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Effect of *CALR* and *JAK2* mutations on the clinical and hematological phenotypes of the disease in patients with myelofibrosis – long-term experience from a single center

M. PALOVA*, T. SZOTKOWSKI, A. HLUSI, K. INDRAK, J. NAVRATILOVA, M. DIVOKA, T. PAPAJIK

Department of Hemato-Oncology, Faculty of Medicine and Dentistry, Palacky University Olomouc and University Hospital Olomouc

**Correspondence: miroslava.palova@fnol.cz*

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Primary myelofibrosis (PMF) is a chronic clonal myeloid disorder. Together with essential thrombocythemia (ET) and polycythemia vera (PV), it belongs to a group of Philadelphia chromosome-negative myeloproliferative neoplasms. An integral part of laboratory tests carried out in this disease group is detecting the presence of mutations in the Janus kinase 2 gene at position 617 (*JAK2* V617F) and in the gene encoding for the receptor for thrombopoietin (myeloproliferative leukemia virus oncogene, *MPL*) found in approximately 60% of PMF patients. The discovery of mutations affecting exon 9 of the calreticulin (*CALR*) gene was of great benefit to the diagnosis of the diseases in *JAK2* V617F and *MPL* unmutated cases. This is a study of the effect of a mutation in the *CALR* gene on the clinical course in patients with primary, post-ET and post-PV myelofibrosis. Analysis of 66 patients (54.5% *JAK2* V617F; 34.8% *CALR*; 6.1% *MPL*; 3.0% triple negative; 1.5% coincidence of *CALR* and *JAK2* V617F) confirmed a different phenotype of the disease in *CALR*-mutated patients as compared with *CALR*-unmutated individuals. Those with *CALR* mutation were significantly younger and had borderline higher platelet counts, less pronounced splenomegaly and less frequent B symptoms at diagnosis. The study suggests that the driver mutation types define variations in the biological basis, clinical manifestations and course of the disease. The presence of *CALR* mutation has been shown to be an independent prognostic favorable factor. Careful risk stratification of these patients is of great importance to adequate therapeutic decision-making and aids in selecting high-risk patients eligible for allogeneic hematopoietic stem cell transplantation which continues to be the only treatment modality for myelofibrosis having curative potential.

Key words: myeloproliferative disease, myelofibrosis, calreticulin (CALR), JAK2 V617F

Primary myelofibrosis (PMF) is classified among chronic Philadelphia chromosome-negative myeloproliferative neoplasms (Ph-MPN), together with essential thrombocythemia (ET) and polycythemia vera (PV); these may progress to myelofibrosis (MF) [1]. The incidence of PMF is 1.5 cases per 100,000 population per year; the median age at diagnosis is 67 years. Approximately 17% of patients are diagnosed before the age of 50 [2, 3]. The incidence of PV is higher in males than in females (2.8 vs. 1.3 cases per 100,000 population per year). The cumulative risk of progression to post-PV MF is 6% after 10 years and 26% after 20 years of disease duration [4, 5]. The incidence of ET is 2.5 cases per 100,000 population per year; the cumulative risk of transformation to post-ET MF is 9% after 15 years of disease duration [6, 7].

Of key importance in the pathogenesis of PMF is dysregulation or constitutional activation of the Janus kinase-signal transducer and activator of transcription (JAK/STAT) signaling pathway, leading to STAT protein autophosphorylation. The phosphorylated forms of STAT proteins translocate into the cell nucleus and participate in activation of transcription of genes regulating cell proliferation, differentiation and apoptosis [8]. This results in cytokine-independent proliferation of hematopoietic stem cells and activation of other signaling pathways involving STAT3, STAT5, MAPK, ERK and PI3K-AKT [8, 9]. The constitutional 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 gene with a regulatory effect on this signaling pathway (*MPL*). Mutation in the calreticulin (*CALR*) gene is also associated with STAT protein activation, but the exact mechanism remains unknown. The signaling pathway activation results in clonal proliferation of atypical

megakaryocytes with more intense production of cytokines and growth factors, leading to enhanced proliferation of non-clonal fibroblasts with subsequent MF. This results in the development of cytopenia, extramedullary hematopoiesis, hepatosplenomegaly and constitutional symptoms such as elevated body temperature, night sweats or weight loss [10–13].

The *JAK2* V617F somatic mutation, discovered in 2005, led to a breakthrough in the classification, diagnosis and understanding of Ph-MPN [14]. However, in only 50–60% of PMF cases the *JAK2* V617F mutation is detected; in another 5–8% of cases, mutation in codons 515 or 505 of the gene encoding for the receptor for thrombopoietin (*MPL*) is detected [12, 13].

After 2003, when Klampfl et al. and Nangalia et al. described recurrent somatic mutations affecting exon 9 of the *CALR* gene occurring in most *JAK2* and *MPL* unmutated patients with PMF, the proportion of patients with a known molecular marker of clonality increased to 80–85% [10, 13]. The *CALR* gene encodes for calreticulin, an endoplasmic reticulum chaperone capable of binding Ca^{2+}. Mutations in the *CALR* gene are a rather heterogeneous group comprising at least 50 different mutations such as short deletions, insertions or substitutions located in exon 9 of the *CALR* gene on chromosome 19. The two most common (in 80% of cases) mutations are 52-bp deletion (*CALR* del52/type1) and 5-bp insertion (*CALR* ins5/type2). Most of them cause a shift in the reading frame, with an alternative reading frame leading to the production of mutant proteins. It is assumed that mutations influence the subcellular localization, stability or function of calreticulin [15, 16]. The present study aimed to determine the association between the mutational status and clinical or laboratory phenotype of the disease in patients with histologically confirmed MF.

Patients and methods

Patients. Included in the study were all 66 patients diagnosed in our center between 1998 and 2016. There were 45 patients diagnosed with PMF (of whom 13 were in the prefibrotic phase of PMF at the time of diagnosis), 10 individuals with post-PV MF and 11 patients with post-ET MF. At the time of diagnosis, all participants met the 2008 WHO classification criteria. The sample comprised 30 males and 36 females with a median age of 55.5 years (range 18–77 years). Written informed consent was obtained from all participants before their genetic material (DNA) was processed and analyzed. Laboratory parameters and clinical data from the time of diagnosis were collected retrospectively. Objectively confirmed thrombotic and hemorrhagic complications were recorded if these occurred at the time of diagnosis or during the follow-up. Thrombotic complications included venous thrombosis (deep venous thrombosis, pulmonary embolism, splanchnic venous thrombosis or cerebral venous sinus thrombosis) and arterial thrombosis (transient ischemic attack, ischemic stroke, acute myocardial infarction and peripheral arterial vascular complications); hemorrhagic complications were gastrointestinal bleeding, hematuria, hemorrhagic stroke and significant bleeding during medical or dental surgery. Transformation to acute leukemia (AL) was diagnosed in accordance with the WHO criteria. Constitutional symptoms included elevated body temperature, weight loss and night sweats. Clinical and laboratory parameters were systematically compared with the patients' mutational status.

Detection of 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 in codon 617 of the *JAK2* gene was studied by allele-specific polymerase chain reaction (AS-PCR). In case of positive findings, real-time PCR was used for quantification and subsequent calculation of percentages of the mutated allele [17]. 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 [10, 18]. Mutations of the *MPL* gene (S505N and W515) were studied by AS-PCR and fragment analysis; the detected mutations were confirmed by Sanger sequencing [12].

Statistical analysis. Relationships between categorical variables were assessed by means of independence tests in contingency tables. For large enough patient counts, chi-square asymptotic test of independence was used, otherwise Fisher's exact factorial test was used. Differences in continuous variables among patient groups were visualized by means of standard box plots and tested by the Kruskal-Wallis test. The test compares the means of the samples in the respective groups, and returns the p-value for the null hypothesis that all samples are drawn from the same population. The Kruskal-Wallis test is a non-parametric vision of the classical one-way ANOVA, and an extension of the Wilcoxon rank sum test to more than two groups. For survival analysis, the non-parametric Kaplan-Meier estimator was used to visualize the survival curves. Difference in survival among groups was tested by means of the log-rank test. All tests were performed at the 0.05 level of significance. The statistical analyses were performed in MATLAB and Statistics Toolbox Release 2013b (MathWorks), and STATISTICA 12 (StatSoft).

Results

At the time of analysis, the median follow-up of patients was 46 months (range 2–221 months); thirteen (20.3%) patients died after a median of 42 months (range 13–173 months) from diagnosis. Three patients progressed to AL after a median of 24 months from diagnosis of high risk PMF or from transformation to post-PV MF. Two patients with AL died after 4–6 months from progression to AL due to infectious complications.

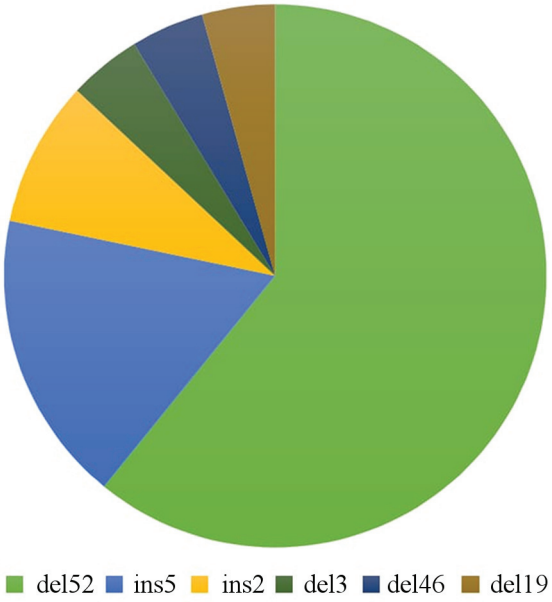

Figure 1. Distribution of types of mutation in the CALR gene. In the sample of 66 patients with PMF, post-ET myelofibrosis and post-PV myelofibrosis, mutation in the CALR gene were found in 34.8%. The distribution of CALR mutations types: type 1 mutation 60.8%, type 2 mutation 17.4% and other types 21.8% (see the graph). PMF – primary myelofibrosis; ET – essential thrombocythemia; PV – polycythemia vera; CALR – calreticulin.

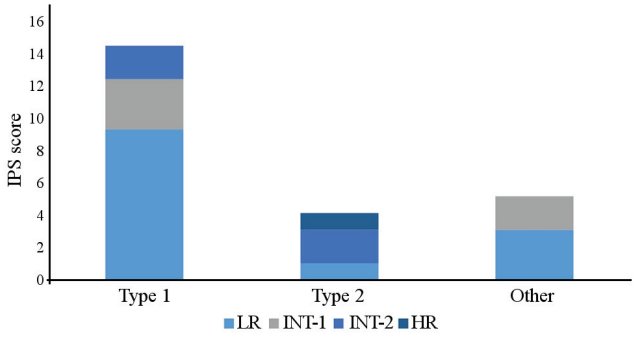

Figure 2. Distribution of IPSS scores at diagnosis depending on the CALR mutation types. The distribution of IPSS scores at diagnosis in the sample of 66 patients with PMF, post-ET myelofibrosis and post-PV myelofibrosis depending on types of mutation in the CALR gene. PMF – primary myelofibrosis; ET – essential thrombocythemia; PV – polycythemia vera; IPSS - International Prognostic Scoring System; CALR – calreticulin.

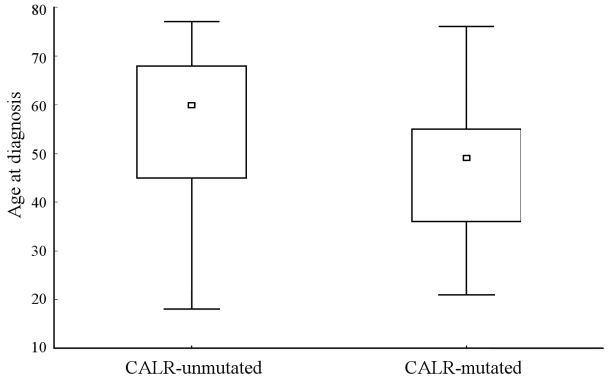

Figure 3. Age at diagnosis depending on the mutational status. In the sample of 66 patients with PMF, post-ET myelofibrosis and post-PV myelofibrosis (24 CALR-mutated, 42 CALR-unmutated), CALR-mutated patients were significantly younger at diagnosis as compared to CALR-unmutated individuals (p=0.009). PMF – primary myelofibrosis; ET – essential thrombocythemia; PV – polycythemia vera; CALR – calreticulin.

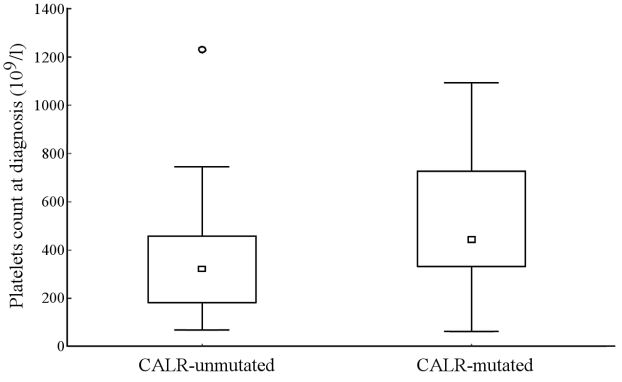

Figure 4. Platelet count at diagnosis depending on the mutational status. In the sample of 66 patients with PMF, post-ET myelofibrosis and post-PV myelofibrosis (24 CALR-mutated, 42 CALR-unmutated), CALR-mutated patients had significantly higher platelet counts (×10⁹/L) at diagnosis as compared to CALR-unmutated individuals (p=0.051). PMF – primary myelofibrosis; ET – essential thrombocythemia; PV – polycythemia vera; CALR – calreticulin.

The driver mutations were distributed as follows: *JAK2* V617F 36 cases (54.5%), *CALR* (exon 9) 23 cases (34.8%) and *MPL* W515 4 cases (6.1%). In the PMF subgroup, the distribution of driver mutations did not differ from the whole group. In one patient, both *JAK2* V617F and *CALR* mutations were detected; by contrast, two patients were triple-negative. Among the *CALR*-mutated cases, 60.8% were of type 1 (14/23), 17.4% were of type 2 (4/23) and 21.8% (5/23) were of less frequent types (Figure 1). The clinical phenotype at the time of diagnosis is summarized in Table 1. The distribution of International Prognostic Scoring System (IPSS) scores at diagnosis depending on types of mutation in the calreticulin (CALR) gene is summarized in Figure 2.

CALR-mutated patients were found to be significantly younger at diagnosis (49 vs 59 years, p=0.009, Figure 3) and had borderline higher platelet counts (445×10^9/L vs 312×10^9/L, p=0.051, Figure 4), much smaller spleen size (2 cm vs 10 cm below the left costal margin, p=0.005, Figure 5) and less frequent B symptoms (4.5% vs 56.5%, p=0.02) at diagnosis as compared with *CALR*-unmutated patients. *CALR*-mutated patients also had lower WBC counts at diagnosis but the difference was not statistically significant. Similarly, there were no statistical differences in the levels of hemoglobin (Hb), hematocrit (Hct), blasts in the

Table 1. Clinical and laboratory phenotypes of patients with myelofibrosis at diagnosis depending on the mutational status (*JAK2* V617F, *CALR*, *MPL*).

	JAK2 V617F	*CALR*	*MPL*
Presence of mutation (%)	54.5	34.8	6.1
Males/ females	13/23	12/11	3/1
Median age at diagnosis (years)	59 (18–77)	49 (23–76)	62.5 (44–68)
Median Hb level at diagnosis (g/L)	127 (78/155)	120 (86–148)	99.5 (90–110)
Median WBC count at diagnosis ($\times 10^9$/L)	11.8 (4.5–37.7)	8.3 (1.5–26.9)	5.9 (1.8–19.2)
Median platelet count at diagnosis ($\times 10^9$/L)	312 (74–1233)	445 (62–1093)	179 (68–342)
Median circulating blasts (%)	0 (0–4)	0 (0–4)	1(0–2)
Median LDH level at diagnosis (μkat/L)	7 (3–30)	5.78 (2.9–19.7)	11.2 (7.9–20.1)
Splenomegaly at diagnosis (median cm below the left costal margin)	10 (0–30)	2 (0–10)	6.5 (3–10)
Presence of B symptoms at diagnosis (%)	56.5	4.5	0
IPSS at diagnosis	no. of patients	no. of patients	no. of patients
Low risk	11	12	0
Intermediate-1 risk	17	7	4
Intermediate-2 risk	8	4	0
High risk	0	0	0
Total number of patients	36	23	4

Comparison of the clinical and laboratory findings and International Prognostic Scoring System (IPSS) scores at diagnosis depending on the mutational status (JAK2 V617/ CALR/ MPL mutated patients). The total number of patients was 66: 36 JAK2V617F positive, 23 CALR positive, 4 MPL positive, 2 triple negative. In one case both JAK2V617F and CALR mutations were detected; 1B symptoms: elevated body temperature, weight loss and night sweats; WBC – white blood cells

Table 2. Distribution of driver mutations in patients with thrombotic complications.

	CALR	Triple negative	*JAK2* V617F	*MPL*
Venous thrombosis (no. of patients)	3	1	8	1
Arterial thrombosis (no. of patients)	3	0	2	0

peripheral blood and lactate dehydrogenase (LDH) between the two subgroups.

Thrombotic complications developed in 27% of patients; 72% of those cases had venous thrombotic complications and the others had arterial complications. Hemorrhagic complications were documented in 1.5% of patients with concomitant moderate thrombocytopenia. In 46% of patients with venous thrombosis and 60% of those with arterial thrombosis, additional risk factors were identified, most frequently acquired protein S deficiency, protein C deficiency and the presence of lupus anticoagulant. The distribution of driver mutations in patients with thrombotic complications is shown in Table 2. Twenty-three percent of patients had a recurrent thrombosis. In all cases, the recurrence occurred in the venous district. This recurrent thrombosis was documented in patients who discontinued oral anticoagulation therapy with vitamin K antagonists (VKA) or in patients treated with VKA, but with non-therapeutic INR (the International normalized ratio). All of these patients had one of the above mentioned additional risk factor.

In *JAK2* V617F-positive patients, the effects of the mutated allele burden on the clinical and laboratory phenotypes of the disease were studied. The median allele burden was 53% (range, 7.7–99%). Patients with a *JAK2* V617F allele burden of more than 50% were found to have significantly more

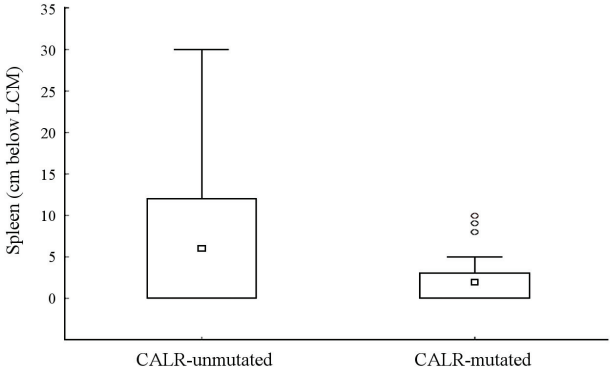

Figure 5. Presence of splenomegaly at diagnosis depending on the mutational status. In the sample of 66 patients with PMF, post-ET myelofibrosis and post-PV myelofibrosis (24 CALR-mutated, 42 CALR-unmutated), CALR-mutated patients had significantly less pronounced splenomegaly (cm below LCM) at diagnosis as compared to CALR-unmutated individuals (p=0.005). PMF – primary myelofibrosis; ET – essential thrombocythemia; PV – polycythemia vera; CALR – calreticulin, LCM – left costal margin.

pronounced splenomegaly (12 cm vs 0 cm below the left costal margin, p=0.0001, Figure 6) and higher WBC counts (16.7×10^9/L vs 8.8×10^9/L, p=0.003, Figure 7) at diagnosis. Differences in the other parameters (Hb, Hct, blasts in the

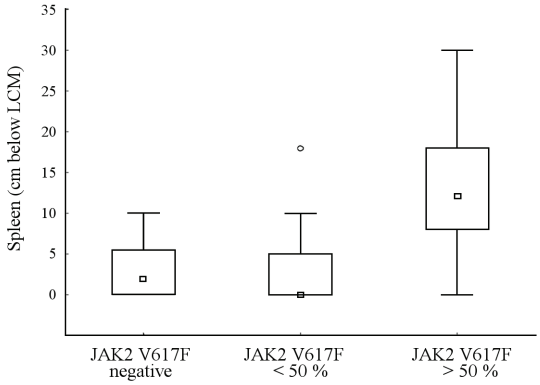

Figure 6. Presence of splenomegaly at diagnosis depending on the JAK2 V617F allele burden. In the sample of 66 patients with MF, 29 individuals were JAK2 V617F negative, 17 patients with a JAK2 V617F allele burden >50%, 17 patients with a JAK2 V617F allele burden <50%, in three patients the quantification was not performed. Significantly more pronounced splenomegaly (cm below LCM) at diagnosis was found in patients with a percentage of the JAK2 V617F mutated allele of more than 50% (p=0.0001). MF – myelofibrosis; JAK2 V617F – mutation in the Janus kinase 2 gene at position 617; LCM - left costal margin.

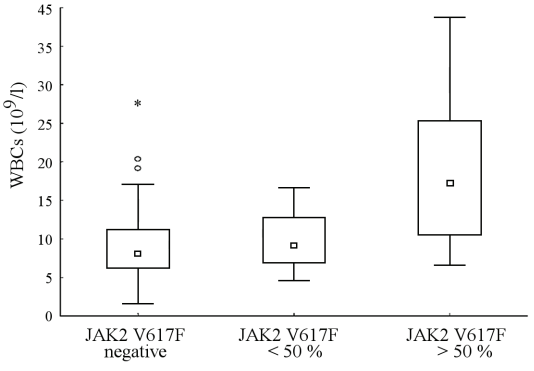

Figure 7. White blood cell count at diagnosis depending on the JAK2 V617F allele burden. In the sample of 66 patients with MF, 29 individuals were JAK2 V617F negative, 17 patients with a JAK2 V617F allele burden >50%, 17 patients with a JAK2 V617F allele burden <50%, in three patients the quantification was not performed. Significantly higher white blood cell counts (WBCs×10^9/L) at diagnosis were found in patients with a percentage of the JAK2 V617F mutated allele of more than 50% (p=0.003). MF – myelofibrosis; JAK2 V617F – mutation in the Janus kinase 2 gene at position 617; WBCs - white blood cells.

Table 3. International Prognostic Scoring System (IPSS)/Dynamic International Prognostic Scoring System (DIPSS) Plus prognostic models.

Risk factors	IPSS	DIPSS Plus
Age >65 years	1	1
B symptoms[1]	1	1
Hemoglobin <100 g/L	1	1
White blood cells >25×10^9/L	1	1
Circulating blasts ≥1%	1	1
Platelets <100×10^9/L		1
Transfusion dependency		1
Unfavorable karyotype[2]		1
Risk group[3]	**OS (months)**	**OS (months)**
Low risk	135	185
Intermediate-1 risk	95	78
Intermediate-2 risk	48	35
High risk	27	16

OS – overall survival; [1]B symptoms: elevated body temperature, weight loss and night sweats; [2]Unfavorable karyotype: complex karyotype, +8,-7/7q-, i(17q), inv(3), -5/5q-, 12p-, 11q23 rearrangement; [3]IPSS: low risk (0 points), intermediate-1 risk (1 point), intermediate-2 risk (2 points), high risk (3-5 points). DIPSS Plus: low risk (0 points), intermediate-1 risk (1 point), intermediate-2 risk (2-3 points), high risk (≥4 points)

Table 4. Risk stratification in MF (Tefferi et al., 2014).

Mutational status	Overall survival (years)
CALR+/ASXL1–	10.4
CALR+/ASXL1+; CALR–/ASXL1–	5.8
CALR–/ASXL1+	2.3

Risk stratification of patients with MF based on the presence of mutations in the calreticulin (*CALR*) or *ASXL1* gene.

peripheral blood, platelets and LDH) were not statistically significant.

The median OS rates were 47 months and 45 months in *CALR*-mutated and *CALR*-unmutated patients, respectively.

Discussion

The percentage of driver mutations in the present study is consistent with those reported by other authors [10, 13, 19]. This cohort of *CALR*-mutated MF patients is characterized by a significantly younger age, less pronounced splenomegaly, less frequent B symptoms and borderline higher platelet count at the time of diagnosis. WBC counts were also lower in *CALR*-mutated cases (statistical significance not achieved). The findings are consistent with previous analyses showing significantly positive effects of the presence of the *CALR* mutation [20–23]. There were no significant differences in the other tested parameters. In addition, some authors (e.g. Rumi et al.) identified lower cumulative incidence rates of anemia in *CALR*-mutated individuals [20]. Consistently with other reports, the present study confirmed comparable distribution of *CALR* mutation variants in MF, with type 1 (*CALR* del52) being the most common mutation. Given the generally relatively small number of *CALR*-mutated patients, relevant statistical assessment of the impact of *CALR* mutation types on disease phenotypes could not be performed. However, multicenter studies suggest that a more favorable phenotype and prognosis is associated with only type 1 *CALR* mutations [21, 24, 25]. A recent study by Tefferi et al. showed different laboratory and clinical findings in MF with type 2 *CALR* mutation (higher WBC count, higher percentage of circulating blasts, more pronounced splenomegaly and OS comparable with *JAK2* V617F-mutated cases).

The differences are likely to be due to different pathogenetic mechanisms of individual *CALR* mutations [24]. Recently, investigators also reported relevant structural and functional differences between proteins encoded by various *CALR* gene variations [26]. However, relatively low numbers of MF cases with type 2 *CALR* mutations in studies published so far prevent deeper understanding of how the *CALR* mutation type affects the OS. A Mayo Clinic study showed the poorest prognosis with a median OS of 2.5 years in triple-negative patients [27]. The rare concomitant presence of mutations in the *JAK2* and *CALR* gene may reflect the presence of two different clones in a patient [27–29].

Consistently with data published by other authors, subanalysis of *JAK2* V617F mutated patients confirmed the association of a mutated allele burden of more than 50% with clinical and laboratory manifestations of the disease (significantly more pronounced splenomegaly and higher WBC count at diagnosis) [30, 31]. The median percentage of mutated alleles was 53%. It must be noted that the two studies showed correlations between shorter OS in PMF and a lower *JAK2* V617F allele burden. The exact biological cause of this remains unclear, but it is assumed that another V617F-negative clone is present that is responsible for the more aggressive disease course [30, 32].

In the present study, thrombotic complications were diagnosed in 27% of patients, with venous thrombosis being more common. In 46% of patients with venous thrombosis and 60% of those with arterial thrombosis, additional risk factors were documented. Twenty-three percent of patients had a recurrent thrombosis after stopping VKA or in case of non-therapeutic INR. Due to the relatively low number of events recorded, a relevant statistical analysis of the effect of the mutational status on the risk of thrombosis could not be performed. However, Rumi et al. claimed that although *CALR* mutation is associated with higher platelet counts in MF, the risk of thrombosis is lower as compared with *JAK2* V617F-mutated individuals. The risk of thromboembolic complications remains lower in patients carrying both type 1 and type 2 mutations of the *CALR* gene [20]. Due to the small number of recorded events, a relevant statistical analysis of difference in OS between the subgroups of *CALR*-mutated patients and their unmutated counterparts could not be performed. However, other multicenter studies showed that *CALR* mutation is associated with better OS of patients, independent of their International Prognostic Scoring System/Dynamic International Prognostic Scoring System scores (Table 3) [20, 27]. Recent studies identified other subclonal mutations in the *ASXL1, SRSF2, EZH2* and *IDH1/2* genes, associated with poor prognosis and a higher risk of leukemic transformation [22, 33, 34]. At the time of performing this study, assessment of these mutations was not available for routine clinical practice. Tefferi et al. published risk stratification of patients based on *CALR/ASXL1* mutational status, with prolonged OS in *CALR*+/*ASXL1*– patients as compared with *CALR*–/*ASXL1*+ patients (Table 4) [35].

Conclusion. Despite its limitations, the present retrospective analysis showed differences in the clinical course of the disease, suggesting differences in its biological basis depending on the type of mutation. All driver mutations lead to persistent activation of the JAK/STAT signaling pathway, but are likely to differ by their specific effect on other metabolic processes [15]. The present analysis confirmed the observation that the presence of *CALR* mutation significantly influences the clinical phenotype and course of the disease. Analyzing *CALR* mutation in routine clinical practice is important for more accurate assessment of an individual risk of the disease and determination of its clonality. The current risk stratification in MF is based on demographic, clinical and hematological parameters [36–38]. Our experiences confirm the previously reported data on the role of driver mutations determining various clinical manifestations and course of MPN. Given the low incidence of the disease, more valid data may only be provided by multicenter collaboration and assembling a large enough cohort of patients. The aim is to develop a prognostic model that takes into account the molecular profile and predicts the treatment response. The presence of *CALR* mutation has a clearly positive effect on the prognosis of MF patients and should be taken into consideration not only in risk stratification of patients but also in clinical studies testing the effectiveness of novel treatment modalities [20, 23]. Last but not least, it is also necessary to elucidate the exact mechanism of *CALR* mutation in the pathogenesis of MF and thus identify a new potential therapeutic goal.

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