ORIGINAL ARTICLE

Thrombin Generation Testing in Patients with Myelofibrosis


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SUMMARY

Background: Primary myelofibrosis (PMF) is a chronic clonal myeloid disorder. Together with essential thrombocytopenia (ET) and polycythemia vera (PV), it belongs to a group of Philadelphia chromosome-negative myeloproliferative neoplasms. Thrombotic events are serious complications negatively influencing the quality and length of these patients’ lives. The confirmed risk factors for venous thromboembolism are age over 60 years, a positive history of thromboembolism, presence of common cardiovascular risks, JAK2 V617F mutation and, according to some authors, leukocytosis. Various opinions on the role of thrombocytemia have been published. The present study was undertaken to evaluate the benefit of thrombin generation test and its potential use in predicting the risk of thrombosis in MF patients.

Methods: The analysis included plasma samples obtained from 36 patients diagnosed with MF in our center from 2004 to 2016 (JAK2 V617-positive 53%; CALR-positive 31%; MPL-positive 14%; triple negative 2%) and a control group comprising 20 healthy volunteer blood donors. Thrombin generation was measured in platelet-rich plasma using the TECHNOTHROMBIN® TGA kit (Technoclone, Austria) and the fully automated system Ceveron® Alpha (Technoclone). The results were correlated with clinical and laboratory parameters of the patients.

Results: There were differences in thrombin generation as expressed by endogenous thrombin potential (ETP) between patients and healthy controls, with ETP being lower in the patient group (p = 0.0003). Analysis confirmed a significant correlation between thrombin generation and platelet counts, with higher thrombin generation in patients with thrombocytemia > 400 x 10^9/L (p = 0.04). ETP values were consistently higher in earlier disease stages and lower in CALR-mutated myelofibrosis.

Conclusions: In MF patients, thrombin generation is mainly influenced by platelet counts and, to a lesser extent, by mutation status, activity, and progression of the disease. Thrombin generation test results have confirmed that thrombocytemia is a potential risk factor for thrombotic complications.


KEY WORDS

Ph-negative myeloproliferative neoplasm, myelofibrosis, thrombin generation test

LIST OF ABBREVIATIONS

WHO - World Health Organization
PMF - primary myelofibrosis
ET - essential thrombocytopenia
PV - polycythemia vera
Ph-MPN - Philadelphia chromosome-negative myeloproliferative neoplasm
MF - myelofibrosis
VTE - venous thromboembolism
CALR - calreticulin
JAK2 V617F - mutation in the Janus kinase 2 gene at position 617
MPL - myeloproliferative leukemia virus oncogene
STAT - signal transducer and activator of transcription
AS-PCR - allele-specific polymerase chain reaction
TGT - thrombin generation test
TG - thrombin generation
ETP - endogenous thrombin potential
Cmax - peak thrombin concentration
PPP - platelet-poor plasma
PRP - platelet-rich plasma
rTF - recombinant tissue factor
APTT - activated partial thromboplastin time
PT - prothrombin time
APC - activated protein C
APC-R - activated protein C resistance
DIPSS - Dynamic International Prognostic Scoring System
ADP - adenosine diphosphate
AA - arachidonic acid
TRAP - thrombin receptor-activating peptide

INTRODUCTION

According to the 2016 WHO classification of hematological malignancies, primary myelofibrosis (PMF), together with essential thrombocythemia (ET) and polycythemia vera (PV), belongs to a group of Philadelphia chromosome-negative myeloproliferative neoplasms (Ph-MPNs) [1].

Epidemiology

The incidence of PMF is 1.5 cases per 100,000 population per year [2]. The annual incidence of PV is higher in males than females, with 2.8 and 1.3 cases per 100,000 population, respectively. The cumulative risk of progression to post-PV myelofibrosis (MF) is 6% after 10 years and 26% after 20 years of disease duration [3,4]. The annual incidence of ET is 2.5 cases per 100,000 population, the cumulative risk of transformation to post-ET MF is 9% after 15 years of disease duration [5,6]. In Ph-MPN patients, thrombotic events are serious complications negatively influencing the quality and length of their lives. The actual incidence of significant arterial thrombosis and venous thromboembolism (VTE) cannot be determined due to heterogeneous methods and patient selection in various studies. The reported rates of arterial and venous thrombosis are 5.5 per 100 patient-years for PV, 1 - 3 per 100 patient-years for ET, and 2 per 100 patient-years for PMF. One-third of cases have venous thrombosis [7].

Pathogenesis

In the pathogenesis of Ph-MPN, an essential role is played by deregulation and constitutive activation of the JAK/STAT signaling pathway. In MF, the consequence is clonal proliferation of atypical megakaryocytes with more intense production of cytokines and growth factors, leading to increased formation of nonclonal fibroblasts with subsequent bone marrow fibrosis and development of cytopenia, extramedullary hematopoiesis, and hepatosplenomegaly. Constitutive activation of the JAK/STAT pathway may be caused either directly by somatic mutation of the JAK2 gene at position V617F or indirectly by mutations in the MPL gene and in the calreticulin (CALR) gene [8]. In 50 - 80% of PMF patients, the JAK2 V617F somatic mutation is detected; another 5 - 8% of cases have mutations in codons 515 or 505 of the gene encoding a receptor for thrombopoietin (MPL). Recurrent somatic mutations affecting exon 9 of the CALR gene occur in most JAK2- and MPL-unmutated patients with PMF [9-11]. The pathogenesis of thrombosis is complex in case of Ph-MPNs. This is related to not only quantitative but also qualitative changes in clonal cells (e.g., persistently increased platelet activation with higher expression of P-selectin, tissue factor, microparticle production, increased amounts of the so-called reticular, or immature, platelets in blood or increased release of proteases from activated neutrophil cleaving proteins C and S). The acquired thrombophilic state associated with Ph-MPNs is of multifactorial etiology. It results from not only the prothrombogenic phenotype of circulating cells of the MPN clone but also the host’s immune response to the presence of the tumor clone with increased production of inflammatory cytokines leading to leukocyte and endothelial activation [7].

Risk factors

According to the European LeukemiaNet, age over 60 years and a positive history of VTE are the main risk factors for thrombosis [12]. In 2015, Tefferi and Barbui revised the system of patients’ stratification based on the risk of thrombosis with respect to age, a history of VTE, the presence of common cardiovascular risks (smoking, diabetes mellitus, hypertension), and JAK2 V617F mutation [13]. It has also been shown in a previous study that leukocytosis is a risk factor too [14]. In recent years, studies have inconsistently identified thrombocytopenia as a risk factor. A study by Czech authors has shown that on average, Ph-MPN patients have higher platelet counts prior to the thrombotic event [15]. By contrast, extreme thrombocytosis (over 1,000 to 1,500 x 10^9/L) is associated with a higher risk of bleeding, particularly if acetylsalicylic acid is concurrently administered; this is due to acquired von Willebrand syndrome. In patients with MF, assessment of an individual’s risk of thrombosis may represent a therapeutic problem. The reasons are not only the frequent coexistence of other acquired risk factors for VTE but also the presence of risk factors for bleeding (e.g., thrombocytopenia, concomitant medication or portal hypertension due to splanchic vein thrombosis). Yet basic coagulation tests (prothrombin time - PT and activated partial thromboplastin time - APTT) yield normal results in
Most MF patients. These tests are unable to reflect all pro- and anti-coagulant reactions affecting thrombin generation, in particular the potential effect of activated platelets and other blood cells. The thrombin generation test (TGT) is assumed to aid in better assessment of the individual potential of thrombin generation in MF patients.

**Thrombin generation test**
Thrombin is a multifunctional central enzyme of the coagulation system that not only promotes the activation of coagulation, but also contributes to inhibition of hemostatic processes and affects fibrinolysis. Monitoring of its generation in time may provide an overall picture of the hemostatic system. The TGT is used to measure thrombin generation in plasma samples after in vitro activation with tissue factor or another trigger and to monitor its concentration over time. The rate of thrombin generation may correlate with hyper- or hypo-coagulable states [16]. This study aimed to determine thrombin generation (TG) in MF patients, analyze the main TGT parameters, compare them with healthy controls, and assess the relationship between clinical and hematological phenotypes of the disease and thrombin generation.

**MATERIALS AND METHODS**
The analysis included 36 patients with MF diagnosed in our center between 2004 and 2016. The sample comprised 24 patients with PMF (of those 11 in the prefibrotic stage), five with post-PV MF, and seven with post-ET MF. All patients met the 2008 WHO classification criteria at the time of diagnosis. There were 13 males and 23 females with a median age of 56 years (range, 25 - 78 years). All participants gave written informed consent to collection and analysis of biological samples and genetic testing (DNA). Objectively confirmed thrombotic complications were recorded if these occurred at diagnosis or during the follow-up. Thrombotic complications included venous thrombosis (deep vein thrombosis, pulmonary embolism, splanchnic vein thrombosis and cerebral venous sinus thrombosis) and arterial thrombosis (transient ischemic attack, ischemic stroke, acute myocardial infarction and peripheral arterial vascular disease). At the time of analysis, a total of 61% of patients had received specific MF treatment, namely hydroxyurea (54%), anagrelide (9%), interferon alfa (4.5%), ruxolitinib (27%), and busulfan (4.5%). Excluded were all patients treated with warfarin or novel anticoagulants; antiplatelet drugs, if administered, were discontinued 7 days prior to performing the TGT; intact platelet function was verified by aggregation tests. All patients with MF and a history of cardiovascular risk factors (hypertension, diabetes mellitus, dyslipidemia) received antihypertension, antidiabetic, and hypolipidemic therapy. The control group comprised 20 healthy volunteer blood donors (15 males and five females) with no history of thrombotic or bleeding episodes, no symptoms of acute infection or chronic inflammatory disease, and not receiving antiplatelet or anticoagulation therapy. None of the female controls used hormonal contraceptives or hormone replacement therapy at the time of analysis. Pre-analysis sample processing and all measurements were performed in an identical way in both groups.

**Blood sampling**
All blood samples were taken in the morning after an overnight fast, as part of regular check-ups, using the standard procedure, that is, drawn from the antecubital vein with minimal tourniquet application. Samples for complete blood count were collected into vacuum tubes containing ethylenediaminetetraacetic acid potassium salt (K3EDTA), those for coagulation tests (PT, APTT, fibrinogen and thrombin time) were drawn into vacuum tubes with 0.5 mL of 3.2% sodium citrate, and blood specimens for platelet aggregometry were taken into a special hirudin tube and one K3EDTA tube. Subsequently, one sample collected into 0.5 mL of 3.2% sodium citrate was centrifuged at 1,000 g for 10 minutes at room temperature. The obtained top fraction (platelet-rich plasma - PRP) was immediately used for the TGT. The other sample drawn into 0.5 mL of 3.2% sodium citrate was centrifuged at 3,000 g for 10 minutes at room temperature. The obtained top fraction (platelet-poor plasma - PPP) was used for the other coagulation tests. Complete blood count was performed with the Sysmex XN 1000 analyzer (Sysmex Corp., Kyoto, Japan) and screening coagulation tests with the ACL TOP 750 CTS system (Werfen, Barcelona, Spain). Platelet function was assessed with the Multiplate® impedance aggregometer (Roche Diagnostics GmbH, Basel, Switzerland) within 1 hour from blood sampling. Platelet aggregation was measured from whole blood according to the manufacturer’s instructions. To test different pathways of platelet aggregation, and their potential dependence on platelet count, aggregation was stimulated by adenosine diphosphate (ADP), arachidonic acid (AA), collagen, ristocetin, and thrombin receptor-activating peptide (TRAP). After platelet activation, a Multiplate® analyzer records impedance between two electrodes during a period of six minutes reflecting platelet aggregation on the surface of the electrodes. Results are reported as area under the curve (AUC), expressed in AU/min [17,18].

**Thrombin generation test**
The test was carried out using the Ceveron Alpha automated coagulation analyzer with fluorescence detection (Technoclone GmbH, Vienna, Austria) and the TECHNOTHRONBIN® TGA kit (Technoclone). The laboratory method described is based on experiences reported in the literature [16,19,20]. Thrombin generation in the sample was initiated with 7.16 pM of recombinant tissue factor (rTF) resuspended in 0.32 µM of phospholipid micelles (containing 2.56 µM of phosphatidylcholine

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and 0.64 μM of phosphatidylserine). The test itself was performed as follows: To 40 μL of the PRP sample, 20 μL of TRIS buffer and 15 μL of 71.6 pM rTF with 3.2 μM of phospholipid micelles were added. Then, 25 μL of the Z-GGR-AMC fluorogenic substrate were added and the reaction itself was initiated with 35 μL of 25 mM CaCl2. The test was calibrated for a thrombin standard of ~800 nmol. Using this standard, calibration points were defined with concentrations of ~200, ~400, and ~800 nmol. With these dilutions, measurements were made according to the following protocol. Into the reaction, 90 μL of adequately diluted thrombin standard were pipetted. The next steps were identical to those used in case of samples. The integrated fluorescence reader itself works with an excitation wavelength of 360 nm and an emission wavelength of 465 nm. Fluorescence is detected at one-minute intervals for 60 minutes at 37°C. Results were automatically analyzed using the Ceveron PC-SW v. 1.4 software, with primarily measured relative fluorescence units being converted into thrombin concentrations in samples using a calibration curve. From the obtained curve, TGT parameters were derived (lag time, peak thrombin concentration - Cmax, area under the curve - endogenous thrombin potential [ETP]). The TGT was performed in the group of MF patients. Excluded were patients with ongoing or recently (< 4 weeks) completed anticoagulation therapy. The control group comprised healthy blood donors. Using MF patients’ history, the relationship between clinical and hematological phenotypes of the disease and thrombin generation was assessed.

Detecting somatic mutations
All patients were tested for the presence of JAK2 V617F, MPL, and CALR gene mutations. All analyses were performed using DNA isolated from peripheral white blood cells (WBCs). In all patients, a mutation at codon 617 of the JAK2 gene was studied by allele-specific polymerase chain reaction (AS-PCR) [21]. In all participants, screening of exon 9 of the CALR gene was performed using fragment analysis. In positive cases, the exact mutation type was identified by Sanger sequencing [11,22]. Mutations of the MPL gene (S505N and W515) were studied by AS-PCR and fragment analysis; the detected mutations were confirmed by Sanger sequencing [10]. Factor V Leiden (G1691A) and factor II (prothrombin) (G20210A) gene mutations were analyzed using the standardized PCR method [23].

Statistical analysis
The associations of individual parameters with TGT indicators were tested by means of univariate analysis. To test the differences in TGT indicators among two or more groups of patients, the Kruskal-Wallis test was used. The Kruskal-Wallis test is a non-parametric method for testing whether samples originate from the same distribution. To assess the relationship between continuous variables (e.g., TGT lag time and leukocyte count), nonparametric correlation was used. All tests were performed in MatLab (MATLAB and Statistics Toolbox Release 2012b, The MathWorks Inc., Natick, MA, USA). The level of significance was set to 0.05.

RESULTS
The distribution of driver mutations was as follows: JAK2 V617F 19 cases (53%), CALR (exon 9) 11 cases (31%) and MPL (W515) five cases (14%); one patient was triple negative. Among CALR-mutated patients, 72% carried type 1 mutation and 27% carried type 3 mutation and other less frequent types; type 2 mutation was not identified in any patient. Venous or arterial thrombosis was documented in 30.5% of patients; of those, 57% of cases were at atypical locations (splanchinic veins). Hemorrhagic complications were documented in 5.5% of cases. At the time of analysis, 67% of patients had splenomegaly with a median size of 10 cm below the left costal margin (range, 2 - 27 cm). In 14% of cases, congenital thrombophilic states were identified (Factor V Leiden mutation and prothrombin gene G20210A mutation). The main characteristics of the study population are summarized in Table 1. To monitor thrombin generation, PRP obtained from MF patients and healthy controls was used. The following three parameters of the obtained curve were assessed: lag time (time to start of thrombin generation), Cmax (peak thrombin concentration) and ETP (area under the curve reflecting the total amount of thrombin generated). The values measured are shown in Table 2. Additional laboratory results obtained in the patient cohort and control group are presented in Table 3. Multiplate® impedance aggregometry (MEA) was tested to identify potential disease-related platelet function abnormalities among the MF group. The group comparison between all MF and controls did not show any statistically significant difference in any of the performed aggregation tests (AA, ADP, TRAP, collagen, ristocetin). In the subgroup of MF with normal platelet count and healthy controls no statistically significant differences were found in any of the tests. Compared to MF without thrombocythemia or healthy control group, MF patients with thrombocytes > 400 x 10^9/L had higher platelet aggregation induced by ADP (320 AU/min vs. 384 AU/min vs. 650 AU/min, p = 0.0002), AA (465 AU/min vs. 472 AU/min vs. 588 AU/min, p = 0.002), and ristocetin (579 AU/min vs. 652 AU/min vs. 1239 AU/min, p = 0.003). No significant difference was observed in TRAP test (577 AU/min vs. 740 AU/min vs. 609 AU/min, p > 0.05). The values are recorded in Table 4. In the patient cohort, platelet counts strongly correlated with aggregation induced by ADP (p < 0.0001), collagen (p < 0.0001), ristocetin (p < 0.05), and AA (p < 0.05). No significant correlation was observed between TRAP induced aggregation and platelet count. Our results show that measurements using available activators (ADP, collagen, ristocetin, AA) were influenced by platelet count.
### Table 1. Clinical and laboratory phenotype and treatment of patients with myelofibrosis at the time of thrombin generation test.

<table>
<thead>
<tr>
<th></th>
<th>prePMF</th>
<th>PMF</th>
<th>post-ETMF</th>
<th>post-PVMF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 11</td>
<td>n = 13</td>
<td>n = 7</td>
<td>n = 5</td>
</tr>
<tr>
<td><strong>Males/females</strong></td>
<td>5/6</td>
<td>7/6</td>
<td>1/6</td>
<td>5/5</td>
</tr>
<tr>
<td><strong>Median age (years)</strong></td>
<td>53</td>
<td>63</td>
<td>51</td>
<td>51</td>
</tr>
<tr>
<td><strong>Splenomegaly (%)</strong></td>
<td>40%</td>
<td>92%</td>
<td>57%</td>
<td>80%</td>
</tr>
<tr>
<td>(median size below the LCM)</td>
<td>(11.5 cm)</td>
<td>(9.5 cm)</td>
<td>(2 cm)</td>
<td>(21 cm)</td>
</tr>
<tr>
<td><strong>Thrombophilic states</strong></td>
<td>0</td>
<td>15</td>
<td>28</td>
<td>0</td>
</tr>
<tr>
<td>(1 congenital thrombophilic states - factor V Leiden gene and factor II (prothrombin) gene mutations.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CVR 2 (%)</strong></td>
<td>0</td>
<td>15</td>
<td>42</td>
<td>20</td>
</tr>
<tr>
<td><strong>History of VTE (%)</strong></td>
<td>27</td>
<td>7</td>
<td>14.2</td>
<td>80</td>
</tr>
<tr>
<td><strong>JAK2 V617F (%)</strong></td>
<td>36.3</td>
<td>54</td>
<td>42.8</td>
<td>0</td>
</tr>
<tr>
<td><strong>MPL (%)</strong></td>
<td>0</td>
<td>30.7</td>
<td>14.2</td>
<td>0</td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Interferon alfa</strong></td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Hydroxyurea</strong></td>
<td>5</td>
<td>6</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><strong>Anagrelide</strong></td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><strong>Ruxolitinib</strong></td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td><strong>Busulfan</strong></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>DIPSS score 3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>LR</strong></td>
<td>10</td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td><strong>INT-1</strong></td>
<td>1</td>
<td>4</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td><strong>INT-2</strong></td>
<td>0</td>
<td>6</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><strong>HR</strong></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

1 congenital thrombophilic states - factor V Leiden gene and factor II (prothrombin) gene mutations. 2 CVR - cardiovascular risk factors (hypertension, diabetes mellitus, dyslipidemia, smoking, overweight).

### Table 2. TGT results in patient with myelofibrosis depending on MF type, progression and mutation status.

<table>
<thead>
<tr>
<th></th>
<th>Lag time (min)</th>
<th>ETP (µmol)</th>
<th>Cmax (µmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>median (range)</td>
<td>median (range)</td>
<td>median (range)</td>
</tr>
<tr>
<td>Controls (n = 20)</td>
<td>3.7 (2.9 - 4.3)</td>
<td>1,876.5 (1,565 - 2,220)</td>
<td>177.5 (134 - 223)</td>
</tr>
<tr>
<td>MF (n = 36)</td>
<td>3.4 (1.9 - 4.3)</td>
<td>1,399 (753.7 - 2,178)</td>
<td>171 (62 - 331.5)</td>
</tr>
<tr>
<td>prePMF (n = 11)</td>
<td>3.4 (2.6 - 4.3)</td>
<td>1,387 (753.7 - 2,178)</td>
<td>165 (62 - 331.5)</td>
</tr>
<tr>
<td>MF (n = 13)</td>
<td>3.1 (2.4 - 3.9)</td>
<td>1,227 (1,129.7 - 2,041)</td>
<td>149 (108.9 - 303)</td>
</tr>
<tr>
<td>CALR- mutated (n = 11)</td>
<td>3.5 (2.9 - 4.3)</td>
<td>1,708 (753.7 - 1987)</td>
<td>164 (62 - 282.9)</td>
</tr>
<tr>
<td>CALR-unmutated (n = 25)</td>
<td>3.4 (1.9 - 4.3)</td>
<td>2,168.4 (1,127 - 875.5)</td>
<td>173.5 (108 - 331)</td>
</tr>
<tr>
<td>DIPSS score 0 (n = 17)</td>
<td>3.4 (2.4 - 3.9)</td>
<td>1,792 (1,257 - 2,178)</td>
<td>200 (114 - 331)</td>
</tr>
<tr>
<td>DIPSS score 1 - 6 (n = 19)</td>
<td>3.3 (1.9 - 4.3)</td>
<td>1,383 (753.7 - 2,028)</td>
<td>142.4 (62 - 290.4)</td>
</tr>
</tbody>
</table>

* p < 0.05 MF patients versus controls; ** p > 0.05 MF patients versus controls. \( ^{1} \) p > 0.05 patients in prefibrotic stage of PMF versus MF. \( ^{2} \) p > 0.05 CALR-mutated patients versus CALR-unmutated. \( ^{3} \) p > 0.05 patients with DIPSS score 0 versus patients with DIPSS score ≥ 1.

TGT - thrombin generation test, ETP - endogenous thrombin potential, Cmax - peak thrombin concentration, MF - myelofibrosis, ET - essential thrombocythemia, PV - polycythemia vera, prePMF/PMF - prefibrotic stage of primary myelofibrosis-primary myelofibrosis, CALR - calreticulin, DIPSS score - Dynamic International Prognostic Scoring System: low risk (0 points), intermediate-1 risk (1 point), intermediate-2 risk (2 - 3 points), high risk (≥ 4 points).
Table 3. Laboratory characteristics of patients with myelofibrosis (n = 36) and controls (n = 20).

<table>
<thead>
<tr>
<th></th>
<th>MF median (range)</th>
<th>Controls median (range)</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quick INR</td>
<td>1.1 (0.9 - 1.58)</td>
<td>0.98 (0.92 - 1.05)</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>Quick (%)</td>
<td>79 (44 - 100)</td>
<td>97 (86 - 100)</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>34.1 (26.8 - 57.6)</td>
<td>27.8 (20.8 - 39)</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>TT (s)</td>
<td>11.3 (10 - 15)</td>
<td>12.3 (10.6 - 13.4)</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>3.46 (1.59 - 6.3)</td>
<td>2.65 (2.11 - 4.35)</td>
<td>p = 0.06</td>
</tr>
<tr>
<td>vWF Ag (%)</td>
<td>126 (46 - 304)</td>
<td>112 (70 - 156)</td>
<td>p = 0.19</td>
</tr>
<tr>
<td>vWF activity (%)</td>
<td>94.2 (35 - 265)</td>
<td>100.5 (63 - 129)</td>
<td>p = 0.72</td>
</tr>
<tr>
<td>Platelets (x 10^9/L)</td>
<td>346 (71 - 882)</td>
<td>236 (163 - 328)</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
<td>112 (77 - 162)</td>
<td>153 (132 - 172)</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>White blood cells (x 10^9/L)</td>
<td>8.3 (3.3 - 37.6)</td>
<td>5.9 (4.53 - 7.55)</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>Blasts in peripheral blood (%)</td>
<td>0 (0 - 12)</td>
<td>0</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>LDH (µcat/L)</td>
<td>7.3 (3.4 - 26)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IL-2R (kU/L)</td>
<td>519 (201 - 1727)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

To test the differences in laboratory characteristics among two groups, the Kruskal-Wallis test was used.

INR - international normalized ratio, APTT - activated partial thromboplastin time, TT - thrombin time, vWF - von Willebrand factor, Ag - antigen, LDH - lactate dehydrogenase, IL-2R - interleukin-2 receptor.

Table 4. Platelet aggregation data and group comparison of the study population.

<table>
<thead>
<tr>
<th></th>
<th>MF</th>
<th>MF with normal PLT</th>
<th>MF with PLT &gt; 400 x 10^9/L</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP (AU/min)</td>
<td>461</td>
<td>320</td>
<td>650 (310 - 859)</td>
<td>384.5</td>
</tr>
<tr>
<td>median (range)</td>
<td>(72 - 1,203)</td>
<td>(132 - 948)</td>
<td>(59 - 740)</td>
<td>(191 - 804)</td>
</tr>
<tr>
<td>Collagen (AU/min)</td>
<td>230</td>
<td>157</td>
<td>436 (59 - 740)</td>
<td>135</td>
</tr>
<tr>
<td>median (range)</td>
<td>(29 - 740)</td>
<td>(35 - 456)</td>
<td>(58 - 420)</td>
<td>(58 - 420)</td>
</tr>
<tr>
<td>Ristocetin (AU/min)</td>
<td>673</td>
<td>579</td>
<td>1239 (120 - 2,065)</td>
<td>652</td>
</tr>
<tr>
<td>median (range)</td>
<td>(55 - 2,065)</td>
<td>(416 - 1,830)</td>
<td>(300 - 1,250)</td>
<td>(300 - 1,250)</td>
</tr>
<tr>
<td>AA (AU/min)</td>
<td>478</td>
<td>465</td>
<td>588 (32 - 987)</td>
<td>472</td>
</tr>
<tr>
<td>median (range)</td>
<td>(32 - 1,084)</td>
<td>(232 - 993)</td>
<td>(257 - 791)</td>
<td>(257 - 791)</td>
</tr>
<tr>
<td>TRAP (AU/min)</td>
<td>582</td>
<td>577</td>
<td>609 (83 - 1,112)</td>
<td>740</td>
</tr>
<tr>
<td>median (range)</td>
<td>(83 - 1,269)</td>
<td>(273 - 1,269)</td>
<td>(543 - 943)</td>
<td>(543 - 943)</td>
</tr>
</tbody>
</table>

Group comparison of all MF patients (n = 36), MF patients with normal PLT (n = 11), MF patients with PLT > 400 x 10^9/L (n = 19) and controls (n = 20). To test the differences in aggregation tests among the groups, the Kruskal-Wallis test was used.

† p > 0.05; MF versus controls. * p > 0.05; MF with normal PLT count versus controls. † † p < 0.05; MF with PLT > 400 x 10^9/L versus controls.

MF - myelofibrosis, PLT - platelets, ADP - adenosin diphosphate, AA - arachidonic acid, TRAP - thrombin receptor-activating peptide.

The study showed a significant difference in thrombin generation between patients and healthy controls. Patients with MF had a shorter lag time (3.4 minutes vs. 3.7 minutes; p = 0.02) and a lower ETP (1,399.0 µmol vs. 1,876.5 µmol; p = 0.0003), see Table 2, Figure 1. In the patient cohort, platelet counts correlated with ETP (p = 0.04) and Cmax (p = 0.004); patients with thrombocythemia > 400 x 10^9/L had significantly higher ETP (1,671.7 µmol vs. 1,381.5 µmol) and Cmax (196.9 µmol vs. 166.0 µmol), see Figures 2 and 3.

There is a clear association between TG and disease progression, and there are also differences in TG between MF subgroups. In the prefibrotic stage of MF and post-ET MF, ETP and Cmax values are consistently higher (Table 2). When comparing TG in patients stratified according to the Dynamic International Prognostic Scoring System (DIPSS), low-risk patients (DIPSS score 0) had higher ETP (1,792 µmol vs. 1,383 µmol) and Cmax (200.0 µmol vs. 142.4 µmol) than those with more advanced disease (DIPSS score 1 - 6) [24]. When
Figure 1. Thrombin generation in patients with myelofibrosis and healthy controls.

Thrombin generation expressed as ETP was significantly lower in patients with myelofibrosis than in healthy controls (1,399 µmol vs. 1,876.5 µmol; p = 0.0003). Measured in platelet-rich plasma.

ETP - endogenous thrombin potential.

Figure 2. Impact of platelet counts on thrombin generation in patients with myelofibrosis.

Platelet counts were correlated with ETP (p = 0.04) and Cmax (p = 0.004); patients with thrombocythemia > 400 x 10^9/L had significantly higher ETP (1,671.7 µmol vs. 1,381.5 µmol) and Cmax (196.9 µmol vs. 166 µmol), suggesting higher thrombin generation. Measured in platelet-rich plasma.

ETP - endogenous thrombin potential, Cmax - peak thrombin concentration.
Figure 3. Impact of platelet counts on thrombin generation in patients with myelofibrosis.

Platelet counts were correlated with ETP (p = 0.04) and Cmax (p = 0.004); patients with thrombocythemia > 400 x 10^9/L had significantly higher ETP (1,671.7 µmol vs. 1,381.5 µmol) and Cmax (196.9 µmol vs. 166 µmol), suggesting higher thrombin generation. Measured in platelet-rich plasma.

ETP - endogenous thrombin potential, Cmax - peak thrombin concentration.

comparing TG in patients stratified according to mutation status of the disease, a longer lag time, lower Cmax and ETP were noted in CALR-mutated MF (the effects were not of statistical significance, probably only due to the limited number of subjects included in the study). Patients with MF and a history of venous or arterial thrombosis did not show significantly higher TG compared to those with a negative history of VTE (p > 0.05). Moreover, there were no differences in TG between patients stratified by their cardiovascular risk (hypertension, diabetes, dyslipidemia, smoking, and overweight). The present study failed to confirm significant correlations between TG and lactate dehydrogenase, IL-2 soluble receptor and hemoglobin levels, total white blood cell count or mature neutrophil count. Similarly, the presence of congenital thrombophilic states (factor V Leiden and prothrombin G20210A gene mutations) or splenomegaly were not associated with higher TG. Neither age nor gender had a significant impact on TGT results.

DISCUSSION

Despite the increased risk of thrombotic complications in MF patients, standard coagulation tests (APTT, PT, activity of coagulation factors and their inhibitors) yield no significant variations in most cases. Monitoring of thrombin generation appears to be a suitable approach to global hemostasis tests capable of reflecting acquired imbalance between procoagulant and anticoagulant factors.

The Multiplate® analyzer can detect platelet dysfunction and therefore has been used as a method for measuring platelet function in whole blood. Comparisons of patients with MF and the healthy control individuals were studied to identify potential platelet function abnormalities among the MF patients. Numerous platelet abnormalities, both quantitative and qualitative, have been identified in ET and PV patients, however none of these findings has been clearly linked to the thrombotic complications [25]. Consistent with literature, the present study confirmed significant correlations between platelet counts and platelet aggregation induced by ADP, collagen, ristocetin, and arachidonic acid. Our results did not show any statistically significant difference in ADP, collagen, ristocetin, AA, and TRAP induced platelet aggregation in all MF patients compared to control subjects [17]. In the analyzed study subpopulation comparing MF patients with normal platelet count to healthy controls no statistically significant differences in any of the performed aggregation tests were detected. On the other hand, MF patients with thrombocythemia > 400 x 10^9/L had significantly higher platelet aggregation than MF without thrombocythemia or control group. These findings suggest that increased aggrega-
tion is due to increased platelet count rather than their dysfunction in our particular MF group. The activation of platelets results in exposure of phosphatidylserine on their surface, which is necessary for platelet procoagulant activity. Activated platelets can facilitate TG by providing a catalytic surface for activation of coagulation factors and hence the platelet surface can play an important role in the production of TG [26]. The present study investigated the potential of TG in MF patients, the ability of TGT to detect deviations from the hemostatic equilibrium, and to identify patients at a high risk for thrombosis. According to the literature, the prothrombogenic state is associated with higher ETP, higher Cmax, and a shorter lag time. The test may be performed using PPP or PRP. Previous studies on the benefit of TGT in Ph-MPNs showing higher TG in the MPN population mostly included patients with PV and ET. The present study analyzed MF patients with significantly lower ETP compared to controls (p = 0.0003). Also, Tripodi et al. and Duchemin et al. reported lower ETP in MPN patients as compared with healthy controls. After modification of the TGT by adding thrombomodulin (activator of protein C on the surface of endothelial cells), the authors found higher ETP in Ph-MPN patients [19,20]. Similarly, Marchetti et al. reported lower ETP in the MPN population as compared with controls. In the Ph-MPN cohort, higher ETP was only detected by the TGT with added exogenous activated protein C (APC). These results suggest acquired APC resistance (APC-R) due to decreased levels of free protein S in patients’ plasma. However, Marchetti et al. failed to confirm APC-R by functional assessment of the anticoagulant capacity of the protein C system using the coagulation method that they did not consider sensitive enough in these cases [27]. In the present study, patients’ lower ETP may be due to possible limitations of the TGT method; however, the effect of specific therapy in most patients cannot be excluded either. Consistent with previous studies, tests using PRP in the present study confirmed significant correlations between platelet counts and TGT parameters (ETP and Cmax) [20,28]. Patients with thrombocythemia had significantly higher TG. The results suggest that MF patients have an increased platelet associated TG potential. By contrast, Olteanu et al. reported no correlation between platelet counts and TG using PPP [29]. It appears that PPP is not a medium fully representing the in vivo hemostatic system where, apart from plasma coagulation factors, activated platelets and other blood cells play an important role in TG. It cannot be reliably established whether higher TG in thrombocythemia patients only reflects higher platelet counts or it is a sign of higher humoral activity of the disease associated with, among others, thrombocythemia.

In the patient cohort, higher TG was observed in those with low risk disease according to the DIPSS score and in the prefibrotic stage of MF. This is consistent with a study reporting the highest TG in newly diagnosed patients [29]. It may be assumed that these patients may be at a higher risk of developing thrombosis compared to those with more advanced disease. Given the low incidence of the condition, the hypothesis needs to be verified by a multicenter study with a sufficiently large cohort of patients. Due to the relatively small number of patients, the present study was unable to provide a statistically relevant assessment of the effects that various types of specific MF treatment have on TG. However, previous analyses found lower TG in Ph-MPN patients receiving hydroxyurea compared to those treated with anagrelide. The extent of reduction of all TGT parameters depended on treatment duration [27,29].

Data from the present study show a trend towards lower TG in CALR-mutated MF (a longer lag time, lower ETP and Cmax; all are correlated with each other; statistical significance was not achieved, probably only because of the limited number of subjects). These findings support results from a study by Rumi et al. reporting a lower risk of thrombosis in CALR-mutated MF compared with JAK2 V617F-mutated. The risk of thromboembolic complications remains lower in patients carrying both type 1 and type 2 mutations of the CALR gene [30]. Marchetti et al. reported higher TG in MPN patients with a positive history of VTE [27]. This was not confirmed by either Olteanu et al. or the present analysis of MF patients [29]. Similarly, the presence of cardiovascular risk factors did not influence the TGT parameters; this may be explained by effective (antihypertensive, antidiabetic, hypolipidemic) therapy of all at-risk patients.

**CONCLUSION**

Given the therapeutic difficulties, there is still a need for identification of additional laboratory markers more accurately reflecting a tendency for thrombosis in MF patients. This is because accurate identification, consistent prevention, and treatment of at-risk individuals may reduce their morbidity and mortality. Significantly higher TG was found in patients with increased platelet counts suggesting a role of thrombocythemia as a risk factor for thrombotic complications. However, there is a high interindividual variability in TG. Whether this “in vitro higher TG” in the MF participants with thrombocythemia is of any clinical significance has to be evaluated in prospective clinical trials. Furthermore, the results indicate that in myelofibrosis, TG depends on mutation status, activity, and progression of the disease, with TG being higher in early disease stages. Lower TG was observed in CALR-mutated MF. The TGT failed to confirm the effect of the other risk factors on TG. Among the studied TGT parameters, lag time appears to be the least beneficial. However, future studies will be needed to find out how accurately the TGT is able to detect patients at risk for thrombosis and whether changes in TGT values induced by MF therapy reflect reduction of these complications in real practice. Due to the low incidence of the condition,
more valid data may only be obtained from a multicenter study with a sufficiently large cohort of patients. The so far inconsistent methods (tests performed using both PRP and PPP; triggers used) make direct comparison of results published by various laboratories impossible. Therefore, an essential precondition for a wider use of TGT in routine clinical practice is standardization of the test protocol and reagents used.

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References:


