Clinical characteristics of essential thrombocythemia patients depend on the mutation status

Abstract
The impact of the mutation status on the clinical course and the outcome of essential thrombocythemia (ET) patients has not yet been completely established. A total of 171 patients with diagnosed ET were tested and subsequently grouped, according to their mutation status – Janus Kinase 2 (JAK2) – 112 patients, calreticulin (CALR) – 36 patients, and thrombopoietin receptor (MPL) – 5 patients. Moreover, 18 individuals were triple-negative (with non-mutated JAK2, CALR, and MPL). CALR-mutated patients preferentially were male, with higher platelets (PLT) counts (mean PLT = 1 002.3) and lower hemoglobin and hematocrit levels at the diagnosis, compared to the JAK2 (mean PLT = 933.6), MPL (mean PLT = 940.8) and triple-negative patients (mean PLT = 822.6) (p = 0.0035). The patients with CALR mutated, and the triple-negative ones had a lower risk of arterial and venous thrombosis (3% and 5.6% cases at the time of diagnosis, respectively) than the patients with JAK2 mutation (7.2%) (p = 0.9210). The overall survival rate did not differ statistically between the groups.

Keywords: essential thrombocythemia, JAK2, CALR, MPL

Introduction
Essential thrombocythemia (ET) belongs to myeloproliferative neoplasms, with an incidence of 2.5 cases per 100,000 inhabitants, and is characterized by an increased risk of thrombosis. The molecular events leading to the development of ET are heterogeneous: 50–60% of the patients with ET carry a mutation in the Janus Kinase 2 (JAK2) gene [1], 3–7% of the patients’ mutation in the thrombopoietin receptor (MPL) gene, and about 20% in the calreticulin (CALR) gene [2, 3]. Thus, molecular confirmation of clonality that supports unambiguously diagnosis of ET is possible nowadays in 80–90% of the patients. The remaining 10% of patients are grouped into the triple-negative category [4, 5]. Molecular heterogeneity of ET may translate to a different clinical course of the disease. Some early reports suggest indeed that such differences exist. Here we present a cohort of 171 ET patients, treated at our institution, aiming at exploring the impact of the mutation status on the clinical course and features of the disease.

Material and methods

Patients
This study included a cohort of 171 patients, diagnosed and treated for ET at the University Clinical Centre at the Medical University of Gdańsk between 2003 and 2014. The median follow-up was 4.7 years. The ET diagnosis was established according to the World Health Organization (WHO) criteria from 2008 [6]. Briefly, post-ET myelofibrosis (pET-MF) was diagnosed consistently with the criteria of the International Working Group of Myelofibrosis Research and Treatment [7]. The transformation into acute leukemia was defined by the WHO criteria [6]. Distribution of the risk for vascular complications was rated as follows: high risk at the age of ≥60 years old and/or previous thrombosis, low risk at the age of <60 years old, and/or no thrombosis history [8]. The prognostic score of thrombosis for essential thrombocythemia (IPSET thrombosis) score was not calculated due to a lack of patients’ cardiovascular risk factors data. Thrombotic events were defined as described by Barosi et al. [7]. The study protocol was approved by the Local Bioethics Committee at the Medical University of Gdansk and was carried out following the Declaration of Helsinki. Informed consent, concerning the use of the clinical data for research purposes, was obtained from all the participants.

Molecular analysis
The JAK2 V617F mutation status was assessed using the amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) described by Jones et al. [9]. The patients with a non-mutated JAK2 gene were further evaluated for CALR exon 9 and MPL exon 10 mutations using high-resolution melting assay and Sanger sequencing [2, 10]. The patients with JAK2, CALR, and MPL mutations and the triple-negative individuals were compared in terms of their demographic characteristics (sex distribution, age at diagnosis of ET), the clinical and laboratory features at the time of diagnosis, the clinical outcome (fibrotic or leukemic transformation), and the overall survival (OS).
The patients were treated according to the national recommendations. Cytoreductive therapy, using hydroxyurea was administered to the high-risk patients, which is with the incidence of arterial or venous thrombosis, elderly, and with comorbidities increasing the risk of a thrombotic event. Other treatment approaches included the use of anagrelide, busulfan, and interferon alfa.

**Statistical analysis**

Normal distributions of the continuous variables were verified by the Kolmogorov-Smirnov test, while their statistical characteristics were presented as arithmetic means, standard deviations (SDs), medians, and ranges. Statistical characteristics of the discrete variables were presented as numbers and percentages. A Student’s t-test or the Mann–Whitney U-test was used for intergroup comparisons of the continuous variables and distributions of the discrete variables. They were compared with the Pearson chi-square test or the Fisher exact test. The power and the direction of the relationships between the presence of mutation types, the demographic, and the clinical characteristics of the ET patients were determined based on the odds ratios (ORs) and their 95% confidence intervals (95% CI), calculated during logistic regression analysis. Survival curves were prepared with the use of the Kaplan-Meier method and compared with the log-rank test. All calculations were carried out using the Statistica10 package (Stat Soft, USA) and the threshold of statistical significance was set at \( p \leq 0.05 \).

**Results**

**Distribution of the mutation status**

The group of 171 ET patients includes 112 (65%) patients with a JAK2 mutation, 36 (21%) with CALR mutation, 5 (3%) with MPL mutation, and 18 (11%) triple-negative cases. Overall, eight types of CALR mutations were detected. In the cohort of the 36 CALR-mutated patients, we found 14 (39%) individuals with a 52 bp deletion (c.1099-1150del – type 1) and 15 (41%) patients with a 5bp insertion (c.1154-1155insTTGTC – type 2). Seven (20%) patients were the carriers of one out of six other mutation types, with potentially three novel variants.

**Demographic characteristics**

The median age at diagnosis between patients with JAK2, CALR, and MPL mutations and of the triple-negative ones were 59, 53, 66, and 52, respectively. The groups did not differ in terms of their age distribution, but there was a tendency toward lower age in the CALR and the triple-negative groups. There were more females (63%) than males in the whole ET group. The preponderance of females to males was preserved in all mutation subgroups except for CALR mutated patients with more males (56%) than females. The difference between the latter group and the JAK2 mutated group reached statistical significance. The demographic, as well as the clinical characteristics, are summarized in table I.

**Laboratory parameters at the time of diagnosis**

The laboratory parameters of all groups, according to the mutation type, are presented in table II. At the time of diagnosis, the JAK2-mutated patients had mean hemoglobin (Hb) and hematocrit (Hct) levels (14.5 ± 1.5 g/dL and 44 ± 4.3 g/dL, respectively), compared to the other groups, and lower PLT counts when compared to the CALR and the triple-negative individuals; however, all Hb and Hct values remained within the normal range limits. There was a trend toward a higher white blood count (WBC) in the JAK2 group, in comparison to the CALR and the triple-negative groups (\( p = 0.1467 \)).

**Vascular complication risk and the observed incidence of vascular complication at diagnosis concerning the mutation status**

More patients with high risk were observed in the JAK2 group (59.1%) than in the CALR and the triple-negative groups (36.7% and 27.8%, respectively). The difference in the distribution of the vascular complication risk between the JAK2, the CALR, and the triple-negative groups was statistically significant (\( p = 0.0264 \)). The incidence of arterial or venous thrombotic events at the diagnosis was observed more often in the JAK2 group than in the other groups (7.2 vs. 5.6 and 3.0% in the triple-negative and the CALR group respectively), but the difference did not reach statistical significance (\( p = 0.9210 \)). No incidence of arterial or venous thrombotic events was observed in the MPL group, possibly due to a low number of individuals (\( n = 5 \)).

**Treatment and the incidence of vascular complications concerning the mutation status and survival**

During the follow-up period, arterial and venous thrombotic complications occurred in 7.2% of the patients from the JAK2 group, whereas in the CALR, the MPL, and the triple-negative groups no thrombotic complications were observed. The patients with CALR or MPL mutation as well as the triple-negative individuals had a lower risk of arterial or venous thrombosis than the patients with JAK2 mutation. Thrombosis-free survival was the highest in the CALR group. Bleeding complications were observed only in the CALR group (3.0%) during the follow-up. Transformation to pET-MF occurred in 2.9% of all patients during the follow-up, including two patients from the JAK2 group, two and one individuals from the CALR and the MPL groups, respectively. No transformation to pET-MF in the triple-negative group was observed. The difference between the groups was statistically significant (\( p = 0.0325 \)). The OS did not differ statistically among the three groups: the JAK2, the CALR, and the triple-negative. Due to a low number of MPL-mutated patients (\( n = 5 \)), the analysis was not performed in this group.

**Clinical characteristic of the CALR subtypes**

We compared clinical and laboratory parameters (Tab. III) between types 1 and 2 CALR subgroups. We found no statistical difference in the demographic characteristic (age and sex), the laboratory
parameters (Hb, Hct, WBC, and PLT count), the thrombotic incidences, the transformation risk, or in the OS.

Discussion

Discoveries of molecular changes in myeloproliferative neoplasms nowadays, allow confirmation of the diagnosis molecularly in 80–90% of ET subjects. Therefore, not only JAK2 but CALR and MPL mutation screening should be performed in every case of a suspected ET [11]. In our study, the molecularly defined ET patients accounted for 89% of the entire cohort. Similar data were presented by other authors [4], though in some studies the percentage of molecularly undefined ET patients was higher [12, 13]. Therefore, it is crucial to perform a precise differential diagnosis, to exclude secondary thrombocytosis in case of a lack of molecular markers. We should also be aware, that even in the case of a confirmed JAK2, CALR, or MPL mutations, other myeloid neoplasms (such as prefibrotic myelofibrosis and refractory anemia with ringed sideroblasts with marked thrombocytosis RARS-T) can mimic ET in their presentation. A trephine biopsy examination (different morphology of megakaryocytes) can help distinguish ET from other myeloid neoplasms [14]. Distribution of the JAK2, the CALR, the MPL, and the triple-negative patients in our cohort was similar to previous reports [4]. However, in the cohorts analyzed by Tefferi et al. [5] and Andrikovics et al. [15], there were more CALR-mutated individuals (32 and 33%, respectively vs. 21%), and less JAK2-mutated patients than in our group. Whereas among the 1150 ET patients, described by Finazzi et al [16], the CALR individuals accounted for only 16% of the entire group. In our CALR cohort, we found slightly more patients with type 2 mutation than those with type 1 (41 vs. 39%). However, this observation is not consistent with other studies [5, 15], which reported the prevalence of a type 1 mutation at 50% of the CALR-mutated patients. Similarly to our studies, there was no difference between the subtypes 1 and 2, in terms of the demographic and the clinical characteristics. Further, population-based studies are necessary to confirm the real mutation distribution in ET patients. Whether the molecular changes could have an impact on the laboratory parameters and the clinical characteristics of ET patients, has remained a matter of debate ever since the CALR mutation discovery. Most researches state that CALR-mutated ET patients are characterized by younger age, higher PLT count, a lower incidence of thrombotic events, and a higher transformation rate to MF, in

### Table I. Clinical features of 171 patients with ET, according to the mutation status

|                      | JAK2+ n = 112 (65.5%) | CALR+ n = 36 (21%) | MPL+ n = 5 (3%) | Triple negative n = 18 (10.5%) | p-value |
|----------------------|-----------------------|--------------------|----------------|-----------------------------|---------|
| **Age at diagnosis** |                       |                    |                |                             |         |
| Mean (SD)            | 58.1 (15.1)           | 54.3 (14.9)        | 66.0 (11.3)    | 51.6 (11.6)                 |         |
| 95% CI               | [55.3; 61.0]          | [49.2; 59.4]       | [48.0; 64.0]   | [45.8; 57.4]                |         |
| Range (min–max)      | 23.0–88.0             | 24.0–82.0          | 54.0–78.0      | 30.0–74.0                   |         |
| Median               | 59.0                  | 53.0               | 66.0           | 52.0                        | 0.0948  |
| **Sex**              |                       |                    |                |                             |         |
| Female               | 74 (66.1%)            | 16 (44.4%)         | 3 (60.0%)      | 14 (77.8%)                  |         |
| Male                 | 38 (33.9%)            | 20 (55.6%)         | 2 (40.0%)      | 4 (22.2%)                   | 0.0594  |
| **Symptoms at diagnosis** |               |                    |                |                             |         |
| None                 | 97 (87.4%)            | 30 (90.9%)         | 5 (100.0%)     | 16 (88.9%)                  |         |
| Thrombotic           | 5 (4.5%)              | 0 (0.0%)           | 0 (0.0%)       | 0 (0.0%)                    |         |
| Cardiovascular       | 3 (2.7%)              | 1 (3.0%)           | 0 (0.0%)       | 1 (5.6%)                    |         |
| Bleeding             | 3 (2.7%)              | 0 (0.0%)           | 0 (0.0%)       | 0 (0.0%)                    |         |
| Others               | 3 (2.7%)              | 2 (6.1%)           | 0 (0.0%)       | 1 (5.6%)                    | 0.9210  |
| **Risk grade**       |                       |                    |                |                             |         |
| High                 | 65 (59.1%)            | 11 (36.7%)         | 2 (50.0%)      | 5 (27.8%)                   |         |
| Low                  | 45 (40.9%)            | 19 (63.3%)         | 2 (50.0%)      | 13 (72.2%)                  | 0.0264  |
| **Complications**    |                       |                    |                |                             |         |
| None                 | 101 (91.0%)           | 32 (97.0%)         | 4 (100.0%)     | 16 (100.0%)                 |         |
| Thrombotic           | 7 (6.3%)              | 0 (0.0%)           | 0 (0.0%)       | 0 (0.0%)                    |         |
| Cardiovascular       | 1 (0.9%)              | 0 (0.0%)           | 0 (0.0%)       | 0 (0.0%)                    |         |
| Bleeding             | 0 (0.0%)              | 1 (3.0%)           | 0 (0.0%)       | 0 (0.0%)                    |         |
| Others               | 2 (1.8%)              | 0 (0.0%)           | 0 (0.0%)       | 0 (0.0%)                    | 0.7070  |
| **Transformation**   |                       |                    |                |                             |         |
| Myelofibrotic        | 2 (1.8%)              | 2 (6.9%)           | 1 (25.0%)      | 0 (0.0%)                    | 0.0325  |
| Leukemic             | 16 (14.8%)            | 2 (6.5%)           | 1 (25.0%)      | 3 (17.6%)                   | 0.5438  |

CALR – calreticulin; ET – essential thrombocythemia; JAK2 – Janus Kinase 2; MPL – thrombopoietin receptor
Our study showed that CALR-mutated patients are characterized by lower Hb and Hct levels, and there is a trend of a lower leucocyte count and a younger age at the diagnosis, compared to the JAK2-mutated subjects. We showed, that the CALR-mutated individuals are characterized by a higher PLT count in comparison to the JAK2-mutated patients. This observation is consistent with other studies already performed and suggests a difference in the biological effects of the mutation status in the JAK2 and the CALR genes [15, 17]. It has been stated that a mutated JAK2 promotes erythroid and granulocyte lineage, whereas a mutated CALR selectivity promotes megakaryocytic proliferation [18]. Interestingly, the CALR-mutated patients, despite a higher PLT count, might be characterized by a lower rate of thrombotic events. This observation was also confirmed in our study group. Passamonti et al. [19] state that new risk assessment factors should be implemented in the risk stratification of ET patients. Moreover, the novel low-risk factors should include the presence of a CALR mutation. However, a study performed by Finazzi et al. [16] on 1150 ET patients showed that a CALR mutation does not have any significant impact on the IPSET thrombosis score. A low number of thrombotic events in CALR-mutated patients raises a question on the role of the PLT in the thrombotic events in ET individuals. More detailed studies are required regarding the role of the level and the activity of the von Willebrand factor, which could be more relevant for thrombosis occurrence than the PLT count [20].

Despite the clinical differences of the JAK2, the CALR, and the triple-negative ET patients, we did not show any variation in the OS. The results presented by other authors are inconsistent. Klamfl et al. [2] showed a survival advantage for the CALR-mutated patients, but

### Table II. Laboratory parameters of 171 patients with ET, according to the mutation status

|          | JAK2+ n = 112 | CALR+ n = 36 | MPL+ n = 5 | Triple negative | p-value |
|----------|---------------|--------------|------------|-----------------|---------|
| **WBC**  |               |              |            |                 |         |
| Mean (SD)| 10.4 (6.3)    | 8.6 (2.6)    | 9.2 (5.5)  | 8.8 (2.7)       |         |
| 95% CI   | [9.2; 11.7]   | [7.6; 9.6]   | [8.4; 10.0]| [7.4; 10.2]     |         |
| Range (min–max) | 3.0–63.0 | 4.4–14.1 | 5.0–17.3 | 2.0–13.5 |         |
| Median   | 9.6           | 8.3          | 7.2        | 8.7             | 0.1467  |
| **Hb**   |               |              |            |                 |         |
| Mean (SD)| 14.5 (1.5)    | 13.7 (1.0)   | 12.8 (1.2) | 13.5 (1.1)      |         |
| 95% CI   | [14.2; 14.8]  | [13.3; 14.0] | [10.9; 14.7]| [12.9; 14.0]    |         |
| Range (min–max) | 9.8–17.7 | 11.5–15.8 | 11.2–14.1 | 11.0–14.9 | 0.0139  |
| Median   | 14.7          | 13.5         | 13.0       | 13.6            | 0.0034  |
| **Hct**  |               |              |            |                 |         |
| Mean (SD)| 44.0 (4.3)    | 41.0 (3.5)   | –          | 39.4 (2.5)      | 0.0002  |
| 95% CI   | [42.9; 45.1]  | [39.4; 42.5] | –          | [37.9; 41.0]    | 0.0043  |
| Range (min–max) | 30.0–52.0 | 32.0–49.0 | –          | 33.0–42.3 |         |
| Median   | 44.0          | 41.0         | –          | 39.0            |         |
| **PLT**  |               |              |            |                 |         |
| Mean (SD)| 933.6 (931.8) | 1,002.3 (259.6) | 940.8 (232.6) | 822.6 (319.8) | 0.0035  |
| 95% CI   | [757.5; 1,109.7] | [911.7; 1,092.9] | [652.0; 1,229.6] | [663.6; 981.6] | 0.0406  |
| Range (min–max) | 491.0–10,000.0 | 452.0–1,624.0 | 563.0–1,335.0 | 475.0–1,399.0 |         |
| Median   | 787.5         | 1,024.0      | 1,006.0    | 652.0           |         |

CALR – calreticulin; ET – essential thrombocythemia; Hct – hematocrit; Hb – hemoglobin; JAK2 – Janus Kinase 2; PLT – platelets; MPL – thrombopoietin receptor; WBC – white blood count

### Table III. Laboratory parameters of 29 patients with types 1 and 2 CALR mutations

|          | 5bp insertion n = 15 | 52bp deletion n = 14 | p-value |
|----------|----------------------|----------------------|---------|
| **WBC**  |                      |                      |         |
| Mean (SD)| 8.2 (2.6)            | 9.1 (2.8)            |         |
| 95% CI   | [6.6; 9.7]           | [7.3; 10.9]          |         |
| Range (min–max) | 4.4–13.3 | 5.4–14.1 |         |         |
| Median   | 7.5                  | 8.6                  | 0.7175  |
| **Hb**   |                      |                      |         |
| Mean (SD)| 22.2 (30.9)          | 14.0 (1.1)           |         |
| 95% CI   | [3.5; 40.9]          | [13.3; 14.7]         |         |
| Range (min–max) | 12.3–125.0 | 12.4–15.8 |         |         |
| Median   | 13.5                 | 14.0                 | 0.4265  |
| **Hct**  |                      |                      |         |
| Mean (SD)| 42.1 (3.5)           | 40.8 (2.6)           |         |
| 95% CI   | [39.4; 44.7]         | [38.6; 43.0]         |         |
| Range (min–max) | 37.7–49.0 | 37.0–45.0 |         |         |
| Median   | 42.0                 | 40.5                 | 0.5790  |
| **PLT**  |                      |                      |         |
| Mean (SD)| 1,036.0 (313.2)      | 952.1 (233.9)        |         |
| 95% CI   | [855.2; 1,216.8]     | [817.0; 1,087.1]     |         |
| Range (min–max) | 452.0–1,624.0 | 662.0–1,415.0 |         |         |
| Median   | 1,052.5              | 889.0                | 0.5035  |

CALR – calreticulin; Hct – hematocrit; Hb – hemoglobin; PLT – platelets; WBC – white blood count

**Comparison to JAK2-mutated patients** [4, 5]. Our study showed that CALR-mutated patients are characterized by lower Hb and Hct levels, and there is a trend of a lower leucocyte count and a younger age at the diagnosis, compared to the JAK2-mutated subjects. We showed, that the CALR-mutated individuals are characterized by a higher PLT count in comparison to the JAK2-mutated patients. This observation is consistent with other studies already performed and suggests a difference in the biological effects of the mutation status in the JAK2 and the CALR genes [15, 17]. It has been stated that a mutated JAK2 promotes erythroid and granulocyte lineage, whereas a mutated CALR selectivity promotes megakaryocytic proliferation [18]. Interestingly, the CALR-mutated patients, despite a higher PLT count, might be characterized by a lower rate of thrombotic events. This observation was also confirmed in our study group. Passamonti et al. [19] state that new risk assessment factors should be implemented in the risk stratification of ET patients. Moreover, the novel low-risk factors should include the presence of a CALR mutation. However, a study performed by Finazzi et al. [16] on 1150 ET patients showed that a CALR mutation does not have any significant impact on the IPSET thrombosis score. A low number of thrombotic events in CALR-mutated patients raises a question on the role of the PLT in the thrombotic events in ET individuals. More detailed studies are required regarding the role of the level and the activity of the von Willebrand factor, which could be more relevant for thrombosis occurrence than the PLT count [20].
Tefferi et al. [5] as well as others [15] did not confirm this observation. The reason for inconsistent data could be the inadequate time of the follow-up. The differences in the clinical courses of the JAK2-mutated and the CALR-mutated patients, especially in terms of the thrombosis risk and the myelofibrotic transformation rate, provides, in our opinion, a rationale for a different therapeutic approach to the high-risk patients. At present, two randomized trials, comparing hydroxyurea and anagrelide in ET patients have been performed. One randomized trial, comparing aspirin combined with either anagrelide or hydroxyurea, showed a lower incidence of fibrotic transformation and arterial thrombosis in the hydroxyurea treated patients [21]. The second one showed no difference between hydroxyurea and anagrelide treated patients, in terms of the fibrotic and the thrombosis rates [22]. However, none of the studies compared the patients concerning the JAK2 and the CALR mutation status. We and others have proved that the mutation status defines the subtypes with substantially different clinical courses. The JAK2-mutated patients represent a different phenotype, with a higher risk of thrombosis and a lower risk of myelofibrotic transformation.

Conclusion

In conclusion, based on ours and other available studies, it can be stated that CALR-mutated patients display a distinct phenotype, compared to JAK2-mutated and MPL-mutated ET patients. Consequently, different therapeutic strategies should probably be implemented for these groups of patients, but further studies are necessary.

Authors’ contributions

WP, AH – contributed to the design of the study. AL – performed the mutation analysis. WP, AM, MB, AL, MC – analyzed the data. All authors – involved in the data collection, edited and approved the final version of the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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Ethics

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments; Uniform requirements for manuscripts submitted to biomedical journals.

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