SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

The long term follow-up of patients with Essential thrombocythemia based on the HUMYPRON registry

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The PhD Defense well be held on 24 January, 2022 at 2:30 p.m.

Live online access well be provided. If you wish to join the discussion, please send an e-mail to the address adamkellner11@gmail.com until 12 p.m. on the previous workday at latest (21 January, 2022). For technical reasons, after the deadline, it will be not possible to join the defanse.

1. Introduction

Essential thrombocythemia is a Philadelphia Negative Chronic Myeloproliferative Neoplasms (MPN). This clonal stem cell disease is characterised by megakaryocytosis of the bone marrow and elevated platelet count. The incidence of this condition in western European countries and in the USA is 0.2-2.3/100,000 people and its prevalence is 38-57/100,000. The average age at diagnosis is 60 years, and the incidence is twice as high in women than in men. There is no specific molecular or genetic marker to ET. In 50-60% of the cases Janus kinase 2 (*JAK2) V617F* activation mutation can be detected. Calreticulin (CALR) mutation appears in 15-32% of the cases, and myeloproliferative leukemia virus oncogene (MPL) mutation is present in 3-4%. CALR positive patients tend to have higher platelet count and can expect better prognosis. 10-20% of the patients are triple negative, where none of these mutations can be detected.

More than 50% of ET cases are diagnosed during routine laboratory examination and show no symptom. The rest of the cases are identified when thrombotic or bleeding complications occure. Thrombocytosis is present in every patient (according to WHO 2016 criteria $\geq 450 \times 10^9$/L), but erythrocytosis and leukocytosis can also be found. ET patients show strong susceptibility for thrombosis. Headache, dizziness, tinnitus, ocular symptoms, erythromelalgia and acroparesthesia are the most common sign of microvascular episodes. Major arterial complications include acute coronary syndrome, stroke and peripheral arterial thrombosis. Superficial and deep venous thrombosis can also occure, splanchic venous thrombosis is particularly typical.

Increased thrombotic risk that we observe among this patients is supposed to be of multifactorial origin. General vascular risk factors (age, obesity, high blood pressure, hyperlipidaemia) and specific conditions also contribute to the developement of a prothrombotic state. The latter include procoagulant factors released by platelets and the endothel, blood hyperviscosity, disturbed endothel regeneration and function, increased acti vation of thrombocytes, monocytes or neutrophil granulocytes. Lipocalin-2 (LNC-2) produced by neutrophil granulocytes and it's possible role in the pathobiology of myeloprolipherative neoplasms has been extensively studied in the past few years. LCN-2 is supposed to damage endothel function via the generation of reactive oxigene species (ROS), and can thus play a role in the developement of the prothrombotic phenotype.

Bleeding complications usually affect the gastrointestinal tract and the upper respiratory system. Extremely high platelet count can lead to secunder von Willebrand-syndrome with bleeding episodes.

2016 WHO classification defines 4 major and 1 minor criteria for ET diagnosis. When elevated platelet count (≥450 x 10^9/L) and typical bone marrow histology are observed, other myeloid neoplasms can be ruled out and JAK2, CALR or MPL mutation are detected, the diagnosis of ET can be estabilished. In triple negative cases a minor criteria (the presence of another clonal marker or the exclusion of reactive thrombocytosis) along the first 3 major criteria has to be fulfilled. Accoring to the above mentioned criteria bone marrow biopsy is necessary to diagnose essential thrombocytemia. Median survival in the whole patient population is 20 years, but among those younger than 60 this value is 33 years. Transformation into myelofibrosis occurs in 0.8-4.9% in 10 years and 4-11% in 15 years. Transformation into acute myeloid leukaemia is observed in 0.7-3% in 10 years and 2.1-5.3% in 15 years.

In 2012 Passamonti et al analysed the data of 867 ET patients and found that 3 distinct risk categories can be identified taking age, thromboembolic anamnesis and white blood cell count into consideration. In this model 2 points were given for age older than 60 years while prior TE event and high WBC meant 1-1 points. The IPSET (International Prognostic Score for ET) risk groups created this way differed significantly from each other in terms of survival. To make a distinction from the IPSET-thrombosis risk analysis this system is called the IPSET-survival model.

Since patient survival is very good and there is no proof that any medication could change the course of disease favourably, today ET treatment is based on the prevention of thromboembolic complications. For this, the identification of risk groups and their appropriate management is crucial.

Landolfi categorizes patients into low, medium, high, and extreme high risk groups based on age, prior thrombotic events, white blood cell and platelet count, as well as general vascular risk factors. He suggests that acetylsalicylate (ASA) treatment is sufficient for patients at low to moderate risk, whereas additional cytoreductive therapy is recommended for patients at high and extreme risk.

The IPSET (International Prognostic Score for Thrombosis in Essential Thrombocythemia) thrombosis risk stratification takes age, previous thrombosis, JAK2 positivity and cardiovascular risk factors into account. Positive thrombotic anamnesis and JAK2 positivity

are more heavily weighted. The incidence of TE events was found to be 1.03% in the low- 2.35% inthe medium- and 3.56% in the high risk group.

Tefferi risk stratification (revised IPSET model for thrombosis) distinguishes four groups (very low, low, medium, and high risk) based on age, prior TE event, and JAK2/MPL mutation status. Cardiovascular (CV) risk factors are not regarded when estabilishing risk groups but are taken into account at the therapeutic recommendation. According to this model there is no need to treat patients at very low risk for thrombosis in the lack of cardiovascular risk factors. If a CV risk factor is present he recommends 1x100 mg ASA per day. Low-risk patients are advised to take 1x100mg or 2x100mg ASA daily depending on CV risk factors. He discourages to give ASA for those with extremely high platelet count ($\geq 1.000x10^9/l$) because of secondary von Willebrand-syndrome and increased bleeding tendency. For patients at moderate risk of thrombosis without CV risk factor 1x100mg ASA is suggested on a daily basis, while in the presence of CV risk factor hydroxyurea is advised in addition to ASA. Patients at high risk for thrombosis are recommended to receive 2x100mg ASA or systemic anticoagulation in addition to hydroxiurea, depending on whether they had arterial or venous TE event.

Previous thrombosis is a highly important aspect according to both Landolfi- and Tefferi risk systems. If thromboembolic anamnesis is positive the patient is classified into the high risk group automatically.

2. Aims

We analysed data of ET patients from the HUMYPRON (Hungarian Myeloproliferative Neoplasm Working Group) registry in order to make correlations regarding the following topics:

1. Thrombotic and bleeding complications:

- analyse the factors that could possibly influence thrombotic tendency, including the investigation the potential role of lipocalin-2;
- test and compare thrombosis risk systems (Landolfi-, IPSET-, R-IPSET model);
- examine the impact of medication (anagrelid vs. hydroxiurea+acetylsalicilic acid) on thrombotic and bleeding complications.

2. Disease progressionand secondary malignancy:

- study disease progression regarding its appearance (MF, AML, MF-AML);
- assess the factors that could possibly efffect the progression of essential thrombocythaemia;
- investigate the impact of medication (anagrelid vs. hydroxiurea+acetylsalicilic acid) on progression-free survival;
- analyse the correlation between the therapeutic choice (anagrelid vs. hydroxiurea+acetylsalicilic acid) and secuder malignancy.

3. Questions regarding overall survival:

- examine prognostic factors and variables that could possibly influence overall survival;
- study the effect of medication (anagrelid vs. hydroxiurea+acetylsalicilic acid) on overall survival;
- analyse the usability of IPSET-survival risk system.

3. Patients and methods

The HUMYPRON registry was founded in 2012 and contains epidemiological, clinical and laboratory data of MPN patients (according to 2008 WHO classification) of 14 hungarian hematology centres. Data was obtained by questioning patients and reviewing hospital documents and than uploaded to the electronic registry. At the start of the study the registry contained the data of 426 PV and 321 ET patients. In our main investigation we analysed 237 patients after excluding those who had missing data or did not meet the therapeutic criteria.

Our analyis has retrospective as well as prospective elements, since we follow up patients and register new finding continuously. Current analysis used the cut-off date of December 2018, which enabled us to work with a median follow-up of 10 years.

ET patients received either anagrelide or hydroxyurea+acetylsalicilic acid therapy. We excluded from the analysis those who switched from one type of medication to the other. Patients treated with anagrelide did not receive arterial thromboprophylaxis because of increased risk of bleeding complications. Venous TE prophylaxis was given to those who had already been anticoagulated due to prior major venous TE event. Patients with thrombophilia (APC resistance, APSA syndrome, etc.) were also excluded from the study. Therapy was choosen according to local protocols. We have no data regarding the motivation of clinicians of different centers in their therapeutic decisions.

For thrombosis risk assessment and risk-adopted medication we used Landolfi, IPSET and R-IPSET models. For evaluating patient surval we relied on IPSET-survival score system.

To classify thrombotic events we used the categories suggeted by Gisslinger:

- Major arterial thrombosis: stroke, myocardial infarction, peripheral arterial thrombosis and splanchnic arterial thromboembolism;
- Minor arterial thrombosis: TIA, angina pectoris, unstable angina, generalized convulsion, erythromelalgia, ocular symptoms and angina abdominalis (transient abdominal ischemia);
- Major venous events: deep venous thrombosis, pulmonary embolism, splanchnic venous thrombosis, pelvic venous thrombosis and other major venous events;

Minor venous episodes were not taken into consideration. When a patient suffered more thromboembolic episodes of the same phenotype we only regarded it once.

We carried out a substudy analysing the possible role of lipocalin-2 in thrombotic complications. We included 37 PV and 37 ET patients from the hospital of Kaposvár. During the investigation non of the patient had actute thromboembolic episode, inflammatory disease, infection or renal failure. We had stopped acetylsalicilic acid and cytoreductive treatment (hydroxyurea, anagrelide) 7 days before sample collection, but we did not change the anticoagulant treatment of those who had previously suffered thrombotic complication. We collected RNA sample from pheripheral blood and carried out qualitative PCR test after reverse transcriotion. We used β-glucoronidase (GUSB) as endogenous control. ΔC_T method was carried out to calculate results, that show LCN2 expression level normalised to GUSB expression.

The study was carried out under licence from ETT-TUKEB in compliance with the principles of Helsinki Declaration. Patients gave written consent to use their data anonymously after being informed of the nature of the study.

Statistical analysis

Kolmogorov- Smirnov test was used to examine the normal distribution of continuous variables (type I error = 10%). Samples with normal distribution were compared with t test for independent samples (test for variance homogeneity: Levene test, type I error = 5%). In cases where the criteria for t test were not fulfilled the Mann-Whitney U test with exact probabilities was carried out. The Mann-Whitney U test was also used to compare ordinal variables. Fisher's exact test or the exact chi-squared (χ^2) test was used to analyse categorical variables. The log-rank test was used to compare time to event variables, as depicted by Kaplan-Meier plots.

Cox regression analysis (forward method) was used to investigate whether treatment (anagrelide vs hydroxyurea+aspirin), age at diagnosis (<60 years vs. >60 years), $JAK2^{V617F}$ positivity (yes or no), gender (male vs. female) and pre-diagnostic thromboembolism (yes or no) had an impact on death or disease progression. The influence of the same variables on post-diagnostic TE events and secondary malignancies was assessed using logistic regression (forward method). We carried out similar analyses to examine the effect of relative LCN2 gene expression on thrombotic events.

Since type I error was not adjusted for multiple testing our results are only descriptive. Statistical analysis was performed using the open-source R statistical software package, version 3.1.2. Statistical tests were interpreted at a significance level of 5%.

4. Results

We analysed data of 237 ET patients with a median follow-up of 10 years (1-29). Mean age at diagnosis was 60.9 years. We observed female dominance, with the ratio of male:female being 1:2. $JAK2^{V617F}$ mutation positivity was detected in 70.5% of the cases.

116 patients received anagrelide therapy and 121 patients were treated with the combination of hydroxyurea+acetylsalicilic acid. There was no significant difference between the two groups in terms of sex, age (<60 years vs. >60 years) mutation status ($JAK2^{V617F}$, CALR, MPL) and thromboembolic anamnesis. Also, in the two therapy groups the proportion of patients falling into the corresponding Landolfi-, IPSET and R-IPSET risk categories was similar. Most patients were classified into the high risk group according to all three model. Using the IPSET-survival risk system most patients were ranked to the medium survival group. However, there was significant difference between the two groups in median age registered at diagnosis (higher in the hydroxyurea+acetylsalicilic acid group), and median time to death/end of follow-up (shorter in the hydroxyurea+acetylsalicilic acid group).

Before diagnosis 63 patient (26.6%) had thromboembolic episode. Most common were the major arterial complications (n=30, 46.1%). Minor arterial TE was registered in 20 (30.8%), major venous TE in 15 (23.1%) cases. After diagnosis 75 patient (32.1%) suffered 90 thromboembolic complications alltogether. Most of the cases were minor arterial episodes (n=44, 48.9%). Major arterial TE was observed in 27 (30.0%) and major venous TE in 19 (21.1%) cases. Minor venous events were not taken into consideration during the median follow-up of 10 years. The earliest TE attack was registered 10 years before diagnosis. The latest thrombosis was observed 19 years after diagnosis of ET. Most of the TE episodes (n=43) occured in the last 3 years prior to diagnosis. The proportion of TE complication before diagnosis was similar in the two therapy groups. After diagnosis there was a marginal difference between the two groups in terms of TE episodes (p=0,052); in the anagrelide group 25%, while in the hydroxyurea+acetylsalicilic acid group 38% of patients suffered TE complication. After the diagnosis of ET significant difference appeared regarding the phenotype of TE events. While minor arterial events occured more often in the hydroxyurea+aspirin group (4,5x more patients, p<0,001), major arterial episodes were observed more frequently in the anagrelide group (1,25x more patients, p=0,049). The

proportion of major arterial and bleeding complications was similar in the two groups – the later was registered in 4-4 cases respectively.

After analysing data of patients who suffered thromboembolic episode after diagnosis (n= 76) we learnd that sex, age (>60 years), time to death/end of follow-up, mutation status, white blood cell- and platelet count, MCH (as an indicator of iron deficiency) or cardiovascular risk factors (diabetes mellitus, dyslipidaemia, high blood pressure) did not have significant impact on subsequent TE events. Logistic regression analysis showed that the only factor that influenced post-diagnostic TE events significantly was thromboembolism before diagnosis ($p<0,001$).

We applied Landolfi-, IPSET- and R-IPSET risk systems on 237 ET patients of the HUPRON registry. In Landolfi's low risk group, 37.5% of patients had a thrombotic event after diagnosis, whereas in the very high risk group we observed TE events in 44.9% of the cases. However, the proportion of TE events in Landolfi's low risk group (37.5%) was higher than what we registered in the medium (23.8%) and high risk (27.8%) groups. Using the IPSET model for thrombosis 18.8% of the patients suffered TE complication in the low risk- and 39.0% in the high risk group. In the intermediate risk category the proportion of patients who had TE episode was less than in the low risk group.According to the R-IPSET risk stratification, 14.3% of patients of the very low risk group suffered thrombosis after diagnosis, and in the high risk group we registered TE episode in 46.0% of the cases. In the intermediate risk group we observed fewer TE events than in the low- or very low risk categories.

It was the R-IPSET high risk category where the ratio of patients suffering TE accident after diagnosis was the highest (46%). In all three risk stratification systems, however, it was the highest risk group in which most TE events occured (Landolfi: 44,9%, IPSET: 39,0%, R-IPSET: 46,0%). We found significant difference between the thrombosis-free survival of patients in the highest risk group and those in the prior risk groups (Landolfi: $p=0.032$, IPSET: $p<0.001$, R-IPSET: $p<0.001$).

Comparing the risk systems we found that the most sensitive indicator of thrombosis is the IPSET high risk group (88.2%). Similarly, the IPSET model has the highest negative predictive value (83.8%). As for specificity and positive predictive value the R-IPSET model proved to be the best. Landolfi risk system turned out to be the weakest in all aspects. Alltogether R-IPSET model seems more balanced than the others, with the sum of

percentages being the highest. This proved to be the strongest risk stratification system in our study.

We registered secondary malignancy in 28 cases. Among those in the anagrelide group 11 (9.5%) patients (all female), in the hydroxyurea+ASA group 17 (14.0%) patients (9 female and 8 male) developed secondary malignancy. This difference is not significant (p=0,854). Gastrointestinal neoplasm developed in 1-1, lung cancer in 1-1, breast cancer in 1-2, skin cancer in 2-3, urogenital neoplasm in 2-4, oropharingeal/laryngeal tumor in 1-2, and lymphoprolipherative malignancy in 1-1 cases of the anagrelide and hydroxiurea+ASA group respectively. Logistic regression analysis proved that no patient characteristic had significant effect on the developement of secondary malignanacy.

Disease progression was proved by bone marrow examination in 33 cases altogether. Out of the 33 patients 10 (8.6%) received anagrelide and 23 (19%) were treated with hydroxiurea+ASA; a difference that is significant (p=0,024). Progression to myelofibrosisba was observed in 31 cases, 10 in the anagrelide and 21 in the hydroxyurea+ASA group. Transformation into AML directly was registered 2 cases, both from the HU+ASA treatment group. Out of those who progressed to myelofibrosis, 6 developed AML, 1 from the anagrelide and 5 from the hydroxyurea+ASA group. We did not find significant difference regarding the phenotype of transformations between the two groups.

Considering the whole patient population (n=237) the median time to progression/follow-up was 10 years in the anagrelide group (n=121) and 8 years in the hydroxyurea+ASA group (n=121) (it ranged from 1-29 years in both groups), which is a significant difference (p=0,004). Regarding only those who progressed (n=33) median time to progression in the anagrelide group was 12,5 years (range: 4-19 év, mean: 11,6 év, standard deviation (SD): 4,35) and in the hydroxiurea+ASA group 7,0 years (range: 2-14 év, mean: 7,7 év, standard deviation (SD): 3,36); also a significant difference (p=0,016).

Progression free survival (PFS) was significantly longer among those who received anagrelide treatment than those treated with hydroxiurea+ASA (log-rank test: p=0,004). Curves of the Kaplan-Meier analysis divided after 5 years.

Cox-regression analysis (multivariate model) showed that applied therapy was the only patient characteristic that significantly influenced progression free survival (p=0,006, hazard ratio: 3.091, 95% confidence intervall 1.387-6.886).

61 patients passed away during follow-up. Significant difference came up between the two groups regarding the number of fatal outcomes, among those treated with anagrelide 20 (17.2%) patients, while in the hydroxiurea+ASA group 41 (33.9%) patients died (p=0,005). Causes of death ranged from disease progression, secondary malignancy, vascular event, heart failure, COPD/respiratory faliure and infection/sepsis to ileus. COPD/respiratory failure was more common in the anagrelide group (3 vs. 1 cases), all other causes were registered more often in the hydroxiurea+ASA group. In 20 cases cause of death remained unknown.

Regarding the whole patient population (n=237) median time to death/follow-up was 10 years in the anagrelide group and 8 years in the hydroxiurea+ASA group (ranging from 1-29 in both groups), a difference that is significant (p=0,011). Considering the 61 fatal cases we did not find significant difference (p=0,510) between the two groups in terms of the median time to death (anagrelide group: median time to death: 8 years, range: 1-27 years, mean: 9,40 years, SD: 6,47; HU+ASA group: median time to death: 8 years, range: 1-27 years, mean: 8,66 years, SD: 5,49).

We observed significantly longer overall survival (OS) in the anagrelide group, than int he hydroxiurea+ASA group (log-rank test: p=0,001). Kaplan-Meier curves divided after about 5 years of follow-up.

With Cox-regression (multivariate model) we found that among the patient characteristics applied therapy (p=0,004), age at diagnosis (p<0,001) and sex (p=0,002) had significant impact on overall survival.

Applying the IPSET-survival score system on the 237 ET patients of the HUMYPRON registry we found marginal difference between the two treatment grops (p=0,059). Significant difference (p=0,007) came up between risk groups regarding fatal cases (n=61): in the IPSET-survival low risk group 5.6%, in the intermediate risk group 21.1%, while in the high risk group 35.4% of the patients passed away.

Studying the whole patient population (n=237) we found that in the low and intermediate risk groups the median time to death/follow-up was 10 years (low risk: range:4-20 years, mean: 10.3 years, SD: 4.2; intermediate risk: range:1-29 years, mean:11.1 years, SD: 5.9), while in the high risk group it was 8 years (range: 1-27 years, mean: 9.4 years, SD: 5.0); the difference is significant (p=0,028). Analysisng the 61 fatal cases, however, we did not find significant

difference between the risk groups regarding median time to death (p=0,262). In the low risk group median time to death was 4 years (1 case), in the intermediate risk group 9,5 years (range: 1-20 years, mean: 9,9 years, SD: 6,1) and in the high risk group 7 years (range: 1-27 years, mean: 8.0 years, SD: 5.4).

Out of the 237 ET patients CALR mutation was detected in 45 cases, 27 in the anagrelid, 18 in the hydroxyurea+acetylsalicilic acid group, a difference that is not significant (p=0,135). We observed male dominance and lower mean age at diagnosis among CALR positive patients, but these differences were not significant. We found no difference between CARL positive and negative cases in terms of the frequency of progression and fatal outcomes, however, significant difference showed up regarding time to progression/death. CALR positive patients had longer progression free- (p=0,017) and overall survival (p=0,021). CALR negative patients suffered TE complications more often (34,4% vs. 22,2%), but this difference did not prove to be significant (p=0,116).

In the lipocalin-2 substudy we analysed the data of 74 patients (37 PV and 37 ET). We found positive TE anamnesis in 35 cases. JAK2-V617F mutation was detected in 100% of PV and 51% of ET patients. Among ET patients average relative LCN2 gene expression was 15.33 (range: 0.47-109.14), while in the PV group this value turned out to be 24.87 (range: 7.38-419). The difference is not significant. Similarly, no significant difference was found when comparing LCN2 relative gene expression levels of JAK2 positive and negative patients.

On the other hand, significant difference showed up between patients with no thrombotic history and those who had previously suffered TE episode. Chosing a cut-off level of 30 in LCN2 gene expression level, odds ratio turned out to be 4,487. Among all patient characteristics it was only LCN2 gene expression level that differed significantly in the two groups (p=0,019).

The groups showing low (n=61) and high (n=13) relative gene expression leves proved to be balanced regarding patient characteristics such as sex, age, diagnosis, JAK2 status, family history of TE events, laboratory parameters, general vascular risk factors and applied therapy. Between those whose LCN2 relative gene expression level exceeded 30 4 ET patients out of the 7 and all 6 PV patients suffered TE complication.

Multivariance analyisis proved that LCN2 level of over 30 is an independent and significant risk factor of thrombotic events (p=0.015, OR: 11.83), the positive predictive value for TE complication is 77%. In the group where LCN2 relative gene expression level did not reach 30 (n=61) TE episode was registered in 25 cases. Thus, low (<30) LCN2 relative gene expression has a negative predictive value of 59%.

5. Discussion

Regarding the whole patient population essential thrombocytopenia has a median survival of 20 years, among the younger patients (<60 years), however, it is 33 years. Mortality and morbidity is determined by thrombotic and bleeding episodes in the first place. Since ET has a good prognosis, today the most important aspect of the treatment is to prevent these complications wihout worsening the course of the disease

It has been proved that a 6 months long anagrelide treatment reduces platelet count and decreases the number of minor- and major thrombotic episodes significantly. The ANAHYDRET study with a median follow-up of 36 months did not find significant difference between ET patients treated with anagrelide and hydroxiurea in terms of major- and minor arterial, venous or bleeding complications. In contrast, the Primary Thrombocythemia 1 (PT-1) study found that within a median follow-up of 39 months the incidence of both arterial thrombosis and severe bleeding was significantly higher in the anagrelide+aspirin, than in the hydroxiurea+aspirin treatment group. The higher proportion of bleeding events along the anagrelide+aspirin treatment is due to the collective inhibition of phosphodiesterase- and cyclooxigenase enzymes, and thus, the synergistic effect of the two active substances. The metaanalysis of the two studies came to the conclusion that thrombotic events were registered with a similar frequency in the anagrelide and hydroxiurea treatment group, whereas major bleeding episodes were less common along hydroxiurea.

We analysed data from a national registry retrospectively (and partially prospectively). Our investigation has the advantage of a substancially longer follow-up compared to the previously introduced studies. After analysing ET patients of the HUMYPRON registry with a median follow-up of 120 months we found that those who received anagrelide threatment suffered marginally less thromboembolic episodes than patients who were treated with the combination of hydroxiurea+acetylsalicilic acid (30 vs. 46; p=0,052). This difference is due to minor arterial events being significantly less common in the anagrelide group (8 vs. 36; p<0,001). We observed marginal difference regarding major arterial attacks, which were slightly less common in the hydroxiurea+ASA group (15 vs. 12; p=0,049). The abscence of acetylsalicilic acid can partially explain the higher incidence of major arterial episodes in the

anagrelide group, just like the low proportion of bleeding complications. The conclusion of our study is that anagrelide has a significant effect on minor arterial episodes. At the same time, no significant difference came up during the 10-year-long follow-up between anagrelide and hydroxyurea+ASA treatment regarding major arterial and bleeding complications.

Our results are concordant with others studies about previous thrombosis being the most important risk factor of later TE events. On the other hand, while other publications identified older age (>60) and $JAK2^{V617F}$ mutation as risk factors for arterial- and male gender for venous thromboembolism, in our analysis there factors did not seem to influence TE episodes after diagnosis of ET. Examining potential thrombotic risk factors on ET patients of the HUMYPRON registry only pre-diagnostic thromboembolism proved to have significant impact on post-diagnostic TE complications ($p < 0{,}001$). This independent and highly significant influence of prior thrombosis on later TE episodes anticipates a prothrombotic phenomenon. Recent studies revealed that CHIP (clonal haematopoiesis of indeterminate potential), that is a preceding condition of many haematological malignancies, is an independent risk factor of the vascular events. According to investigations of the last few years the susceptibility of CHIP patients to vascular events is due to non-driver mutations (ASXL1, TET2, DNMT). Considering the time course of TE episodes it can be assumed that some of the pre-diagnostic TE events may have manifested in the CHIP fase. According to our data we suggest that the thrombotic episodes observed in ET patients are actually attributes of CHIP and not ET itself.

Neutrophlil granulocytes and monocytes derived from clonal haematopoesis produce LCN2 (lipocalin-2) and MMP9 (matrix metalloproteinase-9) that damage the endothel, leading to an increased thrombotic risk. LCN2 has been known as a transport protein for small, hydrophilic molecules. Invetigations of the last few years revealed that it also plays important role in numerous biological processes, such as cell metabolism, inflammatory reaction and immune processes. It's plazma concentration elevates in patients with cancer, inflammatory disease, coronary disease, liver- and kidney malfunction. In animals treated with lipocalin-2 endothel disfunction was observed as a result of oxidative stress and impaired eNOS/NO pathway. A possible explanation for this observation is that lipocalin, as a transport protein can influence the polyamine- homeostasis, and thus, nitrogen- oxid biosynthesis of the endothel.

In a substudy involving 74 PV and ET patients we found that higher relative LCN gene expression corelates significantly with the occurance of thrombotic events.

It's important to emphasize, however, that our results are only descriptive and the cut-off level of 30 has been chosen arbitrarily. To draw further conclusions the examination of a larger patient group and the evaluation of physiological LCN2 gene expression shold be necessary.

Thrombosis risk stratification systems help to classify patients according to thrombotic risk and to chose the optimal therapy. Studying Landolfi-, IPSET- and R-IPSET models we found significant difference in thrombosis-free survival between the highest and the lower risk groups. On the other hand, patients classified to the intermediate risk group suffered less TE events than those in the low risk group in all three models. In our patient population R-IPSET model proved to be the strongest. Landolfi-system turned out to be the least usable, which has the disadvantage of relying on less objective parameters as well (such as smoking). The discrepancy between literature data and our results may arise from differences in the definition of TE events, patient selection and applied therapy.

Landolfi applied his risk stratification system on both ET and PV patients. When creating IPSET thrombosis risk model ET patients were involved according to the 2008 WHO criteria and bone marrow revision was carried out in every case. R-IPSET model was published in 2018 and the latest, 2016 WHO criteria were applied, according to which bone marrow biopsy was performed for every patient. In our study we used 2008 WHO criteria, that only requires bone marrow examination under specific conditions (anaemia, macrocytosis, leukopaenia or signs of extramedullar haematopoesis). As patients came from 14 different centers and no revision was made, there is a possibility that we analysed a slightly different patient population.

Also, the principles of therapy choice that had been used when validating risk models may differ from our national practise. For example, busulphane, a second line therapy suggested by R-IPSET, is not part of the hungarian routine.

In our study we defined thrombotic events according to Gissliger, and analysed major- and minor arterial and major venous episodes. Landolfi-, IPSET- and R-IPSET models, however, only consider major vascular complications, but they included TIA (that we considered a minor event). As the definition of TE episodes is not clear, we might have taken into account some thrombotic events that were excluded when estabilishing the risk models.

A study examining long term complications of ET treatment identified a low incidence of many types of cancers. In our analysis we registered gastrointestinal-, pulmonary-, urogenital-, breast-, skin-, laryngeal-, oropharyngeal and lymphoproliferative malignancies. In

the hydroxizrea+acetylsalicilic acid group we observed secondary malignancy more often than among patients who received anagrelide treatment, though this difference was not significant during the 10-year follow-up (p=0,419).

Progression was observed sooner and twice as often in the hydroxiurea+acetylsalicilic acid group than in the anagrelide group. Transformation into myelofibrosis accounted for majority of these cases; we found only two patients whose disease transformed directly into acute myeloid leukaemia, both in the hydroxiurea+ASA therapy group. Interestingly, secunder transformation (MF-AML) was registered 5 times more often beside hydroxiurea+ASA. Progression free survival of patients treated with anagrelide was significantly longer than of those who received hydroxiurea+acetylsalicilic acid (p=0,004). This difference appeared after about 5 years.

On the contrary, PT-1 study found that the proportion of patients who suffer transformation from ET to myelofibrosis is significantly higher in the anagrelide+aspirin-, than in the hydroxiurea+aspirin treatment group. Other studies following ET patients treated with anagrelide reported no transformation into myelofibrosis, myelodysplastic syndrome or acute myeloid leukaemia at all. As an agent interfering with DNA metabolism, hydroxiurea is potentially leukaemogenic. A study on this topic came to the conclusion that the relationship between hydroxiurea exposition and the developement of AML/MDS was due to differences between patient groups, but it could not rule out for sure the potential leukaemogenic effect of hydroxiurea. Our conclusions are limited by the fact that our therapy groups were not balanced in terms of age, and there also might have been unknown factors- such as genetic mutations- whose potential influence on the developement of AML/MDS could not be taken into consideration.

Studies with a follow-up period of over 10 years report that the life expectancy of ET patients is shorter than that of the whole population. Age and sex are prognostic factors; high age at diagnosis and male gender worsen the chances of survival. Our analysis was in accordance with literature data regarding the relationship between age, sex and survival.

It is known that cases where CARL mutation can be detected have better prognosis than others. A study with a median follow-up of almost 13 years revealed that CALR positive cases are associated with younger age, male gender and lower incidence of recidive TE episodes. Our results are similar, though the differences were not significant. However we found significant difference between CALR mutation positive and negative cases regarding

progression free- (p=0,017) and overall survival (p=0,021). There were more CALR positive cases in the anagrelide group than in the hydroxiurea+acetylsalicilic acid group, but the difference was not significant (23% vs. 15%, p=0,135).

IPSET survival score system proved to be useful on our patient population, showing significant differences between the risk groups regarding survival (p=0,007).

Among patients who received anagrelide longer survival and half as many fatal cases were registered than in the hydroxiurea+acetylsalicilic acid treatment group. This difference appeared after about 5 years of treatment (p=0,001). Patients in the anagrelide group were younger at the time of diagnosis, which is a survival adventage in itself, moreover, IPSET-survival risk evaluation also suggests that patients of the hydroxiurea+ASA group had poorer prognosis. Nevertheless, therapy choice seems significant regarding survival. Our results suggest that anagrelide therapy improves the chances of survival more effectively than hydroxiurea+acetylsalicilic acid.

The strenght of our analysis is that the HUMYPRON registry, as a national database of MPN patients, enables us to follow-up patients using ”real-life” data. Evaluability is limited by the fact that we had to work with previously recorded, and as such, sometimes insufficient data. We have to count with potential misunderstanding between patients and clinicians regarding treatment, complications and risk factors, as well as misreporting. According to current clinical practice patients with different prognosis sometimes received the same therapy. We do not have information about the factors behind therapy choice in different centers, but we assume that it has always happened in accordance with international standards. The outcome of the disease might have been influenced by earlier treatment as well as concomittant medication. The combination of anagrelide and acetylsalicilic acid was always avoided because of the increased risk of bleeding complications. Anticoagulant therapy given as prophylaxis or as part of the treatment can be misguiding for influencing potential complications. At the same time, we do not have information about anticoagulant and other platelet aggregation inhibitor treatments. Also a potential source of mistake is that minor arterial events are harder to define than major arterial complications

Current guidelines for ET treatment classify patients into thrombosis risk groups. For those at intermediate and high risk the combination of hydroxiurea and acetylsalicilic acid is the first line therapy. Anagrelide is only advised after the failure of other therapeutic options,

because it is associated with higher incidence of thrombotic and bleeding complications and fibrotic progression. In our study with a median follow-up of 10 years we found that the incidence of minor arterial episodes decreased, while the rate of major arterial episodes slightly increased in patients who were treated with anagrelide compared to those who received hydoxiurea+ASA. It is important that longer progression free- and overall survival was registered in the anagrelide- than in the hydroxiurea+ASA group

Our findings shold be interpreted together with the limitations mentioned above, however we suggest applying the R-IPSET model for risk-adopted treatment of ET patients, but in contrast with its therapeutic recommendation of using hydroxiurea+acetylsalicilic acid we propose the application of anagrelide therapy, as it proved to be superior.

6. Summary, new results

Essential thrombocythemia is one of the Philadelphia-negative myeloproliferative neolasms. This clonal stem cell disease is characterised by megakaryocytosis of the bone marrow and elevated platelet count. Patient morbidity and mortality is primarily determined by thrombotic and bleeding episodes. Since ET itself has a favorable prognosis, the aim of the therapy is to prevent these complications without worsening the course of the disease.

Analysing the data of 237 ET patients of the HUMYPRON (Hungarian Myeloproliferative Neoplasm Working Group) registry with a median follow-up of 10 years, we came to the following conclusions:

1. Findings regarding thrombotic and bleeding complications:

- thrombotic event before diagnosis turned out to be the only factor that had significant impact on post- diagnositc thrombosis;
- lipocalin-2 relative gene expression significantly corelates with both pre-diagnostic and post-diagnostic TE episodes;
- among thrombotic risk stratification systems (Landolfi-, IPSET-, R-IPSET-) R-IPSET model proved to be the most reliable;
- patients who received anagrelid therapy suffered significantly less minor arterial events but slightly more major arterial events than those threated with the combination of hydroxyurea+acetylsalicilic acid; we found no difference between the two groups in terms of bleeding episodes;

2. Findings regarding disease progression and secondary malignancy:

- it was only the choice of therapy that influenced disease progression significantly;
- in the hydroxyurea+acetylsalicilic acid group we observed disease progression (MF, AML) sooner and with a frequency twice as high as in the anagrelide group; patients threated with anagrelide had significantly longer progression free survival than those who received hydroxyurea+acetylsalicilic acid;

- during follow-up we obsereved many types of secondary malignancies, being more frequent in the hydroxyurea+acetylsalicilic acid group - but this corralation was not found to be significant.

3. Findings reagarding overall survival:

- higher age and male gender came with a less favorable prognosis in terms of survival;
- IPSET survival model proved to be a good predictor of disease outcome, we found significant difference in survival between different risk groups;
- in the group of patients treated with anagrelide we observed half as much fatal outcome and longer survival compared to the hydroxyurea+acetylsalicilic acid group.

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8. Appendix

List of publications:

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Registry number: DEENK//2021.PL
Subject: PhD Publication List

Candidate: Ádám Kellner
Doctoral School: Doctoral School of Clinical Medicine

List of publications related to the dissertation

1. **Kellner, Á.**, Kellner, V., Rajnics, P., Karádi, É., Illés, Á., Udvardy, M., Homor, L., Dombi, P., Herczeg, J., Sipiczki, Z., Győriné, K. V., Egyed, M.: Analysis of Thrombosis Risk Stratification Models Based on 10 Years Follow Up of 237 Essential Thrombocythemia Patients.
J. Blood & Lymph. 11 (2), 1-7, 2021.
DOI: http://dx.doi.org/10.24105/ 2165 7831.11.251

2. **Kellner, Á.**, Dombi, P., Illés, Á., Demeter, J., Homor, L., Ercsei, I., Simon, Z., Karádi, É., Herczeg, J., Győriné, K. V., Gasztonyi, Z., Szerafin, L., Udvardy, M., Egyed, M.: Anagrelide influences thrombotic risk, and prolongs progression-free and overall survival in essential thrombocythaemia vs hydroxyurea plus aspirin.
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   J. Clin. Exp. Oncol. 6 (1), 1-7, 2016.

Total IF of journals (all publications): 5,607
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The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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