Analysis of phenotype and outcome in essential thrombocythemia with *CALR* or *JAK2* mutations

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ABSTRACT

The *JAK2* V617F mutation, the thrombopoietin receptor *MPL* W515K/L mutation and calreticulin (*CALR*) mutations are mutually exclusive in essential thrombocythemia and support a novel molecular categorization of essential thrombocythemia. *CALR* mutations account for approximately 30% of cases of essential thrombocythemia. In a retrospective study, we examined the frequency of *MPL* and *CALR* mutations in *JAK2* V617F-negative cases of essential thrombocythemia (n=103). In addition, we compared the clinical phenotype and outcome of *CALR* mutant cases of essential thrombocythemia with a cohort of *JAK2* V617F-positive essential thrombocythemia (n=57). *CALR*-positive cases represented 63.7% of double-negative cases of essential thrombocythemia, and most carried *CALR* type 1 or type 2 indels. However, we also identified one patient who was positive for both the *JAK2* V617F and the *CALR* mutations. This study revealed that *CALR* mutant essential thrombocythemia is associated with younger age, higher platelet counts, lower erythrocyte counts, leukocyte counts, hemoglobin, and hematocrit, and increased risk of progression to myelofibrosis in comparison with *JAK2* V617F-positive essential thrombocythemia. Analysis of the *CALR* mutant group according to indel type showed that *CALR* type 1 deletion is strongly associated with male gender. *CALR* mutant patients had a better overall survival than *JAK2* V617F-positive patients, in particular patients of age 60 years or younger. In conclusion, this study in a Belgian cohort of patients supports and extends the growing body of evidence that *CALR* mutant cases of essential thrombocythemia are phenotypically distinct from *JAK2* V617F-positive cases, with regards to clinical and hematologic presentation as well as overall survival.

Introduction

The *JAK2* V617F mutation is found in about 50-60% of cases of essential thrombocythemia (ET) while 5-10% of *JAK2* V617F-negative ET patients carry a W515K/L gain-of-function mutation in exon 10 of *MPL*, the thrombopoietin receptor gene.[1-5] Recently, indels in *CALR* have been described in about 70% of cases of *JAK2/MPL* wild-type (double-negative) ET and primary myelofibrosis (PMF), in a mutually exclusive pattern with *JAK2* and *MPL* mutations. *CALR* mutations were not found in other myeloid malignancies, with the exception of the myelodysplastic syndrome, refractory anemia with ring sideroblasts and thrombocytosis.[6,7] *CALR* indels are variable but all cause a +1 frameshift leading to a novel C-terminal lacking the KDEL endoplasmic reticulum targeting sequence. *CALR* mutations have also been identified in familial cases of ET (11%) and PMF (30%).[8]

Several recent studies have analyzed the clinical and hematologic features of ET or PMF patients according to their mutational genotype.[6,7,9-13] These studies have shown that *CALR*-mutated cases are associated with younger age, higher platelet counts, lower erythrocyte counts, lower leukocyte counts, and lower hemoglobin levels compared with *JAK2*-mutated cases. In addition, in most of these studies *CALR* mutations were linked to male gender. However, the impact of the mutations on overall survival seems to differ between cases of ET and PMF.

We retrospectively analyzed a Belgian cohort of 103 *JAK2* V617F-negative ET patients for the presence of *MPL* and *CALR* mutations. In addition, we compared the clinical and hematologic phenotypes of *CALR* mutant cases with those of a control cohort of 57 *JAK2* V617F-positive ET patients, as well as with a group of triple-negative (*JAK2* V617F-negative, *MPL* W515K/L-negative, and *CALR* mutant-negative) patients with ET.

Methods

Patients and clinical information

This study was approved by the local ethical committee of UZ Leuven (study S53745). All patients selected for this study were seen at hematology units of academic or large regional Belgian hospitals between 1980 and 2013. Inclusion criteria were a diagnosis of ET [based on World Health Organization (WHO) 2008 criteria[14]], and the availability of stored bone marrow or peripheral blood for molecular analysis. Given that an initial bone marrow biopsy was not available for all cases, the presence of any two minor criteria for PMF (WHO 2008) was an exclusion criterion for this study, if no bone marrow biopsy was available, thus eliminating all cases with potential PMF.

First, a cohort of 103 *JAK2* V617F-negative ET cases diagnosed between 1980 and 2013 at several Belgian hospitals was collected and analyzed for *CALR* and *MPL* mutations. We also collected a control cohort of 57 *JAK2* V617F-positive ET patients diagnosed between 1987 and 2009 in the University Hospitals Leuven. The median follow-up of the whole cohort of 160 patients was 8 years (range, 1-34 years). Hematologic parameters (platelet counts, erythrocyte counts, leukocyte counts, hemoglobin, and hematocrit) at diagnosis were retrieved as was information on cardiovascular events and complications (arterial thrombosis, and venous events). During follow-up, progression to myelofibrosis, progression to acute myeloid leukemia, need for cytoreductive treatment, and presence of splenomegaly were recorded.

Sequencing

Genomic DNA was extracted from stored bone marrow or blood samples, and analyzed for the *JAK2* V617F mutation by allele-specific polymerase chain reaction testing.[1] The mutational load of *JAK2* V617F was determined with the Ipsogen *JAK2* MutaQuant Kit (Qiagen, the Netherlands). *JAK2* V617F-negative patients were sequenced for *MPL* W515K/L mutations. For the detection of *MPL* exon 10 or *CALR* exon 9 mutations, we applied polymerase chain reaction amplification and Sanger sequencing, using previously described primers.[6] *JAK2* V617F-positive and double-negative ET patients were sequenced for *CALR* indels. Sequencing was done on an ABI PRISM® 3100 Genetic Analyzer and the sequencing traces obtained were analyzed on CLC Main Workbench 6 (CLC bio, Denmark). Indels in *CALR* were labeled in accordance with the study by Klampfl *et al.*[6]

Statistics

The non-parametric Mann-Whitney test was used for numerical comparisons while a chi-square test or Fisher exact test was used for nominal comparisons. The analyses were two-tailed, and *P* values <0.05 were considered statistically significant. Overall survival, leukemia-free survival, and myelofibrosis-free survival were estimated by Kaplan-Meier analysis and compared using the log-rank test. Statistical analyses were performed with SPSS software (version 20).

Results and discussion

First, the *JAK2* V617F-negative group of patients (n=103) diagnosed since 1980 was sequenced for *MPL* W515K/L mutations and *CALR* indels. Sequencing revealed 58 *CALR* mutations, 12 *MPL* W515K/L mutations and no *MPL* or *CALR* mutations in 33 patients (Figure 1). Therefore, 63.7% of *JAK2* V617F- and *MPL* W515K/L-negative patients were carrying an indel in *CALR*, which is in line with previously reported percentages indicating that the percentage of *CALR* mutations in this group of patients is ≥50% making *CALR* the second most recurrently mutated gene in ET.[6,7,9,12] For the comparison of the hematologic and clinical phenotypes, we selected 57 *JAK2* V617F-positive patients from our internal database of patients diagnosed since 1987. In contrast with the first published results stating that *JAK2* V617F and *CALR* mutations are mutually exclusive,[6,7] we identified one *JAK2* V617F ET patient with a type 2 *CALR* indel who was excluded from further statistical analysis. This is, to the best of our knowledge, the fourth ET patient to be reported with both a *CALR* mutation and a *JAK2* V617F mutation. This combination has so far been reported in several cases of myeloproliferative neoplasms.[11,15-18]

Type 1 (c.1092_1143del) and type 2 (c.1154_1155insTTGTC) indels occurred in 35 cases (60.3%) and 18 cases (31%), respectively. The remaining indels included five other types of which three were novel; they were labeled type 41, 42, and 43 in accordance with Klampfl *et al.* (*Online Supplementary Table S1*). Although novel, these types also lead to typical loss of the acidic bases and the gain of a novel basic C-terminal, as with the previously described types.[6] Similar frequencies of type 1 deletion and type 2 insertion in ET have been reported in several other studies. In the first study by Klampfl *et al.* in a cohort of patients with myeloproliferative neoplasms, 53% and 31.7% carried type 1 or type 2, respectively.[6] In later studies, the incidences of type 1 and type 2 in the cohorts studied by Tefferi *et al.*, Rumi *et al.* and Andrikovics *et al.* were 48% and 35%, 46% and 38%, and 50% and 32%, respectively.

The mean age of patients with *CALR* mutations was 51.6±17 years (range, 23 and 84), whereas the mean age of triple-negative patients and *JAK2* V617F-positive patients was 46.6±16.8 years (range, 17-78) and 65±14 years (range, 36-94), respectively. *CALR* mutant patients were younger than *JAK2* V617F-positive ones (*P*<0.001), consistent with previous studies on *JAK2* V617F-positive *versus* *JAK2* V617F-negative myeloproliferative neoplasms.[19,20] Also in agreement with previously published data, the *CALR* mutation-positive group had more males (56.8 %) compared to the *JAK2* V617F-positive group (39.2%).[9,12,13] Patients with a *CALR* mutation also had higher platelet counts (mean ± SD: 989.8±371.3x10⁹/L *versus* 863.4±288.4 x10⁹/L; *P*=0.03), lower erythrocyte counts (4.4±0.9x10¹²/L *versus* 4.9±0.6x10¹²/L; *P*<0.001), lower hemoglobin concentration (13.3±1.8 g/dL *versus* 14.4±1.6 g/dL; *P*=0.002), lower hematocrit (40.2±6.1% *versus* 43.9±4.7%; *P*<0.001) and lower leukocyte counts (8.9±2.6x10⁹/L *versus* 11.6±5.9 x10⁹/L; *P*=0.001) (Table 1). Several patients progressed to myelofibrosis or acute myeloid leukemia during the course of the disease. The percentage of ET patients who progressed to post-ET myelofibrosis was higher in the *CALR*-positive group than in the triple-negative or *JAK2* V617F-positive groups (18%, 0%, and 4%, respectively; *P*=0.01 and *P*=0.05, respectively). Overall these comparisons indicate that *CALR* mutation-positive patients appear to have a higher risk of evolution to myelofibrosis
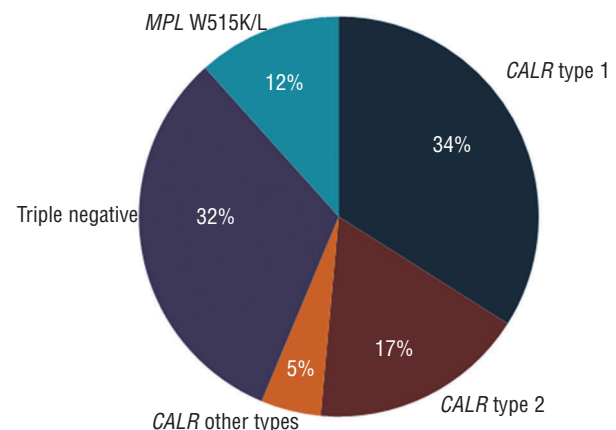
Figure 1. Distribution of *JAK2* V617F-negative ET patients (n=103) according to their mutational status.

Table 1. Hematologic and clinical features of *CALR*-positive *versus JAK2 V617F*-positive and triple-negative patients.

	CALR-positive n=58	*JAK2*-positive n=56	Triple-negative n=33	*CALR*-positive vs. *JAK2*-positive P value	*CALR*-positive vs. triple-negative P value
Age at diagnosis (mean ± SD, range)	51.6 ± 17	65 ± 14.2	46.6 ± 16.8	**<0.001**	0.19
Gender (male)	33/58 (56.8%)	22/56 (39.2%)	20/33	0.059	0.67
Platelets (10⁹/L, mean ± SD)	989.8 ± 371.3	863.4 ± 288.4	939.3 ± 509.6	**0.03**	0.07
Leukocytes (10⁹/L, mean ± SD)	8.9 ± 2.6	11.6 ± 5.9	11.1 ± 6.3	**0.001**	0.1
Erythrocytes (10¹²/L, mean ± SD)	4.4 ± 0.9	4.9 ± 0.6	4.6 ± 1.2	**<0.001**	0.09
Hemoglobin (g/dL, mean ± SD)	13.3 ± 1.8	14.4 ± 1.6	13.9 ± 2.1	**0.002**	0.1
Hematocrit (percentage, mean ± SD)	40.2 ± 6.1	43.9 ± 4.7	41.7 ± 6	**<0.001**	0.14
Cytoreductive treatment	51/56 (91%)	49/56 (88%)	24/32 (75%)	0.76	0.06
Splenomegaly	3/50 (6%)	3/46 (7%)	0/32	1	0.27
Cardiovascular complications at diagnosis	9/55 (16%)	8/55 (15%)	2/32 (6%)	0.79	0.3
Arterial thrombosis	11/55 (20%)	9/54 (17%)	4/31 (13%)	0.65	0.55
Venous event	2/49 (4%)	7/54 (13%)	1/31 (3%)	0.16	1
Death	7/51 (14%)	11/48 (23%)	5/31 (16%)	0.24	0.76
Progression to myelofibrosis	9/51 (18%)	2/50 (4%)	0/31	0.05	**0.01**
Progression to acute myeloid leukemia	4/49 (8%)	3/50 (6%)	0/26	0.71	0.29

Statistically significant values (P<0.05) are shown in bold.

compared to the *JAK2* V617F-positive patients. However, the median follow-up of the *CALR* mutation-positive group (12 years) was longer than that of the *JAK2* V617F-positive group (7 years). The natural history of early/prefibrotic PMF is known to be different from that of true ET, in particular due to higher rates of evolution to overt myelofibrosis and blast transformation.[21] The possibility of early/prefibrotic PMF at diagnosis was specifically ruled out in those evolving to myelofibrosis, based on review of the initial bone marrow biopsy and histopathological criteria for PMF.[14] The bone marrow biopsies showed a proliferation mainly of the megakaryocytic lineage with increased numbers of enlarged, mature megakaryocytes, rather than bizarre highly atypical megakaryocytes. There was no significant increase or left-shift of neutrophil granulopoiesis or erythropoiesis. Previous studies by Nangalia *et al.* and Andrikovics *et al.* also found higher risks of progression among *CALR* mutation-positive *versus JAK2* V617F-positive ET (15% *versus* 6%, P=0.034 and 19% *versus* 2%; P=0.03).[7,22] Other studies found comparable progression rates to myelofibrosis in *CALR* mutation-positive and *JAK2* V617F-positive ET.[12,13,23]

Eighty-six percent of the patients in our study group started on cytoreductive therapy, most commonly hydroxyurea, upon diagnosis. The triple-negative group contained significantly fewer treated patients compared to *CALR* mutation-positive and *JAK2* V617F-positive patients (P=0.04, not shown).

ET is associated with cardiovascular complications including stroke, acute coronary syndrome, transient ischemic attack, pulmonary embolism, abdominal thrombosis, deep-vein thrombosis, and peripheral arterial thrombosis. We assessed whether the mutational status was associated with differences in the incidence of cardiovascular complications at diagnosis and arterial thrombosis or venous events. In our cohort of patients the risks of developing any of these complications did not differ sig-
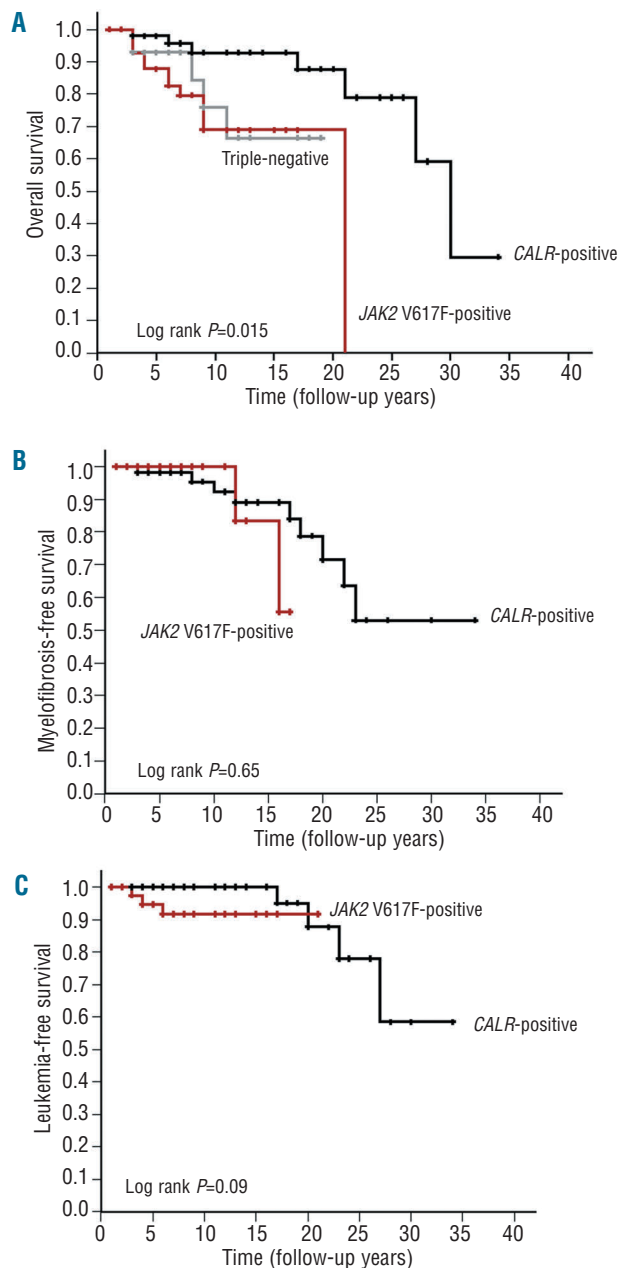
nificantly between the different molecular subtypes.

We also compared the clinical and hematologic data of the *CALR* type 1 and type 2 subgroups and compared them to those of the *JAK2* V617F-positive group (*Online Supplementary Table S2*). Remarkably, the male predilection was confined to the group with *CALR* type 1 mutation, and this group also had a tendency to the highest platelet counts and was associated with younger age compared to the *JAK2* V617F-positive group. Tefferi *et al.* also reported that *CALR* type 1 is associated with male gender. However, they also reported that type 2 is associated with younger age and higher platelet counts,[24] which was not confirmed in our study or by another study published by Andrikovics *et al.*[22]

We divided our *JAK2* V617F-positive group into a group with low mutational load (≤10%; n=11) and another with high mutational load (>10%; n=46), as determined by quantitative polymerase chain reaction. However, no significant differences between the two groups were revealed (*Online Supplementary Table S3*). Given the low number of patients with a low load of *JAK2,* this comparison needs to be conducted again in larger groups.
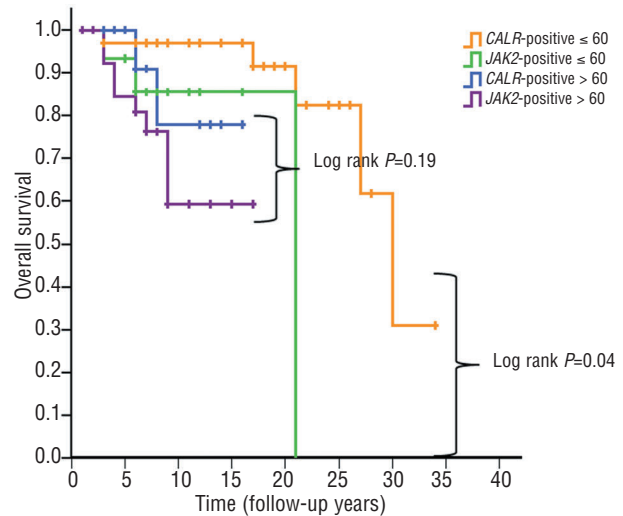
The mean follow-up of the *CALR* mutation-positive, the *JAK2* V617F-positive and the triple-negative groups was 14, 7, and 7 years, respectively. The median overall survivals, as evaluated by Kaplan-Meier analysis, were 30 years (95% confidence interval, 25.4 to 34.5), 21 years (95% confidence interval, not reached), and not reached, respectively (P=0.015) (Figure 2A). Myelofibrosis-free survival (Figure 2B) and leukemia-free survival (Figure 2C) were not significantly different between the *CALR* mutation-positive group and the *JAK2* V617F-positive group (P=0.65 and P=0.09, respectively). In our study, none of the triple-negative patients progressed to develop myelofibrosis or acute myeloid leukemia.

In the first paper by Klampfl *et al.*, the *CALR* mutant group had a better overall survival compared to *JAK2*

Figure 3. Overall survival of the *CALR*-positive and the *JAK2* V617F-positive groups stratified into age groups. Kaplan-Meier estimate according to age groups: greater than 60 and younger or equal to 60 years old.

Figure 2. Overall survival, myelofibrosis-free survival, and leukemia-free survival. Kaplan-Meier estimate of (A) overall survival of *CALR*-positive, *JAK2* V617F-positive, and triple-negative groups, (B) myelofibrosis-free survival and (C) leukemia-free survival of the *CALR*-positive group and *JAK2* V617F-positive group.

ing to age (> 60 or ≤60 years). In the group of patients ≤60 years old, *CALR* mutation-positive patients had a better overall survival than the *JAK2* V617F-positive group ($P=0.04$). On the other hand, in the group of patients older than 60 years there was no difference in the overall survival between the *CALR* mutation-positive group and the *JAK2* V617F-positive group ($P=0.19$) (Figure 3). This indicates that the prognosis of *CALR/JAK2* mutated ET is different only in younger age groups.

We also describe, what is to the best of our knowledge, the fourth ET patient with both a *JAK2* V617F and a *CALR* mutation. In this patient the *JAK2* V617F mutational load assessed by allele-specific polymerase chain reaction analysis was around 0.2%, while the mutational load of *CALR* type 2 estimated by Sanger sequencing was around 40%. Based on the differential mutational loads two scenarios are possible; either the *JAK2* V617F is a subclonal mutation in the major *CALR* mutated clone, or the two mutations arose in two different clones with the *CALR* clone being the larger, with the *JAK2* V617F as a minor clone. Given that no viable frozen material is available, this issue unfortunately cannot be further investigated *in vitro*.

In summary, this study of 160 ET patients confirms that *CALR* mutation-positive ET is phenotypically and biologically distinct from *JAK2* V617F-positive ET, in terms of clinical presentation and disease evolution. We also observed that *CALR* mutant ET was associated with better overall survival in younger ET patients. Our study also suggests the possibility of phenotypic differences within the *CALR* mutant group according to the mutation type, in line with a few other studies.[22,24] Although the analysis of *CALR* mutations is rapidly being implemented in the diagnostic work-up of ET, the molecular subtypes of ET are, at the time of writing, not taken into account in treatment decisions in ET.

V617F-positive cases or *MPL* mutant cases in 311 ET cases and 203 PMF cases.[6] However, subsequent studies by Tefferi *et al.*, Andrikovics *et al.*, and Rumi *et al.* showed better overall survival for *CALR* mutant cases than for *JAK2* V617F-positive cases in PMF,[11,22,25] but not in ET.[8,11,13,22] Tefferi *et al.* addressed this divergence by studying survival in 299 ET cases with long follow-up, but did not find a survival advantage for *CALR*-mutated cases even after adjusting for age (≤ or > 65 years).[12] Since *JAK2* V617F-positive patients are generally older at diagnosis than *CALR* mutation-positive patients, we also divided our patients accord-

Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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