“LUCIAN BLAGA” UNIVERSITY OF SIBIU
FACULTY OF MEDICINE

PhD thesis summary

Contributions to the exploration of the prothrombotic state in Philadelphia-negative chronic myeloproliferative neoplasms

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Contents

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Introduction

Myeloproliferative neoplasms negative for the *BCR-ABL1* fusion gene, named Philadelphia-negative (Ph-MPN), are clonal hematopoietic stem disorders which are strongly affected by disease-related hemostatic complications, esspecially thrombosis. According to the World Health Organization (WHO) classification these include polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF).

The molecular markers together with bone marrow histological features and cytogenetics play an important role in diagnosis but the prognostic value is still under debate. Cytoreductive and anti-platelet treatment are very important in prevention of thrombotic events and the only potential cure is the allogenic stem cell transplant.

The studies conducted so fare proved that the pathogenesis of thrombosis is complex and involves two main mechanisms: the presence of the abnormalities of the blood cells arising from the clonal proliferation of hematopoietic stem cells which express a prothrombotic phenotype and the inflammatory response of the host vascular cells to the insult of cytokines and other mediators released by malignant cells.

Many studies aimed to assess the platelets activation and to determine the biomarkers that would quantify it. Increased levels of cellular activation: expression of P-selectin, platelet-leukocyte aggregates and increased levels of circulating procoagulant microparticles were found in Ph-MPN.

Activated platelets expose their negatively charged phospholipid surface and enhance the thrombin generation, demonstrated *in vivo* through high level of thrombin-antithrombin complex, d-Dimeri and prothrombin fragments 1+2 and increased levels of thrombin generation assay parameters *in vitro*. Studies provided evidences of the involvement of red blood cells, leukocytes and endothelial cells in the increase of hypercoagulable state of blood in Ph-MPN.

The demonstration of the acquired gain-of-function V617F mutation in the tyrosine kinase *JAK2* gene has greatly influenced the diagnostic and therapeutic approch in MPN patients. During the last decade other driven mutations, like *MPL (myeloproliferative leukemia virus oncogene)* and *CALR (calreticulin)* mutations were included in diagnostic criteria for PV, TE and PMF. Some of the studies have reported the correlation of the molecular mutations (especially *JAK2*V617F) and the increasing level of both cellular and plasma activation biomarkers and some have studied the influence of the allele burden on

pathogenesis of vascular events in Ph-MPN. Current drug therapy in PV, ET and PMF, which is neither curative nor capable of prolonging life or preventing disease progression, is primarily directed at prevention of thrombotic complications.

The current clinical research is focused on the development of the prognostic biomarkers and new molecules which will modify the evolution of disease in Ph-MPN. Due to this, I considered that it would be of interest to perform the personal research through hemostasis tests which are new or less used in our country.

The PhD thesis encompasses two sections: the general one in which it is realized a synthesis of the data in the literature referring to Ph-MPN and the special part for personal research.

The Current Knowledge

The current knowledge section is structured in three chapters: ”Chronic myeloproliferative neoplasms”, ”Philadelphia-negative chronic myeloproliferative neoplasms” and ”Platelets, involvement in haemostasis and neoplasms”.

The first chapter defines the MPN and provides the update WHO classification of the myeloproliferative neoplasms.

The second chapter, structured in 7 sub-chapters, represents the preambule of the personal research, as it describes the diseases studied in this thesis and provides an overview, diagnostic criteria, and the present molecular mutations in Ph-MPN. The sub-chapter named ”Pathogenesis of thrombosis in Ph-MPN” synthesises the main mechanisms involved in thrombotic events in MPN. The role of treatment in reducing thrombotic events is presented too.

The third chapter of the thesis, structured in nine short sub-chapters, presents the platelets structure, the role of platelets in haemostasis and coagulation cascade, the platelets mRNAs, the concept of cell-based control of coagulation ”platelet-based coagulation” and the populations of platelet that formed the platelet thrombus. The sub-chapter ”Interactions between platelets and tumor-cells” underlines the contribution of platelet in cancer growth and dissemination, and in the development of the vascular events in malignancies.

Personal research

The most important part of the thesis, is structured in four studies and a pilot study. All of the experiments were performed in the laboratories of County Emergency Hospital, Sibiu and at ”Lucian Blaga” University.

The main pourpose of the research was to prove the existence of the hypercoagulable state in Ph-MPN patients, through thrombin generation assay in platelet rich plasma (PRP) and platelet poor plasma (PPP), expression of P-selectin, and measurement of platelet volume indices.

In the study population, Ph-MPN patients (PV, ET and MF patients), I tried to establish a correlation between tests parameters and the presence of *JAK2*V617F mutation, thrombotic history, cardiovascular risk factors and cytoreductive or antiplatelet therapy.

To fulfill these aims I selected patients diagnosed with Ph-MPN, from both genders. The patients group was heterogeneous and encompassed patients with PV, ET and MF. The diagnosis was established according to the 2001 and 2008 criteria for MPN. The patients diagnosed with MF post PV or post ET were included in MF subgroup along with PMF patients. All patients included in the studies were diagnosed in the Clinic of Hematology from County Emergency Hospital, Sibiu.

The studies were observational studies, case-control, which included a control group formed of healthy subjects (men and women) for each study.

The studies were conducted in consecutive years, and each study had its patients and control groups. To compare the results obtained, the patients were stratified in subgroups: PV, ET, MF, with/without *JAK2*V617F mutation, thrombotic events, cardiovascular risk factors and treatment. Healthy volunteer subjects, without symptoms of acute infection or chronic inflammation diseases, without receiving anti-platelet agents or oral anticoagulants were included as controls.

Working methodology

Evaluation of platelet volume indices

Platelet volume indices, mean platelet volume (MPV), platelet distribution width (PDW) and platelet cell ratio (P-LCR), are a group of parameters which are derived from routine blood counts. MPV is expressed in femtoliters (fl), and is calculated from equation: MPV(fl) = [(plateletcrit % / platelet number x10^3/μL) x 10000]. The other PVI, PDW (fl) and P-LCR are calculated from the platelet size distribution curve. For platelet distribution width

(PDW) the analyzer uses 3 discriminators, 2 moving between 2-6 fl and 12-30 fl, one fixed at 12 fl. The percentage of platelets >12 fl was notified P-LCR.

Thrombin generation assay (TGA)

Thrombin generation assay is a functional assay through which we measure the amount of thrombin generated after activation of coagulation, *in vitro*, with tissue factor or other trigger.

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. The results were automatically calculated by the Ceveron® alpha software and displayed in:

- *Lag time* (phase) (min), from the time point when the TGA reagents are added until the first burst in thrombin formation;
- *Thrombin Peak (nM),* height of thrombin generation, maximal concentration of thrombin formed;
- *Time to peak* (min), peak height time;
- *Velocity index* or peak rate of thrombin generation or slope (nM/min)= the steepest rate of thrombin formation per minute calculated by software as velocity index (VI);
- Area under the curve (AUC) (nM), sum of thrombin concentration from endogenous thrombin potential (ETP);

TGA curve reflects and integrates all pro and anticoagulant reactions that regulate the formation and inhibition of thrombin (initiation, propagation and termination phases of coagulation cascade).

To obtain a thrombin generation (TG) curve from the fluorescence that develops when plasma clots in the presence of a fluorogenic thrombin substrate requires a number of calculation steps that are routinely carried out by the software that comes with the method.

New generation of the device for thrombin generation measurement, Ceveron alpha, owns 4 channels with special fluorometric TGA modules consisting of an UV LED (365nm) for excitation and a photodiode for measurement of the emitted signal that are placed in the pre-heated cuvette rotor.

Evaluation of P-selectin expression by flow cytometry

The use of flow cytometry for the in vitro estimation of platelet function is gaining widespread use. The various advantages of flow cytometric evaluation of platelet function have been comprehensively reviewed. The flow cytometric analysis of platelets is being used increasingly for the detection of agonist-induced alterations in the membrane expression of platelet activation markers.

Flow cytometry rapidly measures the specific characteristics of a large number of individual cells. Before flow cytometric analysis, cells in suspension are fluorescently labeled, typically with a fluorescently conjugated monoclonal antibody (MoAb). In the flow cytometer, the suspended cells pass through the focused laser beam. After fluorescent activation of the fluorophore at the excitation wavelength, a detector processes the emitted fluorescence and light scattering properties of each cell.

Analyses were performed with Cytomics FC 500 flow cytometer Beckman Coulter (argon laser/excitation wavelength at 488nm).

Flow cytometric studies were commenced within 20 minutes from blood collection in whole blood before and after in vitro blood stimulation.

Other laboratory tests

CBC was performed on Sysmex XT 2000i and Sysmex 1000i analyzers and coagulation screening tests were performed on Sysmex CA 1500 coagulation analyzer. Serum glucose and lipid profile parameters were performed on Architect c8000.

Assessment of JAK2V617F mutation was performed by an amplification refractory mutation system polymerase chain reaction (ARMS-PCR) described by Jones et al., and real time PCR described by Larsen, modified.

Statistical analysis

For the statistical analysis we used the program IBM SPSS version 20 and 21 and EPI INFO 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 and Mann-Whitney (MW) test and Median test (Med-T) for groups without normal distribution. The correlation between two continuous variables was made with Pearson correlation or Spearman's rho. For analysis of

variances one uses ANOVA test, Kruskal-Wallis test and for Post-Hoc multiple comparisons one employs Bonferroni and Tamhane`s tests.

The frequent 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) upon parameters studied. We considered the differences statistically significant if $p < 0.05$.

These studies were approved by the Ethics Committee of the Emergency County Hospital Sibiu. All subjects signed an informed consent to enter the study and to donate blood.

Pilot Study. The impact of pre-analytical variable, type of anticoagulant and time delay, on the measurement of mean platelet volume (MPV)

Objectives: The study aimed to demonstrate the influence of anticoagulants and time delay from blood collection on the measurement of the MPV.

Material and Methods: The prospective study comprised two study groups. The first group included 102 Ph-MPN patients and the second one, 57 healthy subjects, volunteers, fallowing the eligibility criteria.

Full blood count (including platelet volume indices) was performed on Sysmex XS 1000i, at 30 minutes and 2 hours after blood collection, through the impedance method from K2EDTA tubs and sodium citrate 3.2% tubs.

Results: The results of the present study has shown that storage of blood sample in anticoagulant based on EDTA resulted in a progressive increase in the MPV.

In this study it was found a significant difference between MPV measured in blood collected in tubs with K2EDTA, compared with those containing sodium citrate 3.2% at 30 minutes and 2 hours. The difference was observed in patients and healthy control groups. MPV from citrated samples revealed significantly smaller MPV.

The values of MPV measured in tubs with K2EDTA at different times, 30 minutes and 2 hours, revealed a significant difference which was not noticed in tubs containing sodium citrate as anticoagulant.

The results did not show a difference of MPV values between patients group and control group from the tubs containing the same type of anticoagulant and analyzed at the same time.

Conclusions: The study demonstrated that time and type of anticoagulants influence the MPV values, measured by impedance based method. Results from the present study showed that storage of blood samples in EDTA based anticoagulant resulted in a progressive increase in the MPV and made EDTA not suitable as anticoagulant for the research on platelet function.

Study no 1. Platelet indices in Philadelphia-negative chronic myeloproliferative neoplasms (Ph-MPN)

Working Hypothesis and objectives: Platelet volume indices (PVI) along with platelet number characterize the platelet population and are provided by hematological analyzer. Recent studies show that platelet from these patients circulate in an activated state and I considerated that this activation state might be correlated with PVI values in patients with PV, ET and MF.

The study aimed to establish if platelet indices (MPV, PDW, P-LCR) issued from automated complete blood count determination are significantly different in Ph-MPNs patients in comparison to healthy subjects and demonstrate a correlation between PVI and the prothrombotic status in patients diagnosed with chronic Ph-MPN.

Material and methods. This prospective study comprised 102 patients diagnosed with Ph-MPN, (36 with PV, 54 with TE, 12 with MF) and one hundred two healthy subjects, volunteers, with study eligibility criteria included as controls. Full blood count (including platelet volume indices) was performed on Sysmex XS 1000i, during the first hour after blood collection through impedance method in K2EDTA tubs.

Results: The study has not highlighted significant differences of MPV, PDW and P-LCR values between the group of patients and healthy subjects, but the distribution of values was wider for patients, especially for the P-LCR in ET subgroup. Patients with MF showed a significantly lower MPV ($p=0.05$) and P-LCR ($p=0.02$).

Patients with thrombotic events did not have statistically different values of platelet volume indices in comparison to those without thrombotic events. Considering the difference of these indices, in carriers of *JAK2*V617F mutation versus patients without this molecular mutation, I found differences that did not reach statistical significance. Patients treated with anagrelide had significantly higher values ($p<0.002$) for PVI compared with patients without anagrelide treatment. No influence of treatment with hydroxyurea (HU) and aspirin was noted for MPV and P-LCR patients' parameters. Only PDW values were significantly lower in

patients treated with cytoreductive and anti-platelet therapies compared with patients without this treatment ($p < 0.05$). The patients receiving statins had significantly lower PVI values than patients without this treatment ($p < 0.05$).

Conclusions: Platelet volume indices measured with the impedance based method, did not show significant differences in Ph-MPN patients in comparison to healthy controls. PVI were increased in patients treated with anagrelide compared with untreated patients. I noticed a moderate correlation between these indices and the presence of *JAK2*V617F mutation. Due to the small number of subjects in the groups, further studies are required to demonstrate a correlation between platelet volume indices and *JAK2* mutation, anagrelide and statins treatments, respectively.

Study no 2. Evaluation of thrombin generation in Philadelphia-negative chronic myeloproliferative neoplasms (Ph-MPN)

Working Hypothesis and objectives: 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. Global tests, such as thrombin generation, were able to detect signs of procoagulant tendency in MPN.

In this study I 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 *JAK2*V617F mutation, thrombotic history, treatment, and cardiovascular risk factors (hypertension, diabetes, dyslipidemia, smoking, overweight) on their TGA parameters.

This is the first report describing the measurement of thrombin generation in Ph-MPN 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 (26 with PV, 51 with ET, 12 with MF). The diagnosis was established according to the 2001 and 2008 criteria for Ph-MPN. The patients who underwent anticoagulant therapies were not included in the study group. Seventy eight healthy volunteer subjects with study eligibility criteria were included as controls.

Thrombin generation test was determined in platelet poor plasma using Technothrombin® TGA reagents kit (Technoclone, Vienna, Austria) for fully automated Ceveron® alpha in accordance with the provider`s recommandations. All the TGT tests were performed by the author of the present study.

Results: In this 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 patients group it was 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 counts and peak thrombin (RS=0.213, p=0.046 Spearman). On the other hand, no significant correlation between actual platelet number and TGA parameters in ET subgroup or other subgroups was noticed.

When I analyzed the TGA parameters for all subgroups of Ph-MPN patients (PV, ET and MF patients) in comparison with the controls, in the PV patients group was 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. Between 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=0.042-MW) in comparison with those who are 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=0.02-MW and p=0.024-MW).

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 *JAK2*V617F 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 in comparison 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) ($p<0.05$).

In the study, TGA parameters means did not differ significantly between men and women and were not influenced by age. Therefore, it seems that neither age nor gender distributions had any influence on the levels of the TGA parameters.

Conclusions: The study has 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 thrombotic risk in these disorders, even during cytoreductive therapy. I 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. It was also found that plasma of patients treated with hydroxyurea generated less thrombin than plasma of those treated with anagrelide. Further studies are required to confirm the findings.

Study no 3. Evaluation of the procoagulant potential of platelets in Philadelphia-negative chronic myeloproliferative neoplasms patients by Thrombin Generation Assay (TGA)

Working Hypothesis and objectives: The previous study results regarding the evaluation of thrombin generation in Ph-MPN patients (PPP), led to this study where my intention was to assess the contribution of platelets in thrombin generation in Ph-MPN. I took into consideration the fact that the platelet rich plasma imitates *in vivo* conditions of the coagulation process and the preactivated platelets of the Ph-MPN patients can increase the level of thrombin generation (TG). The TG expressed by PRP samples has been useful to assess the procoagulant role of platelets in thrombin generation using the fully automated device for TGA measurement Ceveron® alpha.

The TGA parameters lag time, peak thrombin and AUC were evluated in relationship with: presence of *JAK2*V617F mutation, thrombotic history, treatment, and cardiovascular risk factors (hypertension, diabetes, dyslipidemia, smoking, overweight) in their TGA parameters.

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

Material and methods: This prospective study consists of 76 consecutive patients diagnosed with Ph-MPN and sixty healthy volunteer subjects were included as controls. The patients who underwent anticoagulant therapies were not included in our study group.

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

Thrombin generation was determined in platelet rich plasma using Technothrombin® TGA reagents kit (Technoclone, Vienna, Austria) for fully automated Ceveron® alpha and following the provider`s recommandations. All the TGT tests were performed by author of the present study.

Results: Parameters of thrombin generation found significantly increased in patients in comparison with healthy controls were: peak thrombin (p=0.0001-MW), velocity index (p=0.0001-MW) and area under the curve (p=0.008-MW).

In patients group it was noticed a significant weak direct positive correlation between peak thrombin and leukocyte counts (Rs=0.321, p=0.006) and platelet counts (R_S=0.242, p=0.038); VI and leukocyte counts (Rs=0.299, p=0.01) and platelet counts (R_S=0.252, p=0.03) and a significant positive correlation (Pearson) between Ht and peak thrombin (Rs=0.271, p=0.019).

The TGA parameters for all subgroups of Ph-MPN patients (PV, ET and MF patients) in comparison with the control group were analyzed and peak thrombin and VI differed significantly between PV patients and control (p=0.045-T; p=0.023-MW). The maximum velocity of thrombin formation (p=0.0001-MW) peak thrombin (p=0.0001-MW) and AUC (p=0.0001-T) were significantly increased in ET patients in comparison with controls. Between Ph-MPN patient subgroups, only AUC was significantly different (p=0.003-KW).

Patients receiving anagrelide displayed higher values of AUC (p=0.011-T) in comparison with those who are treated with HU. A significant inverse negative correlation (Pearson) between the length of cytoreductive therapies and TGA parameters was noticed for peak thrombin (R= – 0.336, p=0.003) VI (R= – 0.310, p=0.006) and AUC (R= – 0.233, p=0.043).

The carriers of *JAK2*V617F mutation displayed lower values for endogenous thrombin potential (AUC).

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

Thrombin generation parameters did not show significant differences between patients stratified in accordance to the presence of cardiovascular risk factors (hypertension, diabetes, dyslipidemia or smoking) ($p<0.05$). Body mass index (BMI) presented a positive correlation (Spearman) with peak thrombin ($Rs=0.232$, $p=0.044$), VI ($Rs=0.227$, $p=0.048$) and AUC ($Rs=0.237$, $p=0.039$).

The two study groups (controls and Ph-MPN patients) differed in age distribution and gender ratio. A correlation analysis was used to study the influence of age. It was noticed a week correlation ($R<0.25$) between age and TGA parameters which had a tiny influence on TGA parameters variation (<5%).

In the study TGA parameters means did not differ significantly between men and women. Therefore, it seems that neither age nor gender distributions had significant influence on the levels of the TGA parameters.

Conclusions: Platelet rich plasma from Ph-MPN patients, and especially from ET, produced significantly increased heights of thrombin generation in comparison to those of controls. These results may accont for the increase in thrombotic risk in these disorders even during cytoreductive therapy and point out the higher procoagulant potential of platelet in MPN.

In patients` group we noticed significant weak direct positive correlation between peak thrombin and Ht, leukocyte counts and platelet counts, VI and leukocyte and platelet counts. It was provided evidence of the implications of red blood cells, leukocytes and platelets in the increase of hypercoagulable state of blood in these disorders.

It was demonstrated that TGA parameters are inversely correlated with the length of the treatment and TGA could be a useful tool in monitorising the results of cytoreductive therapies and assessing the thrombotic risk. It was also found that plasma of patients treated with hydroxyurea generated less thrombin than plasma of those treated with anagrelide (AUC $p=0.011$). Further studies are required to confirm our findings.

The carriers of *JAK2*V617F mutation displayed lower values for endogenous thrombin potential (AUC).

The body mass index (BMI) is correlated with TGT parameters which indicates that the thrombin generation will increase along with BMI.

Study no 4. Evaluation of P-selectin expression in Philadelphia-negative chronic myeloproliferative neoplasms (Ph- MPN)

Working Hypothesis and objectives: The level of platelet activation and the response of platelet to a variety of specific stimuli in Ph-MPN could be evaluated by flow cytometry.

Previous studies and my personal research have shown a high level of thombin generation in Ph-MPN patients in comparison to healthy subjects. These results and the inverse correlation between *in vitro* trombin-inducible P-selectin and peak thrombin generation potential led to the work of the actual study. The purpose was to evaluate the platelet activation and the degree of responsivity of the platelet from Ph-MPN.

The P-selectin expression (CD62P) was assessed by flow cytometry. Platelet activation was determinated by P-selectin expression, without and after *in vitro* addition of TRAP-6 in both sub-maximal and maximal doses.

The expression of P-selectin was analyzed in comparison with a control group and tried to establish the influence of: presence of *JAK2*V617F mutation, thrombotic history, treatment, and cardiovascular risk factors (hypertension, diabetes, dyslipidemia, smoking, overweight).

Material and methods: This prospective study consists of 68 consecutive patients diagnosed with Ph-MPN (18 with PV, 43 with ET and 7 with MF). Forty seven healthy volunteer subjects, were included as controls. In both study groups the pre-analitical conditions as well as measurement of P-selectin expression were performed identically.

Analyses were performed by Cytomics FC 500 flow cytometer Beckman Coulter. Flow cytometric studies were commenced within 20 minutes of blood collection, in whole blood, before and after *in vitro* platelets stimulation (TRAP-6). The flow cytometer was reset in order to perform platelets acquisition.

For platelet activation studies, the following antibodies were used: anti-CD41b-PC5 (phycoerytrin-Cy5), clone P2 from Beckman Coulter (New Jersey, USA) and anti-CD62P-PE phycoerythrin conjugated), clone CLB-Thromb/6 (Beckman Coulter, New Jersey). Isotype control from the same provider was used. The concentration of all antibodies used has been settled by titration.

Platelet activation was assessed in unstimulated samples (basal samples), or samples stimulated by TRAP-6 (TRAP-6 H-Ser-Phe-Leu-leu-Arg-Asn-OH trifluoroacetate salt; H-8365) (Bachem, Swissland).

Platelet activation studies were performed as previously described. The TRAP-6 concentration for platelet stimulation was settled in a previous experiment dose-response aplied to healthy subjects. It was used both sub-maximal and maximal doses of TRAP-6 in order to obtein a 40-60% P-selectin expression and 80-90% P-selectin expression.

Results were expressed as percentage of cells positive for CD62P from CD41 positive cells population.

Results: The results for expression of P-selectin without and with stimulation were significantly diffrent between patients and controls, without stimulation (p=0.02-MW) and stimulated submaximal (p=0.0001-T) and maximal (p=0.0001 MW).

A significant negative correlation (Spearman) between the length of cytoreductive therapies and the TRAP-6-inducible P-selectin expression (maximal stimulated) was noticed (R= – 0.269, p=0.032) and a positive correlation between the resposivity of patients` platelets after sub-maximal and maximal stimulation (Rs=0.851, p=0.0001) were observed.

We analyzed the espression of P-selectin for all subgroups of Ph-MPN patients (PV, ET and MF patients) in comparison with the controls. In the PV patients group we noticed significant diffrences for the expression of P-selectin without stimulation (p=0.004-MW) submaximal (p=0.006-T) and maximal (p=0.001-T) stimulation. Expression of P-selectin was significantly increased in ET patients in comparison with controls for submaximal and maximal stimulation (p=0.0001-T; p=0.0001-T). MF patients showed a significantly different expression of P-selectin without stimulation and with stimulation in comparison with controls (p=0.017-MW).

Patients receiving HU did not display higher values for expression of P-selectin in comparison with those who are treated with anagrelide. The lengh of the cytoreductive therapies influenced expression of P-selectin. The patients receiving cytoreductive treatment for long time, more than one year, showed a lower expression for P-selectin in comparison with those with recent cytoreductive treatment (less than 1 year) and their platelets were less responsive to the TRAP-6 maximal stimulation (p=0.002-T).

For the carriers of *JAK2*V617F mutation the result did not differ significantly in comparison with WT carriers (p>0.05-T).

Ph-MPN patients with history of thrombosis (arterial or venous thrombosis) did not present significantly higher values in comparison with patients without such thrombotic events (p>0.05-T).

Apart from dyslipidemia, the existence of cardiovascular risk factors (hypertension, diabetes, smoking or overweight) did not influence significantly the expression of P-selectin

among patients ($p<0.05$). The patients with dyslipidemia showed higher level of P-selectin and were less responsive to agonist stimulation *in vitro.* ($p=0.001$-T; $p=0.01$-T).

Therefore, neither age nor gender have any significant influence on the levels of P-selectin expression.

Conclusions: The platelet responsiveness to thrombin-receptor activating peptide-6 (TRAP-6), as determinated by their expression of P-selectin, was significantly lower in Ph-MPN in comparison with controls suggesting *in vivo* ongoing platelet activation. The responsiveness was significantly lower among PV, ET and MF diagnosed groups and controls.

P-selectin expression on platelet surface was significantly lower in basal samples, in Ph-MPN in respect to controls ($p=0.02$). This result was contrary to our expectations and in contradiction with what was previously reported. It is possible that P-selectin on activated platelets was shed and appeared in its soluble form in the circulation.

It was demonstrated a negative correlation between the expression of P-selectin (maximal stimulated) and the lenght of the cytoreductive therapies.

The platelet responsiveness to thrombin-receptor activating peptide-6 (TRAP-6), after maximal stimulation, as determinated by their expression of P-selectin, was reduced in patients with longer therapy than in those with less therapy.

The association of dislypidemia in Ph-MPN conducted to a higher P-selectin expression and the platelets were less responsive to the agonist stimulation in patients with dyslipidemia in comparison with those without dyslipidemia($p=0.001$ și $p=0.01$).

The present study highlighted the inverse correlation between levels of thrombin generated in plasma of Ph-MPN patients and the responsiveness of platelet agonist-stimuleted *in vitro*.

Due to the fact that P-selectin expression on platelet surface was significantly lower in basal conditions (without stimulation) in Ph-MPN, further studies are required to evaluate the level of plasma P-selectin (sP-selectin) and corelate it with the results of present study.

General conclusions

1. Plasma from Ph-MPN patients generated significantly increased levels of thrombin (in PRP and PPP) in comparison with healthy subjects.

2. It has been shown that platelets of patients with Ph-MPN have a higher prothrombotic potential as assessed by the increased thrombin generation produced in PRP.
3. Patients with ET had significantly higher level of thrombin generation potential in comparison with PV and MF patients.
4. The platelet responsiveness to thrombin-receptor activating peptide-6 (TRAP-6), as determined by their expression of P-selectin, was significantly lower in Ph-MPN in comparison with controls.
5. The thrombin generation potential was inversely correlated with TRAP-6 inducible P-selectin. This inverse correlation indicates that platelet are less responsive to further activation in vitro and is associated with platelet ongoing activation in vivo.
6. The cytoteductive therapy influenced the level of thrombin generated, in both TGA studies. Plasma of patients treated with hydroxyurea generated less thrombin than the plasma of those treated with anagrelide. This fact demonstrated the positive influence of HU in decreasing the thrombin generation in Ph-MPN patients.
7. The platelet volume indices (PVI) were significantly higher in patients treated with anagrelide, and this finding might be correlated with the level of thrombin generated by these patients` plasma.
8. The length of the treatment and TGA parameters were inversely correlated with the level of thrombin (peak thrombin, VI, AUC) in both studies for thrombin generation.
9. The platelet responsiveness to thrombin-receptor activating peptide-6 (TRAP-6), after maximal stimulation, as determined by their expression of P-selectin, was lower in patients with long cytoreductive therapy in comparison with those with less long cytoreductive therapy.
10. No influence of treatment with aspirin was noted for TGA parameters, P-selectin expression and PVI values.
11. The patients receiving statins had significantly lower PVI values than patients without this treatment.
12. The association of dyslipidemia in Ph-MPN patients conducted to a higher P-selectin expression and the platelets were less responsive to the agonist stimulation.
13. The association of high body-mass-index (BMI) in Ph-MPN patients showed a positive correlation with TGA parameters carried out in PRP. The increase of BMI will generate higher levels of thrombin.
14. It was noticed a week positive correlation between the number of leukocytes and platelets (in PRP), hematocrit and haemoglobin with TGA parameters (peak

Thrombin and VI). This correlation pointed out the implication of blood cells in thrombin generation.

15. The Ph-MPN patients carriers of JAK2V617F mutation generated less thrombin than wilde type carriers.
16. The platelet volume indices showed a correlation with JAK2V617F presence but without reaching statistical significance.
17. The history of thrombosis in Ph-MPN patients did not significantly influence thrombin generation, P-selectin expression and PVI values.
18. P-selectin expression on platelet surface of Ph-MPN patients was significantly lower in basal samples compared to the controls. This result was contrary to our expectations and in contradiction with what was previously reported. It is possible that P-selectin on activated platelets was shed and appeared in its soluble form in the circulation.
19. Platelet volume indices measured by the impedance based method did not show significant differences in Ph-MPN patients in comparison with healthy controls. I considerated that as a limitation of our methods used to measure PVI (impedance).
20. Age had no inluence in the levels of TGA, expression of P-selectin or the values of PVI.
21. The tests parameters (TGA, P-selectin and PVI) did not differ significantly between men and women.
22. Using K2EDTA as anticoagulant for the measurement of platelet indices resulted in a progressive increase of mean platelet volume (MPV) that might be determined by platelet activation and demonstrated the influence of anticoagulant type and time delay on the measurement of mean platelet volume.
23. The results of this research demonstrated that patients with Ph-MPN display a procoagulant imbalance detectable by thrombin generation assay carried out in PPP and PRP and by flow-cytometry in whole blood. What modulated it was the cytoreductive therapy (HU).
24. As a finale conclusion, I consider that thrombin generation assay brings its contribution in the evaluation of individual prothrombotic state and in the implication of the cytoreductive treatment in reducing the thrombotic event in Ph-MPN patients. The evaluation of cells activation biomarkers and the responsiveness of platelet after agonist stimulation with flow-cytometry brings useful information about the platelet phenotype in these diseases.

Originality and innovative contributions od the PhD thesis

I consider my thesis is original given to:

1. It was for the first time in Romania that the thrombin generation level was evaluated through a functional test of global hemostasis in the Philadelphia-negative myeloproliferative neoplasms.
2. It was for the first time that the results for thrombin generation in the plasma of Ph-MPN were obteined using fully automated thrombin generation device, Ceveron® alpha.
3. This is the first report on the correlation between the length of cytoreductive therapy and thrombin generation in Ph-MPN patients.
4. For the first time the level of thrombin generation was compared with the responsiveness of platelets to agonist in Ph-MPN patients.
5. It the was for the very first time in Romania that I performed the evaluation of the platelet volume indices in Ph-MPN patients.

Innovative contributions:

1. I managed to implement the thrombin generation assay in Romania.
2. The thrombin generation assay parameters were proposed to monitorise the feedback of cytoreductive therapies and the evaluation of individual thrombotic risk in Ph-MPN patients.
3. I implemented the method of quantifying the P-selectin expression in the hemostasis laboratory of County Emergency Hospital, Sibiu.