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Evaluation of thrombin generation in classical Philadelphia-negative myeloproliferative neoplasms

Evaluarea generării trombinei în neoplasmele mieloproliferative Philadelphia- negative

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Abstract

Introduction: Patients with Philadelphia-negative chronic myeloproliferative neoplasms (Ph-MPN), polycytemia vera (PV), essential thrombocythaemia (ET) and primary myelofibrosis (PMF), are prone to develop thrombotic events. We aimed to investigate the coagulation status in their plasma using thrombin generation assay (TGA), a functional global assay, on Ceveron® Alpha.

Materials and methods: The samples were collected from 89 consecutive Ph-negative MPN patients and from 78 controls into K2EDTA and CTAD tubes for blood cell counts, TGA and coagulation screening tests. Thrombin generation was analysed in platelet-poor plasma using Technothrombin® TGA assay kit.

Results: We found a significantly increased peak thrombin generation (p=0.049) and velocity index (VI) (p=0.012) in patients in comparison with controls, especially in ET patients, and a significantly higher values for peak thrombin (p=0.043) and VI (p=0.042) in patients receiving anagrelide in comparison with those treated with hydroxyurea. We also noticed an inverse correlation between the length of cytoreductive therapy and TGA parameters, (peak thrombin R=-0.25, p=0.018, AUC R=-0.257, p=0.015, and VI R=-0.21, p=0.048).

Conclusion: Our results suggest that Ph-MPN patients, and especially those with ET, are predisposed to thrombotic events due to their higher peak thrombin and VI values and their risk may decreases as treatment is longer. Patients treated with hydroxyurea generate less thrombin and could be less prone to develop thrombotic events in comparison with those treated with anagrelide.

Keywords: *thrombin generation, polycythemia vera, essential thrombocythemia, idiopatic myelofibrosis, thrombosis.*

Rezumat

Pacienți diagnosticați cu neoplasm mieloproliferativ cronic Ph-negativ (Ph-MPN), policitemia vera (PV), trombocitemia esențială (TE) și mielofibroza primară (MP) sunt predispuși la complicații trombotice pe parcursul evoluției bolii. În studiul nostru am investigat statusul coagulării în plasma acestor pacienți folosind testul de generare a trombinei (TGT), un test funcțional global, realizat pe analizorul Ceveron® Alpha. Material și metoda: Au fost

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colectate probe de sânge de la 89 pacienți cu Ph-MPN și de la 78 subiecți sănătoși în tuburi conținând K2EDTA și CTAD pentru determinarea hemoleucogramei, a testelor pentru generarea trombinei și a testelor screening de coagulare. Testul pentru generarea trombinei a fost determinant folosind Technothrombin® TGA assay kit.
Rezultate: am demonstrat o diferență semnificativă în generarea trombinei între pacienți și subiecții sănătoși (peak thrombin; p = 0,049 și velocity index (VI); p = 0,012) cu o creștere mai evidentă la pacienții cu trombocitemie esențială precum și valori semnificativ mai mari ale parametrilor TGT, peak thrombin (p = 0,043) și VI (p = 0,042), la pacienții tratați cu anagrelide în comparație cu cei tratați cu hidroxiuree. S-a evidențiat o corelație inversă între parametrii TGT și durata tratamentului citoreductiv (peak thrombin R = -0,25, p = 0,018, AUC R = -0,257, p = 0,015, și VI R = -0,21, p = 0,048).
Concluzii: Rezultatele obținute sugerează că pacienți Ph-MPN și în special cei cu TE pot fi predispuși la tromboze datorită unei generări crescute de trombină, iar riscul ar putea descreşte pe parcursul tratamentului citoreductiv. Pacienții tratați cu hidroxiuree generază mai puțină trombină și ar putea fi mai puțin expuși evenimentelor trombotice în comparație cu cei tratați cu anagrelide.

Cuvinte cheie: *generarea trombinei, policitemia vera, trombocitemia esentiala, mielofibroza primara, tromboza.*

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Introduction

Myeloproliferative neoplasms (MPN) are clonal haematopoietic stem cell disorders characterized by an excessive proliferation of one or more of the myeloid lineage (granulocytic, erythroid, magakaryocytic and mast cells) [1].

According to the World Health Organization (WHO) classification, classical Philadelphia-negative chronic myeloproliferative neoplasms (Ph-MPN) are an operational subcategory of MPN that includes polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) [1,2]. Their natural history is marked by disease-related hemostatic complications like arterial and venous thrombosis, with an incidence ranging from 12%- 39% in PV and from 11%- 25% in ET [3].

The pathogenesis of the hypercoagulable state in Ph-MPN is complex and is related to the abnormalities of blood cells which acquired a prothrombotic phenotype, to the inflammatory response of endothelial cells to the insult of cytokines released by malignant cells, and also related to clinical factors like age, previous history of thrombotic events, and the presence of cardiovascular risk factors [3,4].

A *Jak2* gain of function mutation (*Jak2V617F*) was described in approximately 50% of ET and PMF patients and in almost all PV patients, which leads to a constitutive activation of the enzyme in platelets and neutrophils [5]. Activated platelets directly participate in thrombin generation and activated neutrophils can impair numerous inhibitors of coagulation (including protein C, S, and tissue factor pathway inhibitor) by releasing intragranule-associated proteases [6].

Large meta-analysis studies reported an increased rate of thrombotic events (32% vs 20%) in patients with *Jak2V617F* mutation compared with wild type (WT) counterparts [7].

In spite of their risk of thrombosis, patients with myeloproliferative neoplasms (MPN) show little or no abnormalities of global screening coagulation tests, such as prothrombin time (PT) and activated partial thromboplastin time (APTT). These tests are unable to reflect and integrate all pro- and anticoagulant reactions that regulate the formation and inhibition of thrombin and the effect of platelets and other blood cells [8].

Global tests such as thrombin generation or thromboelastometry can detect signs of procoagulant tendency in MPN [8]. Thrombin generation

assays measure the ability of a plasma sample to generate thrombin following *in vitro* activation of coagulation with tissue factor or another trigger, and then the concentration of thrombin formed over the time is monitored [8,9].

In our study we used a fully automated device for measurements of thrombin generation and aimed to assess the procoagulant activity in the plasma of Ph-MPN patients (PV, ET and MP patients) using a functional global assay, thrombin generation assay (TGA), and to establish the influence of: presence of *Jak2617F* mutation, thrombotic history, treatment, and cardiovascular risk factors (hypertension, diabetes, dyslipidemia, smoking, overweight) on the TGA parameters.

This is the first report describing the measurement of thrombin generation in MPN-Ph negative patients using the fully automated thrombin generation device Ceveron® Alpha.

Material and methods

This prospective study consists of 89 consecutive patients diagnosed with Ph-MPN, admitted to the Hematology Department of Emergency County Clinical Hospital Sibiu and tested between January 2014 and January 2015. The diagnosis was established according to the 2001 and 2008 criteria for Ph-MPN [1,10]. The patients who underwent anticoagulant therapies were not included in our study group.

Seventy-eight healthy volunteer subjects, without history of thrombotic or bleeding events, symptoms of acute infection or chronic inflammatory diseases, without receiving anti-platelet agents or oral anticoagulants were included as controls.

In both groups, pre-analytical treatment, as well as measurement of thrombin generation was performed identically.

Laboratory methods

All blood samples were collected in the morning, after overnight fasting, from the antecubital vein, with the help of a light tourniquet, into vacuum tubes containing potassium-ethylene-diamino-tetra-acetic acid (K2EDTA), for complete blood count (CBC), and in 3.2% sodium CTAD 0.109M tubes (citrate-theophyllin-adenosine-dipyridamole Vacutainers®, glass tubes, 4.5 ml, Beckton Dickinson) for TGA assay and coagulation screening tests (PT, APTT and fibrinogen). The first 5 ml were discharged.

CBC was performed on Sysmex XT 2000i and coagulation screening tests were performed on Sysmex CA 1500 coagulation analyzer.

Blood samples for thrombin generation assay were centrifuged once, within 30 minutes after blood collection, 17 minutes at 2700 x g, at room temperature, with a light brake only, to obtain platelet-poor plasma ($< 1x10^3$ /µL for all plasmas) and aliquots were stored at -20 ºC until testing was performed in series (within one week).

Thrombin generation was determined in platelet-poor plasma using Technothrombin® TGA reagents kit (Technoclone, Vienna, Austria) for fully automated Ceveron® alpha. Technothrombin® TGA reagents for Ceveron® alpha are an assay system used to determine thrombin generation. It is based on monitoring the fluorescence generated by the cleavage of a fluorogenic substrate by thrombin, after activating the coagulation cascade by different concentrations of tissue factor and the negative charged phospholipids in plasma. From the changes in fluorescence in time, the concentration of thrombin (nM) in the sample can be calculated using the respective thrombin calibration curve.

Briefly, thrombin generation was initiated in 40µl citrated plasma by 10 µl trigger reagent (RC low, Technoclone, Vienna, Austria) containing 3.2 µM concentration of phospholipid

micelles (2.56 µM phosphatidylcholine and 0.64 µM phosphatidylserine) and 71.6 pM recombinant human tissue factor (about 0.7 pM in the reaction mixture) in Tris-Hepes-NaCl buffer and 50µl calcium clorure + fluorogenic substrate reagent. Conversion of the substrate, a fluorogenic Z-G-G-R-AMC substrate (Technoclone, Vienna, Austria), and the rate of the thrombin generation were monitored in time resulting in a thrombin formation curve. The results were automatically calculated by Ceveron® alpha software and displayed in: lag time (from the time point when the TGA reagents were added until the first burst in thrombin formation), peak thrombin (maximal concentration of thrombin formed), time to peak (tpeak), slope (the steepest rate of thrombin formation per minute calculated by software as velocity index), and area under the curve (AUC) known as the endogenous thrombin potential (ETP). Reagent, calibrator, and controls were used according to the recommendations of the manufacturer (Technoclone, Vienna, Austria). All samples were analyzed in duplicates.

Assessment of *Jak2V617* mutation was performed by an amplification refractory mutation system polymerase chain reaction (ARMS-PCR) described by Jones et al. [11].

All investigations were approved by the Ethical Committee of the Emergency County Clinical Hospital Sibiu and an informed written consent was obtained from patients and healthy controls.

Statistical analysis

For the statistical analysis we used the IBM SPSS software version 20 and EPI INFO software version 6. Data were considered nominal and quantitative. Nominal variables were characterized by frequency and percentages, and the quantitative variables by mean and standard deviation for normal variables, and median and quartiles for variables without a normal distribution. We used the Kolmogorov-Smirnov test to verify the normal distribution of quantitative variables. The comparison between two groups with normal distribution of quantitative variables was made using the Student T (T) test, Mann-Whitney (MW) test, and Median test (Med-T). The correlation between two continuous variables was made with Pearson correlation or Spearman's rho. For analysis of variances we used One-way Anova test, and for Post-Hoc multiple comparisons the Bonferroni and Tamhane's Te tests. The frequency difference from one nominal variable between two groups was evaluated with the chi-square test (Hi^2). Multiple regression analysis was used to study the influence of certain quantitative variables (age, BMI, length of treatment) on TGA parameters. Values of $p < 0.05$ were considered statistically significant.

Results

The study population consisted of 89 Ph-MPN patients (26 PV, 51 ET, 12 MF) with slightly more female than male subjects, aged 24 to 91**,** and a healthy control group (n = 78; age: 22-77), volunteers, selected based on the criteria mentioned above. Compared to the patients, the control group was younger (median age: 41) and included more females (n = 51, 65%, p = 0.0001).

Patients diagnosed with MF post PV or post ET were included in MF subgroup, along with PMF patients.

The main characteristics of the study population are summarized in **table 1**.

At the time of the enrolment, almost all patients were receiving cytoreductive therapy and/or anti-platelet treatment. All patients with cardiovascular risk factors (hypertension,diabetes, dyslipidemia) underwent treatment with antihypertensive, antidiabetic, statins or others hypolipidemic drugs.

Table 1. The main characteristics of the study population

		Ph-MPN Patients	Healthy Controls	*P-value*
Caracteristics		N = 89	N = 78	
Sex N(%)	Male	49 (55%)	27 (35%)	> 0.0001
	Female	40 (44.9%)	51(65%)	
Age (years)		62.73 ± 13.14	41.42 ± 12.76	0.0001-T
Laboratory data				
WhiteBloodCells (10^3/μL)		8.25 ± 6.28	6.45 ± 1.41	0.067-MW
		6.53 (5.54;9.50)	6.24 (5.35; 7.53)	0.534-MedT
Platelet (10^3/μL)		365.91 ± 227.26	265.59 ± 63.75	0.002-MW
		297.0 (234.0; 440.0)	259.00 (228.00; 293.00)	0.001-MedT
Hemoglobin (g/dL)		13.81 ± 2.45	13.90 ± 1.27	0.768-T
Hematocrit (%)		40.63 ± 7.01	40.44 ± 2.91	0.816-T
Prothrombin time (%)		88.54 ±12.19	99.22 ± 8.46	0.0001-T
APTT (sec)		32.80 ± 3.98	31.04 ± 2.94	0.002-T
Fibrinogen (mg/dl)		320.63 ± 81.28	274.47 ± 44.53	0.0001-T
Medical history				
Diabet mellitus N(%)		8 (8.98%)	0 (0%)	0.018-Hi
Smokers N(%)		11 (12.35%)	11 (14.1%)	0.73-Hi
Hypertension N(%)		38 (42.69%)	7 (9%)	0.000001Hi
Dislypidemia N(%)		25 (28.08%)	23 (29.5%)	0.84-Hi
Overweight N(%)		44 (49.43%)	23 (29.1%)	0.008-Hi
Thrombosis events		25 (28.08%)	0 (0%)	0.000001Hi
Cytoreductive and anti-platelet medication				
Hydroxyurea		18 (20.22%)	0 (0%)	
Hydroxyurea + aspirin		32 (35.95%)	0 (0%)	
Anagrelide		8 (8.98%)	0 (0%)	
Anagrelide + aspirin		14 (15.73%)	0 (0%)	
Anagrelide + hydroxyurea		4 (4.49%)	0 (0%)	
Anagrelide + hydroxyurea + aspirin		6 (6.74%)	0 (0%)	
Aspirin		6 (6.74%)	0 (0%)	

Quantitative variables are expressed as mean ± standard deviation except WhiteBloodCells and Platelet (with deviation from normal law), for which we give also 25%Percentile, median and 75% Percentile; Dichotomous data are shown as n (%).

T = Student T test ; MW = Mann-Whitney test; MedT = Median test; Hi = Yates` corrected Hi^2.

Table 2. Thrombin generation parameters-results

	Lagtime (tlag) (minutes)	Time to Peak (tPeak) (minutes)	Peak Thrombin (nM)	Velocity index (VI) (nM/minute)	AUC (nM)
Controls (n=78)	3.53 ± 0.85	8.86 ± 1.43	145.23 ± 35.58	28.98 ± 11.84	1901.29 ± 300.06
Ph-MPN patients (n=89)	3.73 ± 0.78	8.67 ± 1.38	156.98 ± 40.09*	33.73 ± 13.26*	1914.98 ± 308.35
PV patients (n=26)	3.95 ± 0.78*	9.15 ± 1.27	147.18 ± 27.84	27.8(23.77;31.25)	1865.80 ± 193.10
ET patients (n=51)	3.70 ± 0.79	8.51 ± 1.39	166.35 ± 45.96*	33.7(26.7;41.6)*	1985.68 ± 339.59
MF patients (n=12)	3.44 ± 0.68	8.38 ± 1.52	138.48 ± 22.17	31.4(22.8;37.8)	1721.12 ± 286.03
Jak2V617F negative(n=22)	3.62 ± 0.76	8.43 ± 1.04	168.36 ± 57.0	36.70 ± 15.85	1994.89 ± 412.42
Jak2V617F positive (n=42)	3.74 ± 0.77	8.62 ± 1.43	155.61 ± 33.50	34.06 ± 13.45	1897.90 ± 248.45
Anagrelide treatment (n=22)	3.72 ± 0.85	8.36 ± 1.35	164.4 (140;176.6)†	35.3(29.3;38.77)†	1992.9 ± 216.85
HU treatment (n = 50)	3.86 ± 0.77	8.91 ± 1.48	146.8 (127.2;167.4)	29.5(23.35;39.9)	1903.47 ± 353.64
HU + anagrelide treatment (n=10)	3.48 ± 0.70	8.50 ± 1.17	154.7 (133.7;173.27)	33(27.15;37.8)	1852.93 ± 205.20

The results (mean±SD; median and 25% and 75% Percentile) obtained from healthy controls and patients, according to Jak2V617F status and cytoreductive treatment;

**p < 0.05 Ph-MPN, PV, ET patients versus controls.*

† p < 0.05 Ph-MPN patients treated with anagrelide versus HU and HU + anagrelide.

Only 64 patients were tested for the presence of *Jak2V617F* mutation, 42 of them were carriers (heterozygous n=30 and homozygous n = 12) and 22 patients were found negative for this mutation.

Thrombin generation was determined in platelet poor plasma from patients and from healthy subjects in the control group, after initiating coagulation with hr TF. Results are summarised in **table 2.**

In our study two parameters of thrombin generation were found significantly increased in patients in comparison with healthy controls: peak thrombin (p = 0.049-A) and velocity index (p = 0.012-A). The other TGA parameters did not reveal significant differences.

In the patients group we noticed significant weak direct positive correlation (Pearson) between haemoglobin (Hb) and lag time (R = 0.230, p = 0,03), between Hb and peak thrombin (R = 0.257, p = 0,015), and leukocyte count and peak thrombin (RS = 0.213, p = 0.046 Spearman).

On the other hand, we did not notice any significant correlation between the actual number of platelets and TGA parameters in our TE subgroup or other subgroups.

We analyzed the TGA parameters for all subgroups of Ph-MPN patients (PV patients,

Table 3. Coefficients of the linear regression models for Peak thrombin, VI and AUC

		Unstandardized Coefficients		Standardized Coefficients		
Dependent Variable		B	Std. Error	Beta	t	Sig.
Peak thrombin	(Constant)	171.831	7.431		23.122	.000
	LengthTrait	-2.419	1.006	-.250	-2.405	.018
VI	(Constant)	37.862	2.482		15.255	.000
	LengthTrait	-.673	.336	-.210	-2.003	.048
AUC	(Constant)	2032.623	57.034		35.639	.000
	LengthTrait	-19.176	7.721	-.257	-2.483	.015

Independent variable is LengthTrait (length of the cytoreductive treatment) for the three regression models. We remark from Table 3, a significant inverse negative relation between the length of cytoreductive therapies and TGA parameters (peak thrombin, VI and AUC).

ET patients and MF patients) in comparison with the reference population. In the PV patients group, we noticed a longer lag time (p = 0.03-T). The maximum velocity of thrombin formation (p = 0.002-MW) and peak thrombin (p = 0.004-T) were significantly increased in ET patients.

In Ph-MPN patient subgroups, ET patients displayed a significantly higher peak thrombin and AUC in comparison with MF patients (p = 0.046-T and p = 0.015-T), and a higher VI (p = 0.022-MW) in comparison with PV patients.

Patients receiving anagrelide displayed higher values for peak thrombin (p = 0.043-MW) and VI (p = 0.042MW) in comparison with those who were treated with HU. The AUC values had the same pattern but did not reach a significant difference. The small group of patients (n=10) treated with both HU and anagrelide showed significantly lower values for peak thrombin and VI in comparison with patients treated only with anagrelide (p = 0.02MW and p = 0.024MW).

A significant inverse negative correlation (Pearson) between the length of cytoreductive therapies and TGA parameters was noticed for peak thrombin (R = -0.25, p = 0.018), AUC (R = -0.257, p = 0.015), and VI (R = -0.21, p = 0.048).

The carriers of *Jak2V617F* mutation displayed a lower peak thrombin, VI, and AUC, but the result did not differ significantly in comparison with WT carriers.

Ph-MPN patients with history of thrombosis (arterial or venous thrombosis) did not present higher thrombin generation compared with patients without such thrombotic events (p > 0.05-T).

Thrombin generation parameters did not show significant differences between patients stratified for presence of cardiovascular risk factors (hypertension, diabetes, dyslipidemia, smoking or overweight) (p < 0.05).

The two study groups (controls and Ph-MPN patients) differed in age distribution and gender ratio. Multiple linear regression analysis with backward method was used to study the influence of variables like age, body mass index (BMI), and length of the treatment on the TGA parameters and revealed that only the length of the treatment has a significant influence upon TGA variables (peak thrombin, VI, and AUC) in Ph-MPN patients. The results of these regression models are given in **table 3.**

In our study the mean values of TGA parameters did not differ significantly between men

and women. Therefore, it seems that age and gender distributions do not have a significant influence on the levels of TGA parameters.

Discussion

The study was designed to measure the thrombin generation potential of MPN patients' plasma (PPP) *in vitro* in order to demonstrate a correlation of TGA parameters with the pro-coagulant status of these diseases and to establish the influence of: *Jak2V617F* mutation, thrombotic history, treatment, and cardiovascular risk factors (hypertension, diabetes, dyslipidemia, smoking, overweight).

Under thrombogenic conditions like surgery or malignancy, analyzing the potential to form thrombin could be a useful tool to evaluate the potential of patients to develop thrombosis, and to identify subjects with high risk of recurrent events [12].

Several studies used TGA to explore the underlying mechanism of thrombosis in patients with complex cellular disorders like Ph-MPN, and proved an APC resistance related to reduced protein S levels in MPN patients [6], an acquired thrombomodulin (TM)-resistance [13], or a correlation with the number of microparticles fraction present in plasma of ET and PV patients [13,14].

Modification of the TGA parameters, the short lag time and high ETP or peak thrombin heights point to a pro-thrombotic state [9].

The data from our study showed that MPN patients had a significantly higher peak thrombin and VI in comparison with their healthy controls. Among MPN patients, the ET patient subgroup had the highest peak thrombin and VI values, and the newest diagnosed patient had the highest TGA values of all MPN patients (i.e. between 254.10 nM and 370.20 nM for peak thrombin, and 74.50 and 83.10 nM/min VI). A higher level of microparticles found in the plasma of ET patients and its positive correlation with peak thrombin [14,3] could explain our results. The significantly higher peak thrombin and AUC seen in ET patients in comparison with MF patients could have the same explanation.

In the patients group, we noticed significant weak positive correlation (Pearson) between haemoglobin (Hb) and lag time and the high values of Hb (15.80 ± 1.42 g/dl) could explain the longer lag time found in PV patients' plasma. In addition, we noticed significant week positive direct correlation (Pearson) between leukocyte counts (WBC) and peak thrombin, and this finding is in accordance with the results of the PT-1 trial, the largest multicentric randomized study performed in ET, which found a significant association between WBC count and risk of thrombosis with a nearly linear relationship and no particular threshold at which the risk began to increase [15, 16]. To date, no study has demonstrated a significant correlation between platelet number and thrombosis in PV and ET. In the ECLAP study, neither the currently proposed therapeutic target of 400×10^9/L nor any of the other platelet-count thresholds predicted a higher risk of thrombosis [4]. That is what our study confirmed through our results of TGA parameters.

The significantly lower peak thrombin and VI in patients who received cytoreductive treatment with HU in comparison with those treated with anagrelide were in accordance with the results of Panova-Noeva et al. [17]. In our previous study it has been demonstated that treatment with anagrelide increases mean platelet volume (MPV) and this could influence the generation of thrombin [18]. There are studies that claim the superiority of cytoreductive treatment with HU combined with aspirin in comparison with anagrelide combined with aspirin in reduction of thrombotic events and lowering leukocytes, in ET [19, 20]. However, non-inferiority of anagrelide in preventing ET-related events was demonstrated in a prospective study conducted by Gisslinger et al. [21].

In our study, all TGA parameter values decreased along with the length of the treatment and these findings could be in accordance with a large population study of ET patients where complete response of the therapy rate progressively increased over time to a maximum of 25% after 12 months [20].

The contribution of *Jak2* mutation in thrombotic events is still a matter of debate. The *Jak2V617F* mutation has been consistently reported in various studies as a risk factor for thrombosis in ET and PV, and was recently included in a risk stratification for thrombosis risk in ET patients [7, 22-24]. On the other hand, a study in a *Jak2V617F*-driven mouse model of myeloproliferative neoplasm demonstrated that haemostatic defects are not concomitant with *Jak2V617F* expression, suggesting they are not directly caused by the mutation and emphasize the role of MPN disease phenotype [25]. A greater sensitivity to the cytoreductive therapy with HU was reported in *Jak2V617F* positive ET patients [26], and our study confirms that the patients carriers of *Jak2V617F* mutation generate less thrombin in comparison with the WT counterparts. The other TGA parameter trend was shown in table 2.

In their study, Marchetti et al. claimed that MPN patients with a history of thrombosis (arterial or venous) presented significantly higher peak thrombin values than patients without thrombosis [6], which could not be confirmed by our study. The presence of cardiovascular risk, such as hypertension, diabetes, dyslipidemia, overweight, and smoking in MPN patients did not influence the TGA parameters. This might be because all patients underwent specific treatment.

In our study, age had no effect on thrombin generation as it has been previously suggested [27, 28]. Different reference values between adults and children for all parameters, except one (Tmax) were previously found [29], but there is no need to adjust age when the cohort consists of adult subjects (personal oral communication by professor HB Hemker) [30]. Regarding gender, our results were in accordance with another study that claimed no difference between women and man in TGA parameters [30].

It is known that hereditary thrombophilic states have an important function in the pathogenesis of venous thromboses. The factor V Leiden (FVL) mutation renders factor V insensitive to the actions of activated protein C (aPC), a natural anticoagulant, and increase the risk of venous thrombembolism (VTE) in carriers. A study involving PV and ET subjects demonstrated a higher prevalence of FVL in patients with a history of venous thrombosis than in subjects with no such history (16 versus 3%) [4, 31]. The prevalence of FVL in ET patients in comparison with healthy subjects was slightly lower (1.67% vs. 2.86%) in the study conducted by Kornblihtt LI et al. [32].

The influence of FVL on generating thrombotic events is still a matter of debate in MPN. Some studies found a positive association between the presence of FVL and thrombosis [31, 33, 34], while other studies failed to demonstrate this association [32]. No statistically significant difference has been found in TGA parameters in VTE population among the groups with and without presence of FVL [35, 36]. Only a few patients with thrombotic events in our study experimented venous thrombosis (6/26), thus we consider that the prevalence of FVL in our study group has not influenced our findings.

Limitation:

Our study has some limitations. Thus, for an important number of our patients, we did not have any information about the *Jak2V617F* mutation and we did not perform a screening for inherited thrombophilia in our MPN patients study group.

Moreover, we consider that the number of patients with PV and MF was too small to generate robust results. We need to add that this study, which reflects the particularities of patients in southern Transylvania, is for a single study center.

All our patients were under cytoreductive treatment. The number of patients newly diagnosed during the study was too small for us to analyze separately. It is possible that the coagulation status may be different in the last patients (they may be more prone to thrombosis compared to those treated), but another study to analyse this hypothesis is needed.

Conclusions

1. We have shown that plasma from MPN patients and especially that from ET patients has significantly increased heights of the peak thrombin and VI values, which may account for the increase in thrombotic risk in these disorders, even during cytoreductive therapy.
2. We demonstrated that TGA parameters are inversely correlated with the length of the treatment and TGA could be a useful tool for monitoring the treatment result.
3. We also found that the plasma of patients treated with hydroxyurea generated less thrombin than the plasma of those treated with anagrelide. Further studies are required to confirm our findings.

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Disclosure

The authors state that they have no conflict of interest.

Abbreviation

ARMS- PCR – amplification-refractory mutation system polymerase chain reaction.
AUC – area under the curve.
ET – essential thrombocythemia.
ETP – endogenous thrombin potential.
Jak2 – janus tyrosine kinase 2.
MPN –myeloproliferative neoplasms.
PMF – primary myelofibrosis.
PV – polycythemia vera.
TF – tissue factor.
VI – velocity index.
WHO – world health organizatio.

References:

1. Vardiman JW, Brunning RD, Arber DA, Lebean MM, Porurt A, Telferi A, et al. Introduction and Overview of the Classification of the Myeloid Neoplasms. In: Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, et al. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. 4th edition. IARC, Lyon. 2008; 18-49.
2. Campbell PJ, Green AR. The myeloproliferative disorders. N Engl J Med. 2006 Dec; 355(23):2452-66. DOI: 10.1056/NEJMra063728.
3. Falanga A, Marchetti M. Thrombotic disease in the myeloproliferative neoplasms. Hematology Am Soc Hematol Educ Program. 2012 Dec;2012(1):571-81.
4. Barbui T, Finazzi G, Falanga A. Myeloproliferative neoplasms and thrombosis. Blood. 2013 Mar;122(13):2176-84. DOI: 10.1182/blood-2013-03-460154.
5. Levine RL, Wadleigh M, Cools J, Ebert BL, Wernig G, Huntly BJP, et al. Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. Cancer Cell. 2005 Apr;7(4):387-97. DOI: 10.1016/j.ccr.2005.03.023.

6. Marchetti M, Castoldi E, Spronk HMH, van Oerle R, Baldicci D, Barbui T, et al. Thrombin generation and activated protein C resistance in patients with essential thrombocythemia and polycythemia vera. Blood. 2008 Nov;112(10):4061-8. DOI: 10.1182/blood-2008-06-164087.

7. Lussana F, Caberlon S, Pagani C, Kamphuisen PW, Büller HR, Cattaneo M. Association of V617F Jak2 mutation with the risk of thrombosis among patients with essential thrombocythemia and idiopatic myelofibrosis: a systematic review. Thromb Res. 2009 Sep;124(4):409-17. DOI: 10.1016/j.thromres.2009.02.004.

8. Tripodi A, Chantarangkul V, Gianniello F, Clerici M, Lemma L, Padovan L, et al. Global coagulation in myeloproliferative neoplasms. Annals of Hematology. 2013 Dec;92(12):1633-9. DOI: 10.1007/s00277-013-1834-x.

9. Castoldi E, Rosing J. Thrombin generation tests. Thromb Res. 2011 Feb;127(Suppl3): S21-5. DOI: 10.1016/S0049-3848(11)70007-X.

10. Thiele J, Imbert M, Pierre R, Vardiman JW, Brunning RD, Flandrin G. Chronic idiopathic myelofibrosis. In: Jaffe ES, Harris NL, Stein H, Vardiman JW, eds. WHO Classification of Tumours: Tumours of Haematopoietic and Lymphoid Tissues. Lyon, France: IARC Press; 2001:35–8.

11. Jones AV, Kreil S, Zoi K, Waghorn K, Curtis C, Zang L. et al. Widespread occurrence of the JAK2 V617F mutation in chronic myeloproliferative disorders. Blood. 2005 Sep;106(6):2162-68. DOI: 10.1182/blood-2005-03-1320.

12. Ten Caten H. Thrombin generation in clinical conditions. Thromb Res. 2012 Mar;126(3):367-70. DOI: 10.1016/j.thromres.2011.10.017

13. Duchemin J, Ugo V, Ianotto JC, Lecucq L, Mercier B, Abgrall JF. Increased circulating procoagulant activity and thrombin generation in patients with myeloproliferative neoplasms. Thromb Res. 2010 Sep(3);126:238-42. DOI: 10.1016/j.thromres.2010.06.025.

14. Trappenburg MC, van Schilfgaarde M, Marchetti M, Spronk HM, ten Cate H, Leyte A et al. Elevated procoagulant microparticles expressing endothelial and platelet markers in essential thrombocythemia. Haematologica. 2009 Jul;94(7):911-8. DOI: 10.3324/haematol.13774.

15. Campbell PJ, MacLean C, Beer PA, Buck G, Weathley K, Kiladjian JJ et al. Correlation of blood counts with vascular complications in essential thrombocythemia: analysis of the prospective PT1 cohort. Blood. 2012 Aug;120(7):1409-11. DOI: 10.1182/blood-2012-04-424911.

16. Passamonti F, Rumi E, Pascutto C, Cazzola M, Lazzarini M. Increase in leucocyte count over tome predicts thrombosis in patients with low-risk essential thrombocythemia. Thromb Haemost. 2009 Sep;7(9):1587-9. DOI: 10.1111/j.1538-7836.2009.03531.x.

17. Panova-Noeva M, Marcetti M, Buoro S, Russo L, Leuzzi A, Finazzi G, et al. Jak2V617F mutation and hydroxyurea treatment as determinants of immature platelet parameters in essential thrombocythemia and polycythemia vera patients. Blood. 2011Sep;118(9):2599-601. DOI: 10.1182/blood-2011-02-339655.

18. Olteanu AL, Mihaila RG, Catana AC, Flucus O, Bus C, Mihalache M. Platelet indices in Philadelphia-negative chronic myeloproliferative neoplasms. Rev Romana Med Lab. 2015;23(2):169-77. DOI:10.1515/rrlm-2015-0012.

19. Carrobbio A, Finazzi G, Antonioli E, Vannucchi AM, Barosi G, Ruggeri M, et al. Hydroxyurea in essential thrombocythemia: rate and clinical relevance of responce by European Leukemianet criteria. Blood. 2010 Aug;116(7):1051-5. DOI: 10.1182/blood-2010-03-272179.

20. Harrison CN, Campbell PJ, Buck G, Wheatley K, East CL, Bareford D, et al. Hydroxyurea compared with anagrelide in high risk essential thrombocythemia. N Engl J Med. 2005 Jul;353(1):33-45. DOI: 10.1056/NEJMoa043800.

21. Gisslinger H, Gotic M, Holowiecki J, Penka M, Thiele J,Kvasnicka HM,et al. Anagrelide compared with hydroxyurea in WHO-classified essential thrombocythemia: the ANAHYDRET Study, a randomized controlled trial. Blood. 2013 Mar;121(10):1720–8. DOI: 10.1182/blood-2012-07-443770.

22. Barbui T, Finazzi G, Carrobio A, Thiele J, Passamonti F, Rumi E, et al. Development and validation of an International Prognostic Score of thrombosis in World Health Organization - essential thrombocythemia (IPSET-thrombosis). Blood. 2012 Dec; 120(26):5128-33. DOI: 10.1182/blood-2012-07-444067.

23. Coucelo M, Caetano G, Sevivas T, Almeida Santos S, Fidalgo T, Bento C et al. JAK2V617F allele burden is associated with thrombotic mechanisms activation in polycythemia vera and essential thrombocythemia patients. Int J Hematol. 2014 Jan;99(1):32-40. DOI: 10.1007/s12185-013-1475-9.