JAK2V617F mutation and hydroxyurea treatment as determinants of immature platelet parameters in essential thrombocythemia and polycythemia vera patients

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Running title: Immature platelets in Myeloproliferative Neoplasms

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Abstract

Immature platelets (IPF), which are hemostatically more active than mature platelets, have been found elevated in Essential Thrombocythemia (ET) and Polycythemia Vera (PV), two myeloproliferative neoplasms (MPN) characterized by an increased risk of thrombosis. It is not known whether the IPF levels are influenced by pathogenetic factors including \textit{JAK2V617F} mutational status, or by treatment regimen. To address this point, in 46 ET and 38 PV consecutive patients, we measured IPF and correlated the results to \textit{JAK2V617F} mutation and myelosuppressive treatment with hydroxyurea (HU). This analysis provides two new elements regarding IPF and MPN. The first finding is that the \textit{JAK2V617F} mutation is linked to the quantity of IPF in patients with MPN, which might contribute to the prothrombotic phenotype in these patients. The second finding is that IPF is susceptible to myelosuppressive treatment, which may additionally explain the favourable effect of HU therapy on MPN outcome as well as the associated thrombotic risk.
Introduction

Circulating platelets are heterogeneous in size and structure. A small percentage of circulating platelets (i.e., 2%) are the so-called reticulated or immature platelets (IPF), recently released from the bone marrow. In-vitro studies show that newly formed murine platelets have increased hemostatic activity compared to mature platelets, as demonstrated by the increased response to thrombin and higher expression of surface P-selectin.¹

Elevated numbers of IPF population have been described in two myeloproliferative neoplasms (MPN) characterized by an increased thrombotic risk, i.e., Essential Thrombocythemia (ET) and Polycythemia Vera (PV), as well as in other thrombotic disorders.²⁻⁴ It is not known whether this increase is influenced by pathogenetic factors including JAK2V617F mutational status, or by treatment regimen in ET and PV patients. Therefore, in this study we enrolled ET and PV patients to characterize immature platelet parameters according to JAK2V617F mutation and treatment.

Methods

IPF were measured in whole blood by the fully automated hematology analyser XE-2100 (Sysmex Co., Kobe, Japan) and are described as total IPF count, % of IPF and H-IPF, the latter representing the percentage of platelets within the IPF area with a major amount of m-RNA and thus with the highest fluorescent intensity.⁵⁻⁶ The collection of venous blood samples and JAK2V617F mutation analysis were performed as previously described.⁷ All procedures were approved by the Institutional Review Board of the Ospedali Riuniti, Bergamo, Italy. All statistical analyses were done with SPSS version 15 (SPSS, Chicago, IL). The results are presented as mean ± standard deviation. The intergroup data comparisons were performed using the Student t-test or the nonparametric Mann-Whitney-Wilcoxon U test, according to the distribution of the test variables. All reported P values are two sided with a type I error rate of 5%.
Results and Discussion

This study was conducted in 42 healthy subjects and in a consecutive series of 46 ET and 38 PV patients. Twenty three ET and 18 PV were receiving cytoreductive therapy with hydroxyurea (HU), 35 (16 ET and 19 PV) were on aspirin alone and 8 (7 ET and 1 PV) were not receiving any cytoreductive or aspirin treatment. Twenty three ET patients (50%) and 34 PV patients (89%) have the \textit{JAK2V617F} mutation. The mutation in all ET patients was heterozygous, whereas among PV patients, 27 were heterozygous and 7 were homozygous for the \textit{JAK2V617F} mutation. Two PV patients, negative for \textit{JAK2V617F}, were positive for the \textit{JAK2 exon12} mutation.

Both ET and PV patients had significantly higher platelet and IPF counts compared to controls (p<0.01). In addition, PV patients had significantly (p<0.01) higher hematocrit, white blood cell count, % of IPF and H-IPF compared to both controls and ET patients. Compared to control subjects, \textit{JAK2V617F} positive patients showed significantly (p<0.05) higher platelet (522±260 vs 238±58 x10⁹/L) and IPF count (15±14 vs 5.0±2.0 x10⁹/L), % of IPF (2.8±1.6 vs 2.2±1%) and H-IPF (0.9±0.6 vs 0.6±0.3%), whereas \textit{JAK2V617F} negative patients had significantly higher platelet (594±283 x10⁹/L) and IPF counts (12±8 x10⁹/L). Figure 1 shows the data after grouping the patients according to both \textit{JAK2V617F} mutation and HU treatment. Non-HU \textit{JAK2V617F} positive patients showed significantly (p<0.05) higher platelet count (Figure 1A), IPF count (Figure 1B), % of IPF (Figure 1C) and of H-IPF (Figure 1D) compared to control subjects. In addition, non-HU \textit{JAK2V617F} positive patients showed significantly higher IPF count also compared to non-HU \textit{JAK2V617F} negative patients and HU treated \textit{JAK2V617F} positive and negative patients (Figure 1B). Within non-HU treated subjects, \textit{JAK2V617F} positive patients showed a significantly higher % of H-IPF compared to \textit{JAK2V617F} negative patients (Figure 1D). Finally, within patients negative for \textit{JAK2V617F} mutation, no significant differences in IPF parameters were observed between HU and non-HU treated patients. Differently, within patients positive for \textit{JAK2V617F} mutation, HU
treated patients showed significantly lower (p<0.05) IPF parameters compared to non-HU treated patients (IPF count: 8.7±4 vs 22±18 x10^9/L; IPF%: 2.4±1.0 vs 3.3±2.1).

Multivariate regression analysis adjusted for age, sex, JAK2V617F mutational status and HU therapy confirmed that JAK2V617F mutation is significantly associated (p<0.01) with higher % of IPF (B=0.42) and H-IPF (B=0.38), and IPF count (B=0.7); while HU treatment is significantly associated (p<0.01) with lower % of IPF (B=-0.43) and H-IPF (B=-0.32), and IPF counts (B=-0.45).

The standard flow cytometric measurement of IPF is considered imprecise, time consuming, expensive and most importantly no inter-laboratory consensus of the normal range has been obtained as yet.\textsuperscript{5,8} Therefore, we have evaluated the percentage of IPF in controls and patients by the recently established fully automated and rapid Sysmex XE-2100 method. The new XE-2100 expands the quantitative analytic range to reticulocytes, immature granulocytes and to optical fluorescent platelet count. The reference range in our study for the % of IPF in healthy subjects using this method was 0.7-6% with a mean value of 2.2%, which is in agreement with other studies.\textsuperscript{5,9}

The measurement of IPF has been considered useful in assessing the treatment response and thrombotic risk in patients with thrombocytosis, both primary and secondary.\textsuperscript{10} Increased IPF has also been associated with increased thrombotic risk, i.e. in acute coronary syndromes and in cardioembolic stroke.\textsuperscript{4,11} In addition, increased % of IPF and H-IPF has been associated with increased platelet aggregation in high risk patients with coronary artery disease.\textsuperscript{6} Similarly, renal transplant recipients which are at increased risk for cardiovascular mortality showed higher % of IPF and H-IPF associated with increased platelet aggregation.\textsuperscript{12} Due to an increased platelet turnover, MPN patients have a higher percentage of IPF compared to healthy controls.\textsuperscript{3} In our study we found that the absolute counts of IPF, were significantly (p<0.01) increased in both ET and PV patients compared to controls, while the % of both IPF and H-IPF were significantly increased in PV patients only. The analysis according to JAK2V617F mutational status and HU treatment
showed that non-HU treated MPN mutation positive patients had significantly higher % of IPF and H-IPF compared to control subjects. These findings suggest that JAK2V617F mutation might be an important factor related to the increased % of IPF and H-IPF in MPN patients. The adjusted multivariate analysis demonstrating that JAK2V617F mutation is associated with increased IPF count, % of IPF and H-IPF confirmed our results. Interestingly, our study showed that within patients positive for JAK2V617F mutation, HU-treated patients have significantly lower IPF parameters compared to non-HU treated patients, which was not observed within JAK2V617F negative patients. This finding is in line with a recently published study showing in MPN subjects positive for JAK2V617F mutation an increased chemosensitivity to HU.13

In conclusion, this study provides two new elements regarding the association between IPF and MPN. The first finding is that the JAK2V617F mutation is linked to the quantity of IPF in patients with MPN, which might contribute to the prothrombotic phenotype in these patients. The second finding is that IPF is susceptible to HU treatment, which may additionally explain the favourable effect of this therapy on MPN as well as the associated thrombotic risk. New prospective studies are warranted to evaluate the usefulness of IPF as a cellular marker to predict thrombosis in MPN patients.

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**Authorship**

P.N.M. designed and performed research, collected the clinical data, analyzed and interpreted data, and wrote the paper; M.M. designed research, analyzed and interpreted data, and wrote the paper; A.F. designed research, interpreted data and contributed to writing the paper; H.t.C. contributed to writing the paper; B.S. performed research; L.R. and A.L. performed research and recruited healthy
control subjects; A.R and G.F. contributed to patient recruitment; O.C. contributed to critical review and discussion of the results.

**Conflict-of-interest disclosure:** The authors declare no competing financial interest.
References:

Figure legend

**Figure 1: IPF parameters according to JAK2V617F mutation and therapy**

The data are reported as mean±SD. CTR=Controls; HU=Hydroxyurea; JAK2+=JAK2V617F positive patients; JAK2-=JAK2V617F negative patients. *p<0.05 vs CTR; § p<0.05 vs HU JAK2+; £ p<0.05 vs HU JAK2+, HU JAK2-; &p<0.05 vs non-HU JAK2-, HU JAK2-, HU JAK2+; **p<0.05 vs non-HU JAK2-.
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