




Unexpected low expression of platelet fibrinogen receptor in patients with chronic myeloproliferative neoplasms: how does it change with aspirin?

Alessandro Lucchesi,¹  Silvia Carloni,² Serena De Matteis,²  Martina Ghetti,² Gerardo Musuraca,¹ Monica Poggiaspalla,¹ Accursio F. Augello,¹ Giulio Giordano,³ Pier P. Fattori,¹ Giovanni Martinelli⁴ and Roberta Napolitano² 

¹Hematology Unit, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, ²Biosciences Laboratory, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola, ³Internal Medicine Division, Hematology Service, Regional Hospital "A. Cardarelli", Campobasso and ⁴Scientific Directorate, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola, Italy

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Correspondence: Alessandro Lucchesi, Hematology Unit, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, via Maroncelli 40, 47014 Meldola, Italy.
E-mail: alessandro.lucchesi@irst.emr.it

Patients affected by Philadelphia-negative chronic myeloproliferative neoplasms (MPNs) are considered at high risk of thrombo-haemorrhagic events, but the role of the platelet count in the assessment of the risk of vascular events is still controversial (Bucalossi *et al.*, 1996; Finazzi *et al.*, 1996).

A tight correlation was found between the platelet count and plasma sCD40L (Viillard *et al.*, 2002), which appears to be required for thrombus formation *in vivo* (André *et al.*, 2002). However, sCD40L is increased both in MPNs and reactive thrombocytosis (Viillard *et al.*, 2002). Intravascular aggregates of platelets and leukocytes, mediated by P-selectin on the former and CD11b on the latter, have been observed (Cervantes *et al.*, 2009). The expression of CD11b is even more evident in patients with mutation of the *JAK2* gene Val617Phe (Coucelo *et al.*, 2014). Both of these

Summary

This study was conducted to evaluate the expression of fibrinogen receptors on platelets of Philadelphia-negative chronic myeloproliferative neoplasm (MPN) patients. We collected blood samples from 40 consecutive MPN patients and healthy volunteers. We performed flow cytometry analysis of P-selectin expression and integrin beta-3, activation of glycoprotein (GP) IIb/IIIa and fibrinogen receptor exposure (PAC-1 binding). Surprisingly, we found a very low PAC-1 binding capacity in MPN patients; however, the expression of PAC-1 was almost completely recovered with aspirin intake. We hypothesize that the hypercoagulable states observed in MPN patients could depend on a primarily plasma-driven impairment of fibrin turnover and thrombin generation.

Keywords: platelet fibrinogen receptor, myeloproliferative neoplasms, PAC-1, aspirin.

processes should imply a perpetual — and measurable — platelet activation. Several studies on platelet function have been already proposed. Nevertheless, the mechanisms through which platelets are able to trigger vascular events are not yet adequately clarified. A refined method for the determination of platelet activation appears to be the use of platelet PAC-1 antibody, able to identify the expression of the fibrinogen receptor of platelet glycoprotein IIb/IIIa (Lu & Malinauskas, 2011). This expression is indeed unique in the process of platelet activation, and yet rarely analyzed. One of the few reports demonstrating platelet fibrinogen receptor expression in MPN patients documented a decrease in PAC-1 binding, despite a normal expression of GP IIb/IIIa. This finding was more marked in myelofibrosis (MF) than in essential thrombocythaemia (ET) or polycythaemia

vera (PV) (Jensen *et al.*, 2000). Moreover, since the exposure of platelet fibrinogen receptor seems to be influenced by turbulence in blood flow, we estimated that its evaluation could provide important biological evidence to explain some clinical manifestations, such as microvascular disturbances.

Patients and Methods

After obtaining informed consent from patients and the approval of the local Ethics Committee, blood samples from 40 consecutive MPNs patients who never received cytoreductive agents were obtained. Clinical characteristics of patients are reported in Table I. In all, 28/40 patients were receiving a continuative antiplatelet prophylaxis with low-dose aspirin (ASA, 75–100 mg) at the time of collection, while 12/40 of them were not on such therapy.

Flow cytometric analyses were performed using a FACS-Canto flow cytometer (Becton–Dickinson, Franklin Lakes, NJ, USA) and 50 000 events were recorded for each sample. Our aim was to verify the expression of platelet fibrinogen receptors (PFRs) in the two different groups of patients compared to healthy volunteers, using whole blood flow cytometry. In each experiment sodium citrate and heparin tubes were collected from the same patient (positive control of platelet activation). Within 10 min from blood sampling, 5 μ l of whole blood from each tube was incubated for 20 min at room temperature in the dark with a saturated concentration of CD61 peridinin-chlorophyll proteins (PerCP), CD62P phycoerythrin (PE) and PAC-1 fluorescein isothiocyanate (FITC). Positive control was also incubated with PAC-1 in

the presence of Arg–Gly–Asp–Ser (RGDS) in order to test the specific antibody binding. Samples were fixed with paraformaldehyde 1% for 30 min at 4°C in the dark and analyzed on a flow cytometer. Prism 8.0.1 (GraphPad, San Diego, CA, USA) was used for statistical analysis. To compare continuous response variables between two groups a Mann–Whitney *U*-test was performed. $P < 0.05$ was considered as statistically significant. To measure the linear correlation between two variables the Pearson correlation coefficient was used.

Results

Surprisingly, we were able to verify a very low binding of PAC-1 to platelets in patients with MPNs not receiving cytoreduction nor antiplatelet agents if compared to that observed in healthy subjects (35.3 ± 12.9 vs 65.3 ± 24.2 respectively, $P = 0.008$). Conversely, the use of aspirin seems to restore the expression of platelet fibrinogen receptor, as PAC-1's binding capacity is comparable to that of healthy volunteers (56.7 ± 18.7) (Fig 1). No difference was found with respect to the *JAK2* Val617Phe mutation and its allele burden (data not shown). Interestingly, by focusing on the group of patients under antiplatelet prophylaxis and with no history of thrombosis, it was found that subjects with persistent microcirculatory disorders (MD) show a higher PAC-1 binding capacity if compared to the asymptomatic ones (67.7 ± 17.8 vs 51.7 ± 14.0 respectively, $P = 0.030$) (Fig 2).

Discussion

The expression of fibrinogen receptors has already been previously evaluated in patients with microcirculation disorders similar to those observed in MPNs (Raynaud's phenomenon). Interestingly, in the paper by Polidoro *et al.* (2012), particularly high values of PAC-1 expression were reported, but with an inverse relation to the circulating levels of thromboxane B2. The authors hypothesized a possible downregulation of GP IIb/IIIa as a response to chronic

Table I. Patients and characteristics.

MPNs	
No. patients	40
Gender	
Male	17 (42.5)
Female	23 (57.5)
Median age, years (range)	64 (32–86)
Pathology	
(A) Essential thrombocythaemia	22/40
Platelet count $10^9/l$ (mean \pm SD)	691.18 ± 264.40
Haematocrit (mean \pm SD)	41.00 ± 5.50
<i>JAK2</i> allele burden (mean \pm SD)	17.67 ± 15.46
CALR mut exon 9 – Type 2	2/22 (9%)
(B) Polycythaemia vera	13/40
Platelet count $10^9/l$ (mean \pm SD)	415.08 ± 176.96
Haematocrit (mean \pm SD)	48.93 ± 3.06
<i>JAK2</i> allele burden (mean \pm SD)	47.85 ± 34.18
CALR mut exon 9 – Type 2	0/13 (0%)
(C) Myelofibrosis	5/40
Platelet count $10^9/l$ (mean \pm SD)	461.60 ± 32.18
Haematocrit (mean \pm SD)	43.64 ± 7.73
<i>JAK2</i> allele burden (mean \pm SD)	12.63 ± 11.74
CALR mut exon 9 – Type 2	1/5 (20%)

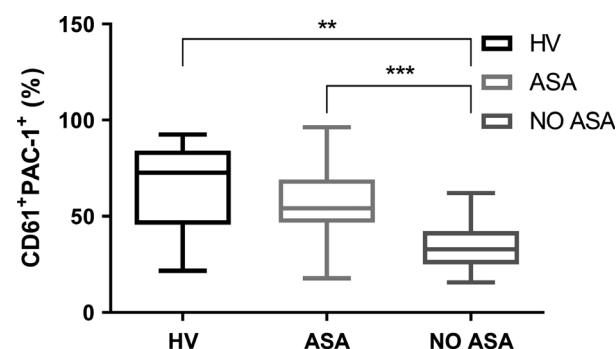


Fig 1. PAC-1 frequency in whole blood obtained from healthy volunteers (HV, $n = 10$) and myeloproliferative neoplasm (MPN) patients treated or not with low-dose aspirin (ASA, $n = 28$ and NO ASA, $n = 12$). ** $P = 0.008$, *** $P < 0.001$.

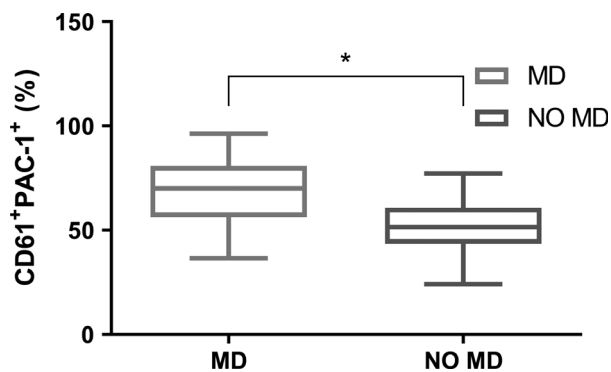


Fig 2. PAC-1 frequency in whole blood obtained from MPN patients stratified according to the presence of microcirculatory disorders (MD, $n = 13$ and NO MD, $n = 9$). * $P < 0.05$.

exposure to thromboxanes. In MPNs, platelet counts are usually high, as well as the rate of vascular events. Moreover, since MPNs are characterized by high turbulence of blood flow — and consequently by an increase in endothelial shear stress — the PAC-1-binding capacity was expected likely to be high, by analogy to Raynaud's phenomenon. As a matter of fact, there is already evidence of how the binding between fibrinogen and GP IIb/IIIa is important in shear stress-induced platelet aggregation (SIPA). Inhibitors of thromboxane synthesis seem able to counteract SIPA (Ikeda *et al.*, 1988; Floyd & Ferro, 1988). Surprisingly, we observed that in untreated MPNs large amounts of platelets are resting in a conformation that is unable to bind fibrinogen, as demonstrated by the low PAC-1 expression in cytofluorometry. This lack of activity can be reversed by administering ASA, which is also known for its fibrinolytic and hypoprothrombinemic effects. We hypothesize that the hypercoagulable states observed in these patients could depend on a primarily plasma-driven impairment of fibrin turnover and thrombin generation. A study by Moore *et al.* (2013) found an impaired fibrinogen binding in ET patients, despite normal levels of GP IIb/IIIa receptors. In the same study, interestingly, the authors describe enhanced Protease-Activated Receptor-1 (PAR1)-mediated expression of GP IIb/IIIa after thrombopoietin stimulation, followed by the disappearance of fibrinogen binding sites. Starting from these observations, we can speculate that an increased generation of thrombin in

MPN patients could secondarily lead to PAR1 activation, determining both a major conversion of fibrinogen into fibrin and the disappearance of fibrinogen binding sites on platelets with a reduced PAC-1 expression. As is well known, PAR receptors are expressed in platelets, endothelium, and smooth muscle, contributing to both normal and pathological haemostasis (Leger *et al.*, 2006). PAR1 activation could thus be a key element for the pathogenesis of thrombosis in MPNs. The results of our experiment are also similar to those observed in patients with pre-infarct angina, where a reduced expression of PAC-1 is described (Scalone *et al.*, 2013), and to what occurs months after an acute ST segment elevation myocardial infarction, with a progressive increase in the expression of the platelet fibrinogen receptor despite dual antiplatelet therapy (Scalone *et al.*, 2011). Indeed, in acute cardiovascular diseases the role of plasma procoagulants such as tissue factor was considered fundamental (Steffel *et al.*, 2006). Finally, PAC-1 could at the same time be a good marker of aspirin resistance in patients experiencing microcirculatory disorders. Further investigations are continuing in our group.

Conflict of Interest

The authors declare no potential conflicts of interest.

Author contributions

AL devised and directed the research project, and wrote the paper. SC and RN designed and performed experiments, analysed data and co-wrote the paper. SDM and MG performed experiments and contributed to improve the methodology. AL, G Musuraca, MP, AFA and PPF worked on patient enrollment and provided clinical data. GG and G Martinelli contributed to the interpretation of the results. All authors provided critical feedback and approved the final version of the manuscript.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Patients and characteristics.

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