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ORIGINAL ARTICLE

GENETIC POLYMORPHISMS OF HEMOSTATIC FACTORS AND THROMBOTIC RISK IN NON *BCR-ABL* MYELOPROLIFERATIVE NEOPLASMS: A PILOT STUDY

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ABSTRACT

The most important complications of Philadelphia-negagive (non *BCR-ABL*) myeloproliferative neoplasms (MPNs) are vascular events. Our aim was to evaluate the effects of single nucleotide polymorphisms (SNPs), platelet glycoproteins (GPs) (Ia/IIa, Ibα, IIb/IIIa and VI), von Willebrand factor (vWF), coagulation factor VII (FVII), β-fibrinogen, and the risk of thrombosis in patients with non *BCR-ABL* MPNs at the Lithuanian University of Health Sciences. Kaunas, Lithuania. Genotyping was done for 108 patients. The TT genotype of the *GP* Ia/IIa c.807C>T polymorphism was more frequently found in the group of MPN patients with arterial thrombosis compared to MPN patients who were thrombosis-free [26.5 *vs.* 11.5%, p = 0.049; odds ratio (OR) 2.68; 95% confidence interval (95% CI) 1.01-7.38]. The CT genotype of the β-fibrinogen c.-148C>T polymorphism occurred more frequently in MPN patients with arterial, and total thrombosis compared to the wild or homozygous genotype (57.7 *vs.* 40.0 *vs.* 12.5%; p = 0.027), (64.7 *vs.* 44.4 *vs.* 25%; p = 0.032), respectively. The carrier state for the c.-323P10 variant of *FVII* SNP (summation of P10/10 and P0/10) was more frequent in MPN patients with thrombosis compared to the wild-type genotype carriers (71.4 *vs.* 43.4%; p = 0.049; OR 3.26; 95% CI 1.01-11.31). The coexistence of heterozygous β-fibrinogen c.-148C>T and *FVII* c.-323P0/10 SNP, increased the risk of arterial thrombosis (21.1 vs. 3.7%, p = 0.008; OR 6.93; 95% CI 1.38-34.80). The TT genotype of *GP* Ia/IIa c.807C>T, the CT genotype of β-fibrinogen c.-148C>T and *FVII* c.-323P0/10 SNP could be associated with risk of thrombosis in MPN patients.

Keywords: Genetic polymorphism; Myeloproliferative neoplasia; Thrombosis.

INTRODUCTION

The group of Philadelphia-negative (non *BCR-ABL*) myeloproliferative neoplasms (MPNs), polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF), are known for their different phenotypes but similar complications. The most important of these are vascular events. The incidence of thrombotic complications varies between disease types. They occur in 7.2-15.0% patients with PMF, in 19.0-32.0% patients with ET, and in 30.0-41.0% patients with PV [1-6]. The recent series from Enblom *et al.* [7], showed that 66.0% of these occurred prior to diagnosis. *Janus kinase 2* (*JAK2*) (p.V617F) and the recently discovered *Calreticulin* (*CALR*) mutation are important in the genesis of thrombosis, as the former increases, and the latter decreases the risk of thrombosis [8-11]. Blood cells, interaction between them, and the activation of coagulation factors also play a role in the pathogenesis of thrombosis in non *BCR-ABL* MPNs. This also includes platelets, as they are important in clot formation and thrombosis. Platelet membrane glycoproteins (GPs) are essential in platelet adhesion and aggregation [12,13]. The main role in the above-mentioned processes is played by GP Ib/IX-V that binds to the von Wilebrand factor (vWF) after endothelial cells are damaged [14]. After this process, platelets become activated and promote conformational changes of GP IIb/IIIa that further facilitates the binding of fibrinogen and vWF to the subendothelial layer

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[15]. Glycoproteins Ia/IIa and GP VI interact directly with collagen [12]. Platelet-specific polymorphisms in the GP have shown an association with an increased risk of thrombosis in patients with non *BCR-ABL* MPNs. The PIA1/2 allele of GP IIb/IIIa, and its relation to arterial thrombosis in patients with PV have been described in previous studies [16]. The tissue factor and coagulation factor VIIa (FVII) complex is another initiator of the coagulation cascade, which contacts with platelets, resulting in the generation of thrombin on platelet surfaces [17]. The influence of FVII single nucleotide polymorphisms (SNPs) on arterial thrombosis in ET patients has recently been described [18]. The main purpose of our study was to evaluate the effects of different SNPs: platelet GP (c.807C>T of GP Ia/IIa, c.-5T>C of GP Ibα Kozak, GP Ibα variable numbers of tandem repeats (VNTR), GP Ibα c.5792C>T, GP IIb/IIIa PIA 1/2 allele, c.13254T>C of GP VI), vWF c.24/1282A>G, FVII c.-323P0/10, and β-fibrinogen c.-148C>T on the risk of thrombosis in patients with PV, ET, and PMF at the Institute of Oncology of the Lithuanian University of Health Sciences, Kaunas, Lithuania.

MATERIALS AND METHODS

Patients. This retrospective study included 108 patients. The diagnosis of ET, PV, and PMF was established between 2000 and 2014 at the Department of Hematology of the Institute of Oncology, the Lithuanian University of Health Sciences, Kaunas, Lithuania. Patients who were diagnosed before 2008 were reviewed according to the WHO diagnostic criteria. From a total of 108 patients, 60 (55.6%) patients had ET, 41 (38.0%) patients had PV, and seven (6.5%) were PMF patients. Detailed medical information was collected including the date of diagnosis, the patient's age, sex, body mass index (BMI), cardiovascular risk factors (smoking, diabetes mellitus, arterial hypertension, and ischemic heart disease), splenomegaly and findings of hematological analyses. We gathered the data on white blood cell (WBC) counts, monocyte, basophile, and platelet counts, medium platelet volume, hemoglobin (Hb), erythrocyte count, hematocrit or packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular Hb (MCH) at the time of the diagnosis, as well as *JAK-2* p.V617F that was performed from 2009. For *JAK-2* p.V617F negative patients, *CALR* mutation status was performed in 2015. History of previous thrombosis was collected as well. Arterial or venous thrombosis, such as ischemic stroke, myocardial infarction, transient ischemic attack, unstable angina, deep vein thrombosis (DVT) of the legs, thrombosis of abdominal veins, and thrombosis of the pulmonary artery, were defined as vascular events. All comparisons were performed between the thrombosis and the thrombosis-free groups for all ET, PV and PMF patients. This study was conducted with the permission of the regional biomedical research ethics committee and in accordance with good clinical and laboratory practices and the principles of the Declaration of Helsinki. A signed consent form was obtained from all participants.

Genotyping. Venous blood samples were drawn in vacutainers containing EDTA as anticoagulant. Genomic DNA was isolated from peripheral blood leukocytes, using the commercially available DNA extraction kit, according to the manufacturer's recommendations (Thermo Fisher Scientific Baltics, Vilnius, Lithuania). Primer sequences for genotyping for the detection of the c.807C>T polymorphism of GP Ia/IIa, c.-5T>C polymorphism of GP Ibα Kozak, GP Ibα polymorphism VNTR, GP Ibα c.5792C>T (HPA-2), PIA1/2 polymorphism in GP IIb/IIIa, the von Willebrand factor (vWF) c.24/1282A>G, the FVII c.-323P0 /10 polymorphism, β-fibrinogen c.-148C>T polymorphism, c.13254T>C polymorphism of GP VI in Table 1.

Statistical Analyses. The Statistical Package for the Social Sciences (IBM SPSS Statistics) version 22 was used for the association analyses. Categorical variables were described by the frequency of their values. Quantitative variables were described by mean and standard deviation (SD) or median and the sample width (minimum-maximum). The χ^2 test was used for categorical variables. Student's *t*-test or Mann-Whitney *U* test was used for the analysis of quantitative variables. The logistic regression analysis distinguished thrombotic risk factors significantly affecting the possibility of thrombosis in the study group. The differences were considered to be statistically significant if the calculated *p* value was less than the chosen significance level a = 0.05 (*p* value <0.05).

RESULTS

In total, 108 patients were analyzed. The main clinical characteristics of patients with non *BCR-ABL* MPNs in our population are depicted in Table 2. Patients with thrombosis were older [mean age 66.98 years (SD = 13.42) *vs.* 60.17 years (SD = 15.58) $p = 0.016$], and had a lower median value of MCV and MCH at the time of diagnosis [83.00 (range 70-100) *vs.* 87.00 (range 65-101), $p = 0.021$, and 27.0 (range 17-34) *vs.* 28.0 (range 23-33), $p = 0.048$, respectively]. In the non *BCR-ABL* MPN thrombosis group, patients were predominantly female (54.8 *vs.* 45.7%), and more patients were positive for the *JAK2* p.V617F mutation (52.6 *vs.* 47.4%, $p = 0.002$). Concerning

Table 1. Primer sequences, restriction enzymes used for genotyping, and length of polymerase chain reaction-restriction fragment length polymorphism products [19-22].

Substitution	Primer Sequence (5'>3')	Restriction Enzyme	DNA Fragment (bp)	
			Wild Type Allele	Polymorphic Allele
GP Ia/IIa c.807C>T	F: GTG TTT AAC TTG AAG ACA TAT R: ACC TTG CAT ATT GAA TTG CTT	*Taq*I	92; 23	115
GP Ibα c.-5T>C	F: GGC GAG TGT AAG GCA TCA GG R: ACA CTT CAC ATG GAC TGG AT	*Ava*II	223; 26	249
GP Ibα VNTR	F: ACA CTT CAC ATG GAC TCC AT R: GGG TCA TTT CTG GAG CTC TC	–		A-520; B-480; C-440; D-400
GP Ibα c.5792C>T (HPA-2)	F: GCC AGC CAC CTA GAA GTG AA R: AAA AGC AAA AGG CAG GAG GT	*Hha*I; *Bsa*HI	245; 170; 116; 45	286; 245; 45
GP IIb/IIIa PIA1/2	F: TTC TGA TTG CTG GAC TTC TCT T R: TCT CTC CCC ATG GCA AAG AGT	*Msp*I	223; 39; 6	173; 50; 39; 6
GP VI c.13254T>C	F: ACA TCC ACA ACA GTC CAG TG R: ATC GAG AAG TCT AGG CAG AG	*Hpa*I	120; 112; 47	112; 95; 47; 25
vWF c.24/1282A>G	F: AAG CCA GGA TTA GAA CCC GAG TCG R: AAC TCC ATG GTT CTG GAT GTG GCG TTC	*Kpn*I	276; 406	682
FVII c.-323P0/10	F: TCG CAT GAT TGC TAT GGG AC R: GTT GAC ATT CCC CAT GGG AC	*Eco*RI	284; 74	284; 82
β-Fibrinogen c.-148C>T	F: GAA CAT TTT ACC TTA TGT GAA TTA AGG R: GAA GCT CCA AGA AAC CAT CC	*Hind*III	290; 194; 185	379; 290

F: forward primer; R: reverse primer.

Table 2. Clinical characteristics.

Characteristics	Patients With Thrombosis (*n* = 57)	Patients Without Thrombosis (*n* = 51)	*p* Value
Age: mean (SD)	66.98 (13.42)	60.17 (15.58)	0.016[a]
Males: *n* (%) Females: *n* (%)	21 (45.7) 34 (54.8)	25 (54.3) 28 (45.2)	0.96[b]
Disease duration: months: median (min-max)	28.0 (8.0-184.0)	30.0 (14.0-180.0)	0.70[c]
Spleen size, cm: median (min-max)	14.60 (4.88)	14.18 (3.37)	0.87[c]
Hb (g/dL): mean (SD)	14.68 (2.99)	15.34 (3.05)	0.26[a]
RBC count (10^{12}/L): median (min-max)	5.19 (2.75-7.34)	5.17 (3.21-8.04)	0.87[c]
PCV (L/L): median (min-max)	0.45 (0.22-0.63)	0.45 (0.31-0.64)	0.50[c]
MCV (fL): median (min-max)	83.0 (70.0-100.0)	87.0 (65.0-101.0)	0.02[c]
MCH (pg): median (min-max)	27.0 (17.0-34.0)	28.0 (23.0-33.0)	0.048[c]
Platelet count (10^9/L): median (min-max)	553.0 (21.0-1554.0)	581.0 (164.0-1700.0)	0.65[c]
WBC count (10^9/L): mean (SD)	9.34 (1.46)	9.46 (1.34)	0.71[a]
Leukocyte count (10^9/L): median (min-max)	10.14 (4.58-24.67)	10.15 (4.00-22.00)	0.64[c]
Monocyte count (10^9/L)L median (min-max)	0.60 (0.10-1.39)	0.60 (0.20-2.68)	0.80[c]
Basophils (10^9/L): median (min-max)	0.09 (0.00-0.36)	0.08 (0.00-0.52)	0.62[c]
BMI: mean (SD)	26.56 (4.47)	27.67 (5.04)	0.30[a]
Diabetes mellitus: *n* (%)	7 (70.0)	3 (30.0)	0.11[b]
Smoking: *n* (%)	4 (50.0)	4 (50.0)	0.75[b]
JAK-2: *n* (%)	41 (52.6)	37 (47.4)	0.002[b]
CALR: *n* (%)	1 (11.1)	8 (88.9)	0.03[b]

SD: standard deviation; Hb: hemoglobin; RBC count: red blood cell count; PCV: packed cell volume; MCV: mean corpuscular volume; MCH: mean corpuscular Hb; WBC count: white blood cell count; BMI: body mass index.

[a] *t*-Test for two independent samples.
[b] ζ^2 Test for independence (homogeneity) of two features.
[c] Non-parametric Mann-Whitney *U* test.

patients with ET and PMF, 11.1% of them were *CALR*-positive in the thrombosis group compared to 88.9% of the thrombosis-free patients ($p = 0.033$). Other clinical characteristics were similar.

Arterial thrombosis was more frequent in the ET/PMF group than in the PV group (73.5 *vs.* 26.5%, $p = 0.03$) (Table 3). Forty-five (42.6%) patients had experienced an arterial thrombotic event, 10 (9.3%) patients had experienced a venous event, and four (3.7%) patients had both arterial and venous thrombosis.

Genotype distributions and allele frequencies of the studied polymorphisms in patients with thrombotic complications according to the type of thrombosis are summarized in Table 4. Our data showed a higher prevalence of GP Ia/IIa c.807C>T polymorphism of the TT genotype than the CC wild-type or the heterozygous CT genotype in MPN patients with arterial thrombosis (65.0 *vs.* 38.2 *vs.* 42.6%, $p = 0.09$), respectively. The analysis of the c.807 C>T polymorphism TT genotype separately showed that it was significantly more frequent in the MPN patient group with arterial thrombosis compared to the MPN group of thrombosis-free patients [26.5 *vs.* 11.5%, $p = 0.049$; odds ratio (OR) 2.68; 95% confidence interval (95% CI) 1.01-7.38]. The CT genotype of the β-fibrinogen (c.-148C>T polymorphism) in MPN patients with arterial and arterial and venous thrombosis occurred significantly more frequently compared to the CC wild-type or the homozygous TT genotype (57.7 *vs.* 40.0 *vs.* 12.5%; $p = 0.027$) and (64.7 *vs.* 44.4 *vs.* 25.0%, $p = 0.032$), respectively. The carrier state for the c.-323P10 variant of FVII SNP (summation of P10/10 and P0/10) was significantly more frequent in MPN patients with arterial and venous thrombosis compared to wild-type genotype carriers (71.4 *vs.* 43.4%, $p = 0.049$; OR 3.26; 95% CI 1.01-11.31). It maintained a borderline significance when analyzed in the arterial thrombosis subgroup only (69.2 *vs.* 38.2%, $p = 0.06$).

The coexistence of both heterozygous genotypes of β-fibrinogen c.-148C>T and FVII c.-323P0/10 SNP in-

Table 3. Thrombotic complications.

	Thrombosis: *n* (%)		
	ET/PMF	**PV**	***p* Value[a]**
Arterial:	36 (73.5)	13 (26.5)	0.03
Cardiac (myocardial infarction)	11	4	NS
Neurological (TIA and ischemic stroke)	20	8	NS
Peripheral arterial thrombosis	5	1	NS
Venous:	7 (50.0)	7 (50.0)	0.36
Deep venous thrombosis	4	3	NS
Pulmonary artery thrombosis	0	1	NS
Splanchnic vein thrombosis	3	3	NS

ET: essential thrombocythemia; PMF: primary myelofibrosis; PV: polycythemia vera; NS: not significant; TIA: transient ischemic attack.
[a] ζ^2 Test for independence (homogeneity) of two features.

Table 4. Distribution of polymorphisms in arterial and venous thrombosis.

Polymorphisms	**GP IIa/IIIa PIA1/2**						**GP Ia/IIa c.807C>T**		
Genotypes	TT	TC	CC	CC	CT	TT	CC	CT	TT
Arterial *n* (%)	34 (47.2)	12 (48.0)	2 (28.6)	44 (46.3)	3 (37.5)	1 (50.0)	13 (38.2)	23 (42.6)	13 (65.0)
Venous *n* (%)	10 (13.2)	1 (4.0)	2 (28.6)	12 (12.6)	0 (0.0)	1 (50.0)	5 (14.7)	7 (13.0)	2 (10.0)

Polymorphisms	**GP Ibα VNTR**			**GP Ibα c.4T>C Kozak**			**GP VI c.13254T>C**		
Genotypes	CC	CD	DD	TT	TC	CC	TT	TC	CC
Arterial *n* (%)	41 (46.3)	4 (57.1)	4 (66.7)	38 (48.1)	11 (40.7)	0 (0.0)	43 (46.7)	6 (37.5)	0 (0.0)
Venous *n* (%)	10 (10.6)	2 (28.6)	1 (16.7)	11 (13.9)	3 (11.1)	0 (0.0)	11 (12.0)	3 (18.8)	0 (0.0)

Polymorphisms	**β-Fibrinogen c.-148C>T**			**FVII c.-323P0/10**			**vWF c.24/1282A>G**		
Genotypes	CC	CT	TT	P0/P0	P0/10	P10/10	AA	AG	GG
Arterial *n* (%)	**18 (40.0)**	**30 (57.7)[a]**	**1 (12.5)**	**29 (38.2)**	**9 (69.2)[b]**	0 (0.0)	39 (41.1)	0 (0.0)	0 (0.0)
Arterial *n* (%)	5 (11.1)	7 (13.5)	1 (12.5)	8 (10.5)	2 (15.4)	1 (100.0)	12 (12.6)	0 (0.0)	0 (0.0)

FVII: coagulation factor VII; vWF: von Willebrand factor.
Data are presented as *n* (number) (%) in arterial and venous thrombosis groups. Comparisons reaching statistical significance are bold.
[a] $p = 0.027$.
[b] p Exact = 0.049 in total thrombosis.

creased the risk of arterial thrombosis in MNP patients (21.1 *vs.* 3.7%, $p = 0.008$; OR 6.93; 95% CI 1.38 – 34.80). In the univariate analysis, no statistically significant association was found between the remainder of the tested polymorphisms and thrombosis.

In the multivariate analysis performed on arterial thrombosis considering TT genotype of c.807C>T GP Ia/IIa, CT genotype of β-fibrinogen c.-148C>T, a carrier state for c.-323P10 variant of FVII (summation of P10/10 and P0/10) SNPs, MCV, and age, two of three SNPs (TT genotype of c.807C>T GP Ia/IIa and CT genotype of β-fibrinogen c.-148C>T) as well as MCV and age, retained statistical significance (Table 5). A test of the full model against a constant only model was statistically significant, indicating that the predictors as a set reliably distinguished between MPN patients with arterial thrombosis and those without thrombosis ($\xi^2 = 21.82$, $p < 0.001$ with df = 4). Nagelkerke's R^2 was 0.33. The overall prediction success was 72.4% (62.2% for thrombosis and 80.0% for no thrombosis).

Table 5. Multivariate logistic regression.

Variable	OR	*p* Value	95% CI-OR
TT of GP Ia/IIa c.807C>T	3.83	0.032	1.13-13.03
CT of β-Fibrinogen c.-148C>T	2.72	0.042	1.04-7.12
c.-323P0/10 + c.-323P10/10 of FVII	3.44	0.08	0.86-13.69
Age	1.03	0.041	1.001-1.068
MCV	0.96	0.003	0.94-0.97

OR: odds ratio; 95% CI: 95% confidence interval; MCV: mean corpuscular volume.

Separate analyses of different arterial thrombotic events showed more frequent CT genotype of β-fibrinogen c.-148C>T SNP in MPN patients with ischemic stroke compared to other arterial vascular events; however, the results were of borderline significance (75.0 *vs.* 50.0%, $p = 0.06$). The c.-323P0/10 plus c.-323P10/10 genotype of FVII SNP was statistically significantly more frequent in the ischemic stroke group compared to the thrombosis-free group (33.3 *vs.* 12.2%, $p = 0.04$).

DISCUSSION

The main objective of this study was to evaluate whether SNPs located in genes of platelet glycoprotein Ia/IIa (c.807C>T), glycoprotein Ibα (VNTR; c.5T>C Kozak; c.5792 C>T), glycoprotein IIb/IIIa (PIA1/2), glycoprotein VI (c.13254T>C), vWF (c.24/1282A>G), coagulation FVII (c.-323P0/10), and β-fibrinogen chain (c.-148 C>T) could be associated with risk of thrombosis in patients with non *BCR-ABL* MPNs. The analysis of nine different SNPs provided evidence that the TT genotype (c.807C>T) of GP Ia/IIa, CT genotype (c.-148C>T) of β-fibrinogen chain and coagulation FVII (c.-323P0/10) SNP represent an additional risk factor for thrombosis in patients with non *BCR-ABL* MPNs.

The c.807C>T SNP of GP Ia/IIa was also studied by Afshar-Kharghan *et al.* [16] in ET and PV patients. The investigators did not identify any associations between this SNP and thrombotic complications in a cohort of 86 ET and PV patients [16]. The homozygous state (TT genotype) of the above-mentioned polymorphism was identified as an additional risk factor for arterial thrombosis in patients with the antiphospholipid syndrome in the studies of Jimenez *et al.* [23] and Yonal *et al.* [24]. Our results suggest that the homozygous state of c.807C>T GP Ia/IIa SNP may increase the risk of arterial thrombosis in non *BCR-ABL* MPNs. A meta-analysis of 66,155 cases that was published by scientists from China did not show any significant relation between GP Ia/IIa c.807C>T polymorphism and coronary artery disease [25]. In addition, German scientists revealed a modulatory effect of the aforementioned polymorphism on thrombosis development, which can be region- and race-dependent [26].

The impact of coagulation FVII c.-323P0/10 SNP has been recently reviewed by Buxhofer-Ausch *et al.* [18]. The investigators screened 105 patients with ET meeting the 2008 WHO criteria and 62 patients with early PMF. The c.-323P10 variant of the coagulation FVII showed a statistically significant association with total and arterial thrombosis in univariate as well as in multivariate analysis for patients with ET, but not for those with early PMF [18]. However, the investigators considered that solid data are lacking to explain the mechanism of the relation of c.-323P0 /P10 FVII polymorphism with thrombosis. The authors admit the importance of further experiments to evaluate the influence of the aforementioned SNP on the risk of thrombosis [18]. Our cohort of patients was smaller, but it also included a similar number of patients with ET. We also extended the non *BCR-ABL* MPN cohort to the whole WHO-confirmed non *BCR-ABL* MPN population. The majority of cases of the heterozygous variant of c.-323P0 /P10 FVII SNP were found in the ET cohort,

compared to the PV cohort, which is in agreement with data published by Buxhofer-Ausch *et al.* [18]. We also observed a higher prevalence of this polymorphism in non *BCR-ABL* MPN patients with arterial and total thrombosis. However, the majority of cases of the c.-323P0/10 FVII SNP retained borderline significance on the multivariate analysis of our non *BCR-ABL* MPN patient cohort. Our study revealed that the coexistence of both heterozygous genotypes in c.-323P0/P10 FVII and β-fibrinogen c.-148C>T SNP was related to arterial thrombosis in non *BCR-ABL* MPN patients with the OR of 6.93 and the relative risk of 2.19 for those who were double heterozygous in this study. To the best of our knowledge, a common effect of those two SNPs in non *BCR-ABL* MPN patients has never been analyzed before. Although the exact pathophysiological mechanism is unclear, we can only speculate that the simultaneous occurrence of two or more prothrombotic SNPs can activate the hemostatic cascade that further predisposes the development of thrombosis in non *BCR-ABL* MPNs.

Our results showed a tendency of a higher frequency of the β-fibrinogen CT genotype (c.-148C>T SNP) in patients with total and arterial thrombosis than in non *BCR-ABL* MPN patients who did not experience vascular events. The effect of this SNP was evaluated in a large population of Chinese patients with ischemic stroke and cerebral infarction, but not in non *BCR-ABL* MPNs [27,28]. There was also evidence that β-fibrinogen c.-148C>T SNP is functional and associated with elevated plasma fibrinogen levels [29]. The carriers of the heterozygous genotype of this SNP showed a trend toward a higher risk of ischemic stroke in our cohort, although, considering the small number of patients, it was of borderline significance. This gave rise to a hypothesis that different single nucleotide poly-morphisms could be potential confounders in vascular-specific events.

The age of non *BCR-ABL* MPN patients who experienced thrombotic events is already a well-known risk factor for thrombosis [2,30]. Our results also confirmed older age as a risk factor for thrombosis. We observed lower MCV and MCH indices in the thrombosis group compared to the thrombosis-free group. However, this difference could be due to the fact that three distinct non *BCR-ABL* MPN subtypes were studied in this cohort. Differently from Carobbio *et al.* [30], we were not able to reveal any cardiovascular risk factor as a predictor for thrombosis, probably due to the lower prevalence of cardiovascular risk factors in our cohort. The *JAK2* p.V617F mutation is considered to be a risk factor for thrombosis in non *BCR-ABL* MPN patients. This was observed in many studies. *JAK2* p.V617F mutation is also included in the International Prognostic Score of thrombosis in WHO-essential throm-bocythemia (IPSET-thromobosis) model for ET patients [31]. The results of our study also confirmed that this mutation was a risk factor for thrombosis in non *BCR-ABL* MPN patients. Conversely, *Calreticulin* mutation was associated with a decreased risk of thrombosis in ET and PMF patients in our cohort.

Polymorphisms of the factor V Leiden (FVL) and the prothrombin gene *G20210A* were recognized as risk factors for venous thrombosis from 1994 [32]. They were also investigated in the thrombotic approach of myeloproliferative neoplasms. Ruggeri *et al.* [33] retrospectively investigated FVL in a cohort of 304 ET and PV patients. The study results revealed that the prevalence of FVL mutation in PV and ET patients was similar to that observed in the normal population. Moreover, the FVL mutation was not associated with arterial or venous thrombosis. However, the authors stated that the FVL mutation is associated with the risk of venous thrombosis recurrences [33]. Schwarz *et al.* [34] recognized the FVL mutation to be a significant additional risk factor in the occurrence of venous thrombosis in a prospective analysis of 1179 ET patients. De Stefano *et al.* [35] investigated MPN patients younger than 60 years of age. The study revealed that the risk of thrombosis was increased when FVL and prothrombin gene *G20210A* mutation coexists with the *JAK2* p.V617F mutation in ET patients [35]. Similar results were demonstrated by Tevet *et al.* [32] in 192 patients with MPN. According to them, the thrombotic risk was higher in the *JAK2* p.V617F mutation subgroup and it was further increased by the presence of the FVL mutation [36].

Unfortunately, we did not analyze the aforementioned mutations. However it would be interesting to investigate these thrombophilic factors in conjunction with our studied platelet receptor polymorphisms. Moreover, our study had a few limitations, such as small group size, the absence of controls, and non *BCR-ABL* MPN heterogeneity, as three types of the disease were analyzed. We also did not measure FVII or plasma fibrinogen levels in our patients.

In conclusion, this study was focused on polymorphisms that were located in genes coding different players of the primary and secondary hemostatic system, which reflects several pathophysiological paths from platelet plug to fibrin formation. This study analyzed a large number of SNPs in non *BCR-ABL* MPN patients, bringing up-to-date evidence of what platelet and coagulation factors can contribute to thrombotic complications. The coexistence of several different polymorphisms as well as vascular-specific event polymorphisms could be a clue for further investigations in order to delineate the pathophysiology of thrombosis in non *BCR-ABL* MPN patients.

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