjects and Methods

A case–control study conducted on 53 patients whose recruited from those attending the outpatient clinic of the Clinical Hematology Department in Al-Imamain Al-Khadimiyan Medical City, and the National Hematology Center, College of Medicine, Al-Mustansyria University, Baghdad. The study was approved by the Medical Ethics Committee Institutional Review Board of the College of Medicine, Al-Nahrain University (MA10-17/1/2016) and an ethical consent for participation in the study was ensured from all participants recruited.

Patients were examined by a consultant in clinical hematology before being considered eligible for the purpose of the study. PV patients should meet the WHO published consensus criteria in 2008 for the diagnosis of Philadelphia-negative myeloproliferative neoplasms.[14] The criteria for the diagnosis of PV include 2 major criteria and 3 minor criteria. The major criteria include hemoglobin (Hb) 18.5 g/dL in men, 16.5 g/dL in women, or other evidence of increased red cell volume; and the presence of *JAK2*^{V617F} or other functionally similar mutation such as *JAK2* exon 12 mutations. The minor criteria include bone marrow biopsy specimen showing hypercellularity for age with trilineage growth (panmyelosis) with prominent erythroid, granulocytic, and megakaryocytic proliferation; serum erythropoietin (EPO) level below the reference range for normal; and endogenous erythroid colony formation *in vitro*.

The diagnosis requires the presence of both major criteria and 1 minor criterion or the presence of the first major criterion together with 2 minor criteria.[15]

The initial data for those already diagnosed with PV cases were retrieved and reviewed from their casefiles while it was collected by direct interview for the new patients.

Secondary polycythemia is defined as an absolute increase in RBC mass that is caused by enhanced stimulation of RBC production governed by EPO overproduction.[16]

Diabetic patients with long-term complications, patients with a history of hereditary thrombophilia or coagulopathy disorders, acute inflammatory conditions, connective tissue diseases, acquired cause for thrombotic complication were excluded from the study.

The demographic data of the patients and healthy control groups are shown in Table 1. Patients with PV were divided into subgroups as follows: Group 1: The newly diagnosed PV (10 patients); Group 2: Those with thrombotic complications (7 patients); and Group 3: Those with long-standing disease and under follow-up (13 patients) in whom the diagnosis was established before the time of the current study. These patients were kept under their regular treatment of hydroxyurea with or without venesection.

Methods

Six milliliter of venous blood samples were aspirated from the antecubital vein and used as follows:

1. Two milliliter mixed with anticoagulant ethylenediaminetetraacetic acid (EDTA) and stored at −20°C for the quantitative assessment of *JAK2*^{V617F} mutation in PV patients. The DNA was extracted using the gSYNCTM DNA Mini Kit (Geneaid, Korea). Real-time polymerase chain reaction (PCR) was done based on the measurement of accumulated PCR product through a TaqMan probe which is a small oligonucleotide labeled with

Table 1: Hematological data of polycythemia patients and control subjects

Parameter	Polycythemia patients		Control subjects (n=30)
	Primary (n=30)	Secondary (n=23)	
Hb (g %)	14.36±2.52	18.15±1.4*	14.46±1.49
PCV (%)	44.91±7.19	54.43±2.65*	43.64±4.28
WBC ×10^3	11.11±6.49#	9.34±4.17	7.94±2.32
RBC ×10^6	5.59±1.4	6.16±0.41‡	5.13±0.46
MCV (fL)	82.97±13.63	86.65±6.08	87.58±4.29
Platelets ×10^3	379.3±242.96†	231.43±56.71	257.13±61.6

*P<0.001 (secondary vs. primary polycythemia and control), #P=0.035 (primary vs. secondary polycythemia and control), ‡P=0.001 (secondary vs. primary polycythemia and control), †P=0.01 (primary vs. secondary polycythemia and control). Hb=Hemoglobin, PCV=Packed cell volume, WBC=White blood cell, RBC=Red blood cell, MCV=Mean corpuscular volume

two different fluorescent dyes. In the 5′ end a reporter dye (FAM [6-carboxyfluorescein]) is attached and at the 3′ end, a quencher dye (CHQ [Black Hole Quencher]) is attached
2. Two milliliter mixed with EDTA in a test tube for studying neutrophil CD11b and platelet CD62P by flow cytometry within 6 h
3. For every subject in the study, complete blood count was done including Hb level, Hct, WBC and RBC count, mean corpuscular volume (MCV), and platelets count
4. One milliliter of the blood into a centrifuge gel tube (clot activator tube). The serum was collected for estimation of EPO. The blood allowed to clot for 10 min and then centrifuged at 4000 rpm for 5 min. The serum then removed into an eppendorf tube and stored in a deep freezer at −60°C until the time of analysis. The samples which show hemolysis were discarded.

Statistical analysis

Data analysis was performed using the Statistical Package for the Social Science software (version 16.0, IBM Inc., Chicago, USA) with categorical variables were expressed as percentages. Pearson's Chi-squared test was used to compare percentages of different groups where there were categorical variables. Analysis of variance was used to compare the continuous variables. The association between different variables was examined with binary correlation test. The causal relationships between some variables were investigated with regression test. A value of $P < 0.05$ was considered statistically significant.

Results

Hematological parameters

In patients with secondary polycythemia, the Hb level was significantly higher ($P < 0.001$) as compared to those with PV and the control group. Moreover, no significant difference was noticed between the latter two groups. The Hct was significantly different among the three studied groups ($P < 0.001$). The WBC and platelet counts were significantly higher in patients with PV as compared to patients with secondary polycythemia and control group ($P = 0.035$; $P = 0.01$; respectively). No significant difference was found between the latter two groups.

In addition, the RBC count was significantly higher ($P = 0.001$) in patients with secondary polycythemia as compared to patients with PV and control group and no significant difference was observed between the latter two groups. He MCV level was more or less the same among the three studied groups [Table 1].

The Hb level, Hct, and RBC count were significantly higher in Group 1 than Group 2 and Group 3 PV patients ($P = 0.035$; $P = 0.001$; and $P = 0.032$; respectively). Meanwhile, no significant difference was found when compared to Group 1 and Group 2.

With regard to WBC count, it was significantly higher ($P = 0.031$) in Group 2 as compared to Group 1 PV patients. On the other hand, no significant difference was found between Group 3 and Group 2 and between Group 3 and Group 1 PV patients. Similarly, no significant difference was found between the three subgroups concerning MCV and platelet count [Table 2].

Figures 1 and 2 show the sample of the effect of light scatter on the CD11b and CD62P expressions on the cell membrane of neutrophil and platelets of a patient with PV, secondary polycythemia, and the normal subject group.

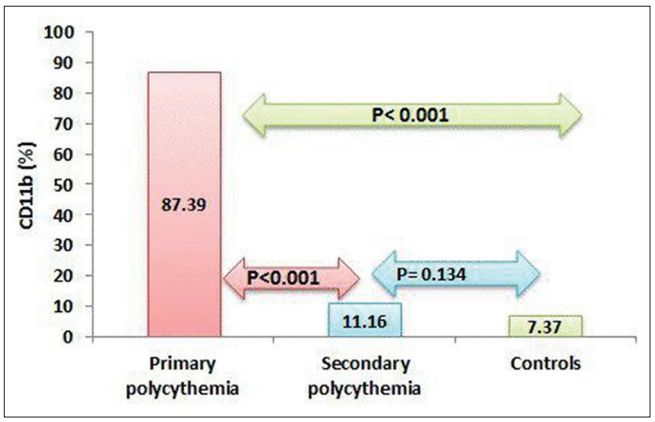

Figure 1: The percentage of CD11b expression in the polycythemia subgroups

Table 2: Hematological data of the patients with polycythemia vera subgroups

Parameter	PV patients		
	Group 1 (new diagnosis) (*n*=10)	Group 2 (PV with thrombosis) (*n*=7)	Group 3 (longstanding PV) (*n*=13)
Hb (g %)	15.93±1.44[#]	12.98±2.26	13.89±2.8
PCV (%)	51.1±3.03[†]	39.8±4.54	42.9±7.49
WBC ×10^3	12.89±7.17	14.4±7.68[##]	7.97±3.75
RBC ×10^6	6.54±0.9[‡]	5.07±1.06	5.13±1.64
MCV (fL)	79.39±12.18	79.42±10.82	87.62±15.42
Platelets ×10^3	441.9±226.83	473.86±354.55	280.23±148.66

[#]*P*=0.035 (Group 1 vs. Group 2 and 3), [†]*P*=0.001 (Group 1 vs. Group 2 and 3), [##]*P*=0.031 (Group 2 vs. Group 3), [‡]*P*=0.032 (Group 1 vs. Group 2 and 3).
Group 1=Newly diagnosed, Group 2=With thrombosis, Group 3=Longstanding disease, PV=Polycythemia vera, Hb=Hemoglobin, PCV=Packed cell volume, WBC=White blood cell, RBC=Red blood cell, MCV=Mean corpuscular volume

Neutrophil CD11b expression was reported in 87.39% of PV patients which is significantly higher ($P < 0.001$) than 11.16% and 7.37% of the secondary polycythemia and controls, respectively). No significant difference was demonstrated between the latter two groups [Figure 1].

Similarly, platelet CD62P expression in PV patients was found in 30.15%, which is significantly higher ($P < 0.001$) than 8.18% and 7.77% of the secondary polycythemia and controls, respectively). No significant difference was demonstrated between the latter two groups [Figure 2].

JAK2 mutation and flow cytometry in polycythemia vera subgroups

Figure 3 illustrates the percentage of *JAK2* mutation rate in PV subgroups. The mutation rate was significantly higher ($P = 0.050$) in Group 2 (68.11%) as compared to Group 1 (47.67%), with no significant difference ($P = 0.141$) when compared to Group 3 (53.32%). No significant difference was noticed between Group 1 and Group 3 ($P = 0.523$).

Regarding the CD11b, as it is present in Figure 4, the percentage of expression was not significantly different between the three groups (82.2% vs. 91.99% vs. 88.91%).

Furthermore, the results of CD62P expression in the three PV subgroups are shown in Figure 5. They were 30.74% in Group 1, 31.39% in Group 2, and 29.02% in Group 3.

Relationships between different data parameters

In patients with PV, a positive correlation was demonstrated between *JAK2* and each of CD11b and CD62P ($P = 0.008$, $P = 0.000$, respectively) as shown in Figures 6 and 7.

Discussion

Blood cell count data

The estimated Hb level, Hct, and RBC count were lower in patients with PV when compared to the secondary polycythemias. These results were anticipated as the mainstay of treatment being to lower Hct level to than the normal level (<45%) and eventually to diminish the morbidity and mortality that accompanied thrombosis complication. In this study, two-thirds (20:30) of PV patients were established cases, and they were on 2 g per daily of hydroxycarbamide in addition to the repeated venesection, which is now considered to be the standard PV treatment.[17,18]

Within polycythemia vera subgroups

The Hb level, Hct and RBC count were higher in Group 1

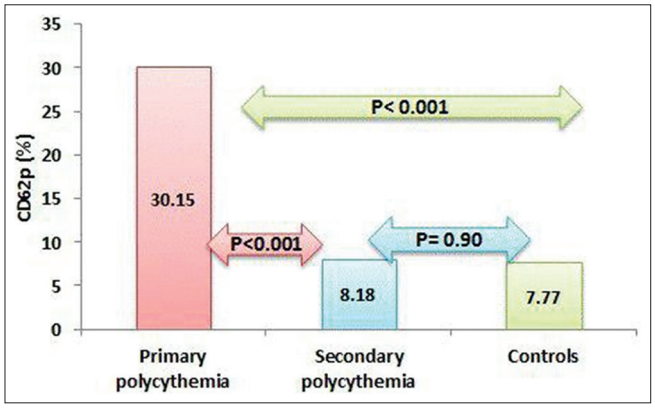

Figure 2: The percentage of CD62P expression in polycythemia subgroups

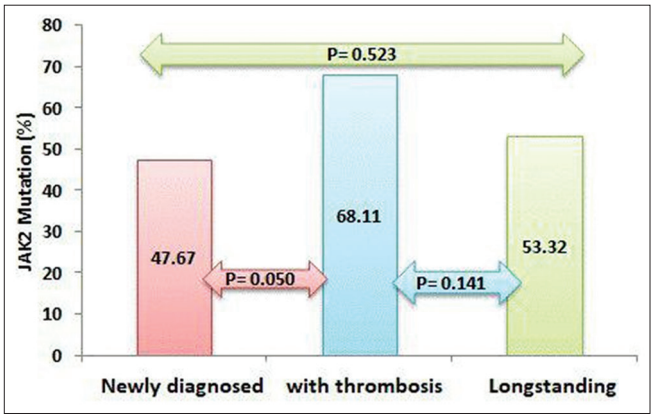

Figure 3: Mutation rate of JAK2 in all patients with polycythemia vera subgroups

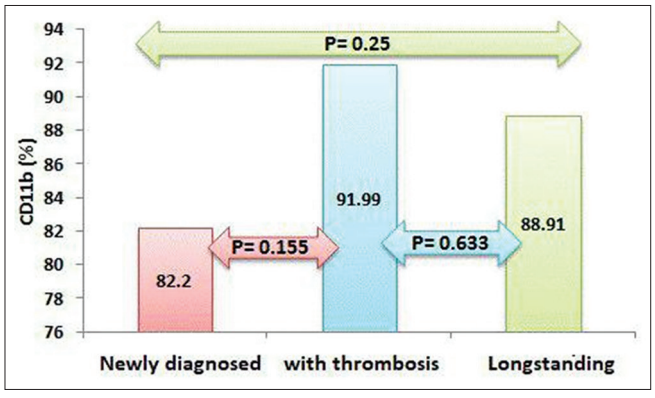

Figure 4: CD11b expression in all patients with polycythemia vera subgroups

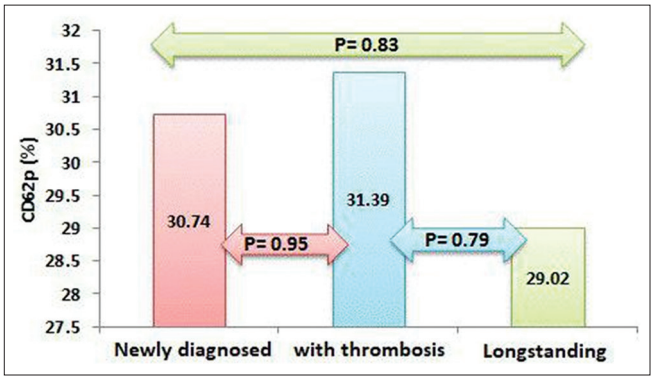

Figure 5: CD62p expression in all patients with polycythemia vera subgroups

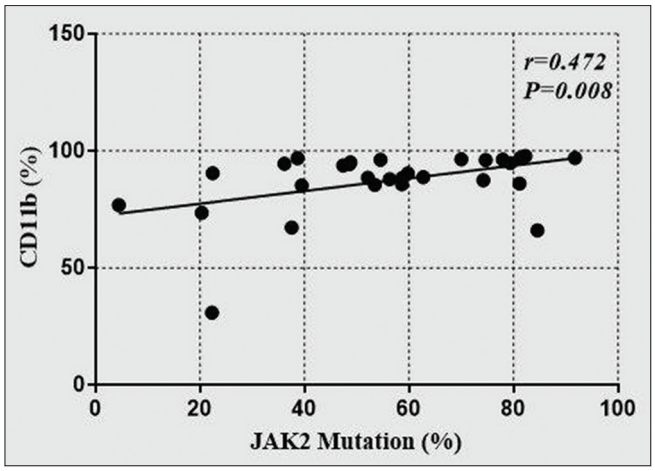

Figure 6: The correlation between JAK2 mutation rate and CD11b percentage in polycythemia vera group

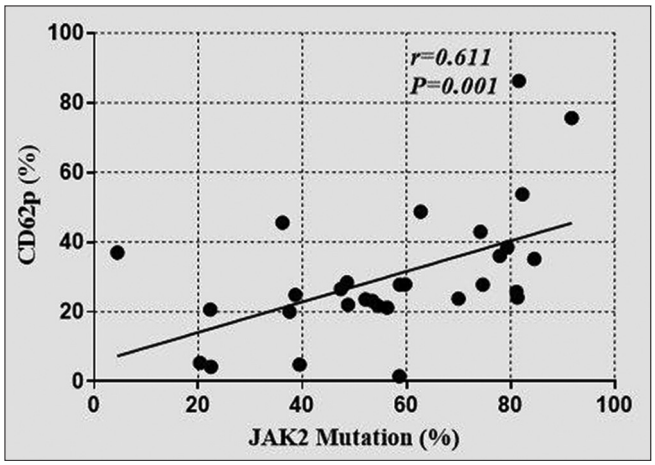

Figure 7: The correlation between JAK2 mutation rate and CD62p percentage in polycythemia Vera group

of PV patients when compared to patients of Groups 2 and 3 who were already on hydroxyurea treatment.[19] The control of Hct is reflected on by the normalization of Hb level, Hc t percentage, and RBCs count.

PV patients of Group 2 demonstrated higher WBC count when compared to Group 3 and 1, which could be attributed to the acute phase reaction and postinflammatory response resulted from thrombosis.[20-22] On the other hand, the lower WBC count in Group 3 may result from the effect of long-term treatment which causes normalization of hematological parameters.

Blood cell activation

CD11b and CD62P expression

The PV patients expressed high neutrophil CD11b and platelet CD62P. A similar finding was also reported by other researchers.[23-26] The higher functional levels of CD11b on the WBC surface reflect an activated WBC. CD62P expression is a critical stage for blood aggregation, neutrophil activation, and inflammatory processes.[27] The activated polymorphonuclear (PMN) leukocytes can bind to platelets in a dynamic process.[28] Blood cell activation plus interaction with endothelial cells, and the coagulation system represent the pathogenetic mechanism of thrombosis in PV.[29]

Furthermore, the interplay between activated PMN leukocytes and activated platelets produces leukocyte/platelet-mixed aggregates, which are considered a sensitive marker of platelet activation (in PV patients, about half of leukocytes circulate while platelets held to their membrane).[11,23,30]

Similarly, after platelet activation, anionic phosphatidylserine exposes on their surface, offering a catalytic surface for the production of thrombin which auxiliary augments platelet activation. This thrombin production brought about by platelets shown to be increased and coupled with platelet activation, particularly in PV carriers with *JAK2*^{V617F} mutation.[24,31-33]

The study demonstrates a nonsignificant difference in the expression of neutrophil CD11b and platelets CD62P between secondary polycythemia patients and control groups. This may explain, at least partially, the mechanisms that causes qualitative as well as quantitative changes in leukocytes and platelets in PV which enhances their pro-coagulant tendency are not the same as in the pathogenesis of secondary polycythemia. This would suggest that erythrocytosis alone is not responsible for the increased thrombosis in PV.[34]

Within the PV subgroups, *JAK2* mutation rate was high in those with a history of thrombosis followed by the newly diagnosed patients versus those with longstanding disease. Despite the limited number of patients within each subgroup, this offers an insight into the mechanisms of thrombosis in MPN and favor the hypothesis of an increased hypercoagulable condition in *JAK2*^{V617F} carriers. This hypothesis goes in conformity with that declared by Falanga *et al*.[35]

The percentage of *JAK2* mutation rate in those with thrombosis equals to 68.11%, a proportion similar to that of other researchers;[8,36] who found a *JAK2*^{V617F} allele burden threshold of 50%–75% may classify patients at high risk for thrombotic events. Accordingly, with the increment of the allele burden, the frequency of thrombosis gradually increased, with the greatest rate of vascular complications in those with an allele burden >50%.[8]

Within PV subgroups, the percentage of CD11b and CD62P expressions were not significantly different with relatively higher percentages of both cluster of differentiation (CD) markers within thrombosis

subgroup. Postinflammatory phase is expected to fade after 6 weeks from acute-phase reaction.[31] This can either explain the insignificantly higher level of CD markers in PV patients with thrombosis or it might be due to the relatively short detection time of these markers in the circulating blood.[37,38] Nevertheless, CD11b result coincides with Murphy and Tall[39] who stated that leukocytosis remains the strongest risk factor for thrombosis in people with PV. Moreover, those patients were already under regular treatment.

Correlation of cluster of differentiation markers with JAK2 mutation rate

A positive correlation was demonstrated between *JAK2*^{V617F} mutation rate percent and each of CD11b and CD62P. This result was also noticed by other researchers[24,40,41] who implied that platelet activation is moderated by the *JAK2* mutation, and consequently, P-selectin stimulates leukocytes recruitment and activation at the sites of endothelial damage, thus facilitates the formation of platelet-leukocyte complexes. Accordingly, and through P-selectin overexpression existence, *JAK2* mutation ensue a prothrombotic state.

Accordingly, these results speculates a reliable relationship between the *JAK2* mutation rate in PV patients and their propensity to thrombosis, and eventually support the role of this mutation in the development of cellular alterations in a hematopoietic system that ultimately aids in prothrombotic tendency.

In summary, our study showed an overexpression of neutrophilic CD11b and platelets CD62P in patients with PV which can be considered as marker of procoagulant activity in relation to blood cells. These events relate blood cell activations to inflammation and this last, in turn, play a substantial role in the thrombotic risk of myeloproliferative neoplasms. The aforementioned mechanisms of prothrombosis in PV are not existing in pathogenesis of secondary polycythemia.

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Conflicts of interest

There are no conflicts of interest.

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