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ORIGINAL ARTICLE

Efficacy of Antiplatelet Therapy in Myeloproliferative Diseases in Relation to Immature Platelets and Thrombosis

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SUMMARY

Background: **Patients with myeloproliferative diseases have an increased risk and incidence of thrombotic complications. The mechanisms involved in the pathogenesis of this acquired thrombophilic state are multifactorial. One mechanism reported in the literature is increased thrombopoiesis and with it associated increase of young, immature platelets in peripheral blood. This increased washout of new platelets, which are unaffected by antiplatelet drugs, may lead to a reduced response to antiplatelet therapy.**
Methods: **In our study, we aimed to monitor the efficacy of the standard of antiplatelet therapy with 100 mg of acetylsalicylic acid in relation to immature platelet size fraction and platelet count in a group of 86 patients with myeloproliferative diseases. The efficacy of antiplatelet therapy was investigated by the ASPItest method on the multiplate impedance aggregometer.**
Results: **The results demonstrated that the efficacy of low-dose acetylsalicylic acid treatment was independent of immature platelet fraction size (0.2600, $p = 0.0156$) and absolute platelet count (0.4505, $p < 0.0001$). Detailed analysis of the cohort showed no association between the incidence of thrombotic complications and the ASPItest value, nor was there an association between the incidence of thrombotic complications and the size of the immature platelet fraction or the total platelet count.**
Conclusions: **The mechanisms not assessable by impedance aggregometry are involved in the development of thrombotic complications in patients with MPD; the value of the ASPItest does not provide comprehensive information about thrombotic risk.**
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INTRODUCTION

Myeloproliferative disorders (MPDs) are clonal diseases caused by malignant transformation of hematopoietic stem cells, leading to uncontrolled proliferation and subsequent differentiation of one hematopoietic lineage, while inducing less pronounced proliferation of blood cells of other lineages.

MPDs are categorized according to the presence of the Philadelphia chromosome, which distinguishes between Ph-positive and Ph-negative diseases. Ph-negative MPDs include polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). The hallmark of these diseases is the presence of characteristic mutational changes in genes such as JAK-2V617F (JAK2) mutation, calreticulin (CALR) mutation, and MPL W515L/K (MPL). In a significantly smaller proportion of cases, MPD is not accompanied by any of these mutations.

Patients diagnosed with myeloproliferative disease (MPD) have an elevated risk and incidence of thrombotic complications, with reported incidence of thrombosis ranging from 12 - 39% in PV and 11 - 25% in ET [1]. Indeed, thrombosis may be the first symptom leading to the diagnosis of MPD. The majority of thrombotic complications are classified as arterial thrombosis, accounting for 60 - 70% of cases [2]. The typical manifestations of arterial thrombosis include myocardial infarction, stroke, and peripheral artery occlusion. Deep venous thrombosis manifests in various forms, including deep vein thrombosis of the lower extremities, upper extremities, pulmonary embolism, visceral vein thrombosis, splanchnic veins, and Budd-Chiari syndrome. The incidence of venous thrombosis is more frequent in patients with polycythemia vera, where it accounts for up to one-third of thrombotic complications. Platelet thrombi lead to occlusion of small blood vessels, which are clinically manifested as erythromelalgia, transient ischemic attack (TIA), headache, vertigo, visual and hearing disturbances, recurrent miscarriages, and fetal developmental disorders. Risk factors for thrombosis include advanced age (over 60 years), a personal or family history of thrombotic events, the presence of a JAK2 mutation, and cardiovascular risk factors.

The mechanisms involved in the pathogenesis of this acquired thrombophilic condition are multifactorial. Platelet-related factors include the release of microparticles from platelets [3] and increased thrombopoiesis associated with ongoing MPD, a condition that is particularly exacerbated in patients with JAK2 mutations[2]. Immature platelets, newly released from the bone marrow, exhibit heightened hemostatic activity in comparison to their mature counterpart and have increased prothrombotic potential. The immature platelet fraction (IPF) parameter is a quantitative metric that quantifies the number of young platelets.

In MPDs, platelets have been observed to exhibit increased expression of P-selectin and tissue factor while concurrently demonstrating diminished responsiveness to ADP and epinephrine stimulation [4,5]. The potential for laboratory artefacts, resulting from the dilution of platelet-rich plasma, to influence these measurements has been a subject of consideration [5]. Consequently, the impedance aggregometry method was selected for the present study.

Further evidence for platelet activation in these diseases is provided by elevated levels of beta-thromboglobulin, platelet factor 4 in plasma, and thromboxane A2 metabolites in urine [6,7]. Activation of the platelet surface by phosphatidylserine provides a catalytic surface for thrombin generation and the process of secondary hemostasis.

In the treatment of patients diagnosed with MPDs, standard antiplatelet therapy is employed, comprising low-dose (50 - 100 mg daily) acetylsalicylic acid (ASA) or, in the event of acetylsalicylic acid intolerance, clopidogrel at a dose of 75 mg daily. Increased washout of new platelets not affected by antiplatelet drugs may lead to a reduced response to antiplatelet therapy [8,9]. Some studies have confirmed a more consistent platelet inhibition when antiplatelet therapy is administered with ASA twice daily versus once daily [10].

The primary objective of the present study was to verify the hypothesis that immature platelet fraction value is elevated in patients diagnosed with Ph-negative myeloproliferative diseases. The further objectives of this study were to determine whether elevated immature platelet fraction values result in reduced efficacy of antiplatelet therapy and whether reduced efficacy of antiplatelet therapy increases the incidence of thrombotic complications.

MATERIALS AND METHODS

Patients

The prospective observational study was conducted between November 2022 and April 2024. The study population comprised patients over the age of 18 years who were dispensed in the hematology outpatient department of the University Hospital Pilsen with Ph-negative MPD (PV, ET, PMF, unspecified MPD, both newly diagnosed and not treated with cytoreductive therapy, and those treated for a long time).

A total of 94 patients were enrolled in the study, and 86 patients treated with a standard dose of 100 mg of ASA were selected for statistical analysis. Patients treated with clopidogrel due to ASA intolerance and patients receiving a different dose of ASA were excluded from the statistical evaluation.

This study was conducted ethically in accordance with the World Medical Association Declaration of Helsinki, and the protocol was reviewed and approved by the Ethical Committee of the University Hospital and the Faculty of Medicine, Charles University in Pilsen, Czech Republic, on February 2nd, 2023, approval number 48/23.

All patients participating in the study completed and signed the participant informed consent form. This was a non-interventional cohort study, and collection and storage of data were performed by the investigators directly involved in the patients' care using current techniques to ensure privacy.

Table 1. Patient characteristics.

Gender	**male**	**40% (n = 34)**
	female	**60% (n = 52)**
Age	**20 - 95 years**	
Diagnosis	**ET**	**52% (n = 45)**
	PV	**22% (n = 19)**
	PMF	**6% (n = 5)**
	ET/PMF	**6% (n = 5)**
	ET/PV	**7% (n = 6)**
	unspecified	**7% (n = 6)**
Mutation status	**JAK2 mutation V617F**	**79% (n = 68)**
	CALR	**14% (n = 12)**
	MPL	**1% (n =1)**
	no mutation	**6% (n = 5)**
Treatment	**Hydroxyurea**	**56% (n = 48)**
	Peginterferon alfa-2a	**20% (n = 17)**
	Anagrelid	**19% (n = 16)**
	Hydroxyurea + peginterferon alfa-2a	**2% (n = 2)**
	Hydroxyurea + anagrelid	**2% (n = 2)**
	only ASA	**1% (n = 1)**

Table 2. ASPItest [U] value in relation to the incidence of thrombotic events.

	ASPItest [U] median (min - max value)
No thrombotic event	**36.0 (7 - 142)**
Thrombotic event	**35.5 (11 - 100)**
Thrombotic event before MPD diagnosis	**35.0 (11 - 100)**
Thrombotic event after MPD diagnosis	**31.5 (22 - 53)**

Sample collection

Blood for platelet function testing by impedance aggregometry was collected into a tube containing the anticoagulant hirudin (15 IU/mL), a direct thrombin inhibitor. Samples were collected by the same nurses as during routine recruitment. Each sample collected was immediately and gently inverted six times and transported to the laboratory as quickly as possible. All samples were processed within 2 hours of collection.

For the IPF test, which is part of the blood count analysis, blood was drawn into a 2 mL tube containing the anticoagulant K_3EDTA. Immediately after collection, the sample was gently inverted six times and transported to the laboratory as quickly as possible. All samples were processed within 4 hours of collection.

Laboratory methods

Platelet aggregation

Samples were analyzed on a Multiplate impedance aggregometer (Roche, Basel, Switzerland) using the ASPItest method. The aggregation reaction was initiated by adding the inducer, arachidonic acid, at a final concentration of 15 mM. Platelet adhesion and aggregation were monitored through increasing impedance and expressed as aggregation units, i.e., U.

An ASPItest cutoff value ≤ 30 U was used to assess the efficacy of ASA treatment (Roche, Multiplate® analyzer, cutoff values ADPtest and ASPItest, version 2.0, 2014). Platelet aggregation results below the cutoff value were assessed as sufficiently effective antiplatelet treatment, and results above the cutoff value as laboratory ineffective antiplatelet treatment.

IPF (immature platelet fraction)

IPF is part of the complete blood count procedure, which is conducted on the Sysmex XN 1000 analyzer. It is an add-on method determined in the fluorescence channel of the analyzer by fluorescence flow cytometry. The reference range for the absolute IPF count in healthy adults, as recommended by the manufacturer of the analyzer used, is 2.5 - 17.8 x 10^9/L.

Statistics

Statistical analysis was performed using GraphPad Prism 9.0 software (GraphPad Software, San Diego, CA, USA). The normality of the data was tested and, according to the results, the non-parametric test, Spearman's correlation analysis, was used.

RESULTS

In the studied cohort of patients with MPDs, an increased absolute number of IPF demonstrated to be in accordance with the literature. A total of 69% (59/86) of patients had values above the upper reference range (median 23.8 x 10^9/L, min - max value 3.2 - 74.5 x 10^9/L, reference range 2.5 - 17.8 x 10^9/L). Absolute platelet counts were also elevated in 59% (51/86) of patients (median 421 x 10^9/L, min - max value 95 - 972 x 10^9/L, reference range 150 - 400 x 10^9/L).

Spearman's correlation test was conducted, revealing a very weak correlation between absolute IPF count and ASPItest value (0.2600, $p = 0.0156$) and a weak correlation between platelet count and ASPItest value (0.4505, $p < 0.0001$). Consequently, the efficacy of antiplatelet therapy is independent of both the absolute IPF count and the total platelet count.

The evaluation of the ASPItest results in relation to the

Table 3. Absolute IPF value [10^9/L] and platelet count (PLT) [10^9/L] in relation to the incidence of thrombotic events.

	Absolute IPF value [10^9/L] (reference range 2.5 - 17.8 x 10^9/L) median (min - max value)	Absolute PLT value [10^9/L] (reference range 150 - 400 x 10^9/L) median (min - max value)
No thrombotic event	22.7 (4.0 - 74.5)	447 (158 - 972)
Thrombotic event	26.0 (3.2 - 6.2)	390 (203 - 855)
Thrombotic event before MPD diagnosis	30.2 (3.2 - 66.2)	465 (203 - 855)
Thrombotic event after MPD diagnosis	19.5 (10.4 - 45.0)	314 (95 - 406)

Table 4. Incidence of thrombotic events in relation to the mutation status.

Thrombotic event	Mutation
No thrombotic event - 64% (n = 55)	JAK2 - 73% (40/55)
	CALR - 18% (10/55)
	MPL - 2% (1/55)
	no mutation - 7% (4/55)
Thrombotic event - 36% (n = 31)	
Thrombotic event before MPD diagnosis - 30% (n = 26)	JAK2 - 92% (24/26)
	CALR - 4% (1/26)
	no mutation - 4% (1/26)
Thrombotic event after MPD diagnosis - 8% (n = 7)	JAK2 - 71% (5/7)
	CALR - 29% (2/7)

Table 5. Incidence of thrombotic events in individual causal mutations.

	n	No thrombotic event	Thrombotic event before MPD diagnosis	Thrombotic event after MPD diagnosis	Thrombotic event before and after MPD diagnosis
JAK2	68	40	24	5	1
CALR	12	10	1	2	1
MPL	1	1	0	0	0
No mutation	5	4	1	0	0

incidence of thrombotic events revealed no difference between the patient groups experiencing and not experiencing thrombotic events, as demonstrated in Table 2 and Figure 1.

Two patients exhibited thrombotic events prior to and following diagnosis; one patient with JAK2 mutation (ASPI test value 53 U) and one with CALR mutation (ASPI test value 39 U).

Similarly, when the absolute values of IPF and platelet count were compared, no difference was observed in the group with and without thrombotic event, as illustrated in Table 3. and Figure 2.

The analysis of the cohort in terms of the representation of individual thrombotic complications in relation to the time of diagnosis revealed that before diagnosis of MPDs deep vein thrombosis, thrombophlebitis/phlebothrombosis, or pulmonary embolism was present in six patients, stroke or TIA was present in seven patients, lower limb ischemia with acute vascular occlusion was present in six patients, ischemic heart disease was present in seven patients, and retinal vein thrombosis was present in one patient. One patient with ischemic heart

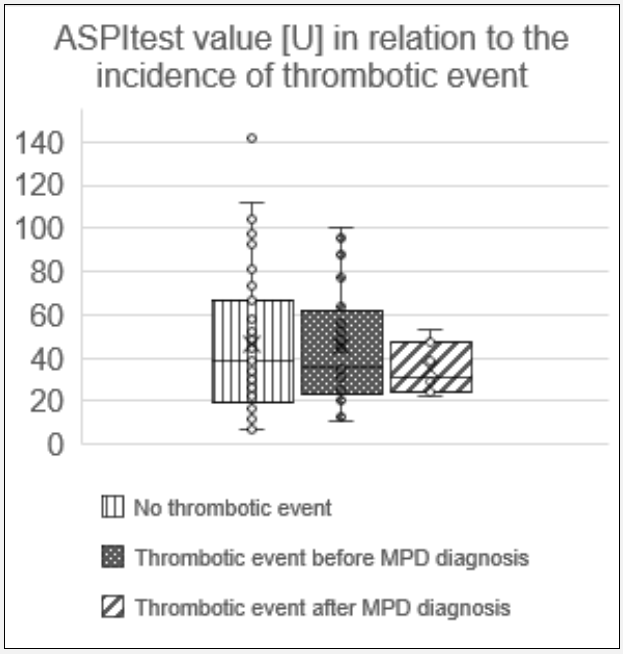

Figure 1. ASPItest [U] value in relation to the incidence of thrombotic events.

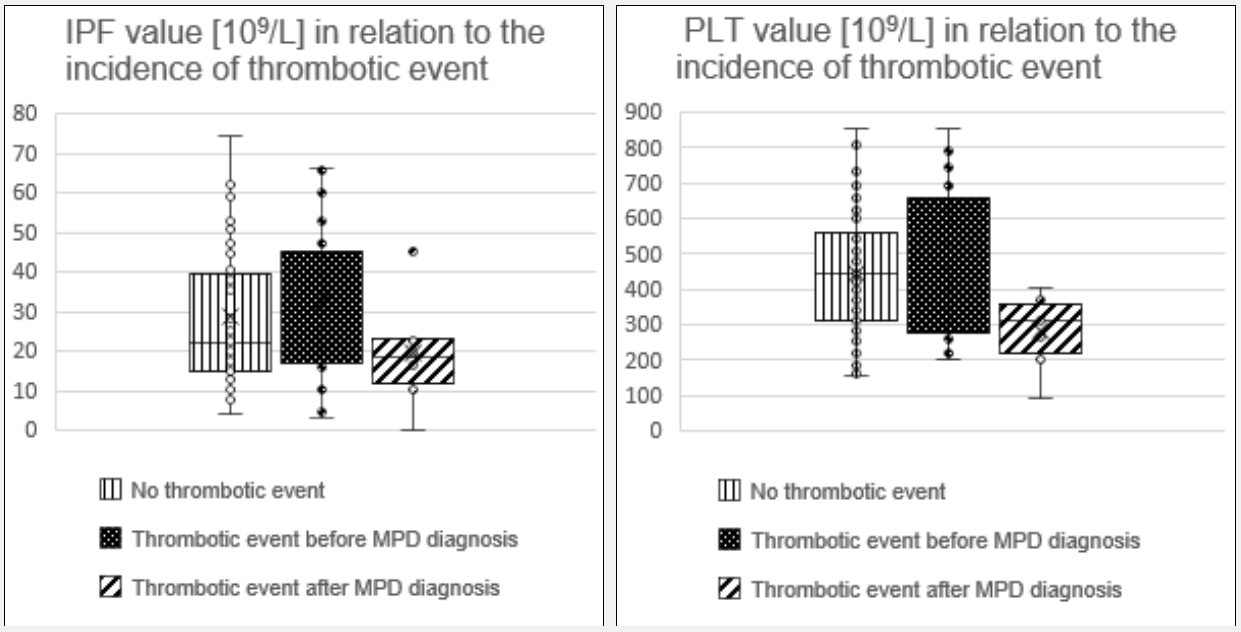

Figure 2. Absolute IPF value [10^9/L] and platelet count (PLT) value [10^9/L] in relation to the incidence of thrombotic complication.

disease had lower limb phlebothrombosis, and one patient with ischemic heart disease had stroke and lower limb phlebothrombosis.
Following the diagnosis of MPD, the most prevalent thrombotic event was the manifestation of ischemic heart disease in four patients, one patient experienced a TIA, one patient developed thrombophlebitis, and one patient sequentially experienced a TIA and thrombophlebitis.
Incidence of thrombotic complications for each causal mutation is demonstrated in Table 5.

DISCUSSION

Patients diagnosed with Ph-negative MPD have an elevated risk of thrombotic complications [1]. In the present cohort, 64% of patients did not experience thrombotic complications, while 36% of patients did experience such complications before or following the diagnosis of MPD. The present findings are consistent with the existing literature [2], the highest prevalence of thrombotic events was observed in JAK2 mutation carriers. The most prevalent thrombotic complications are observed in the arterial circulation, including ischemic heart disease, stroke, TIA, and acute vascular occlusion in lower limb ischemic disease. Thrombotic complications within the venous system are less frequent. The potential causes of thrombotic complications have been extensively reviewed in the literature. The involvement of platelets in the pathogenesis of small-vessel occlusion is suggested by the clinical response to acetylsalicylic acid administration, namely the rapid alleviation of erythromelalgia symptoms [11], and the reduced risk of cardiovascular events (acute myocardial infarction, stroke and death from cardiovascular causes) in patients with polycythemia vera treated with acetylsalicylic acid [12].
In our study, the focus was directed towards contemporary blood count parameters that have been shown to be indicative of platelet production in patients; the specific parameters under scrutiny included the fraction of immature platelets, the total platelet count, and their correlation with the efficacy of antiplatelet therapy. The study revealed an elevated absolute number of immature platelets within the study cohort; however, the efficacy of antiplatelet therapy did not appear to be contingent upon these platelet parameters or the total platelet count. The mean ASPItest value in the study group of patients with MPD is above the cutoff for effective antiplatelet therapy; however, the ASPItest value does not differ between the groups of patients with and without clinical thrombotic events. Although the standard dose of COX-1-blocking ASA did not result in a decrease in ASPItest below the desired value of 30 U, a reduction in the incidence of thrombotic complications was observed. Consequently, the increased incidence of thrombotic complications in the MPD group cannot be attributed to an insufficient effect of standard low-dose acetylsalicylic acid therapy, suggesting the involvement of an alternative mechanism.
Thus, the primary cause of thrombotic complications is not the quantity, but rather the alteration in platelet behavior. This is induced by the overall inflammatory response of the organism to cytokines and other substances released from malignant cells [2]. On clonal blood cells (platelets, erythrocytes, and leukocytes), there is an increased expression of the prothrombotic phenotype, increased expression of adhesive molecules, and then the formation of leukocyte and platelet aggregates. Endothelial integrity is compromised, and procoagulant microparticles are produced. Activated platelets and microparticles move phosphatidylserine from the inner membrane layer to the surface, providing a catalytic surface for thrombin formation. Furthermore, the process of thrombin formation is known to be enhanced by acquired resistance to activated protein C. In addition, the number of clonal platelets has been shown to decrease in association with cytoreductive therapy, which would be consistent with a lower incidence of thrombotic complications in treated patients after a diagnosis of MPD.

CONCLUSION

It is evident that mechanisms not assessable by impedance aggregometry are involved in the development of thrombotic complications in patients with MPD; the value of the ASPItest does not provide comprehensive information about thrombotic risk. However, low-dose antiplatelet therapy with acetylsalicylic acid is sufficiently effective in preventing thrombotic events in patients at all stages of MPD, regardless of the absolute number of immature platelets or total platelet count.

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Declaration of Interest:
All authors declare that they have no conflict of interest.

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