Distinct clinical characteristics of myeloproliferative neoplasms with calreticulin mutations

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ABSTRACT

Somatic insertions/deletions in the calreticulin gene have recently been discovered to be causative alterations in myeloproliferative neoplasms. A combination of qualitative and quantitative allele-specific polymerase chain reaction, fragment-sizing, high resolution melting and Sanger-sequencing was applied for the detection of three driver mutations (in Janus kinase 2, calreticulin and myeloproliferative leukemia virus oncogene genes) in 289 cases of essential thrombocythemia and 99 cases of primary myelofibrosis. In essential thrombocythemia, 154 (53%) Janus kinase 2 V617F, 96 (33%) calreticulin, 9 (3%) myeloproliferative leukemia virus oncogene gene mutation-positive and 30 triple-negative (11%) cases were identified, while in primary myelofibrosis 56 (57%) Janus kinase 2 V617F, 25 (25%) calreticulin, 7 (7%) myeloproliferative leukemia virus oncogene gene mutation-positive and 11 (11%) triple-negative cases were identified. Patients positive for the calreticulin mutation were younger and had higher platelet counts compared to Janus kinase 2 mutation-positive counterparts. Calreticulin mutation-positive patients with essential thrombocythemia showed a lower risk of developing venous thrombosis, but no difference in overall survival. Calreticulin mutation-positive patients with primary myelofibrosis had a better overall survival compared to that of the Janus kinase 2 mutation-positive (P=0.04) or triple-negative cases (P=0.01). Type 2 calreticulin mutation occurred more frequently in essential thrombocythemia than in primary myelofibrosis (P=0.049). In essential thrombocythemia, the calreticulin mutational load was higher than the Janus kinase 2 mutational load (P<0.001), and increased gradually in advanced stages. Calreticulin mutational load influenced blood counts even at the time point of diagnosis in essential thrombocythemia. We confirm that calreticulin mutation is associated with distinct clinical characteristics and explored relationships between mutation type, load and clinical outcome.

Introduction

The characterization of the genetic background of *BCR-ABL1*-negative chronic myeloproliferative neoplasms (MPN) was greatly advanced by the discovery that the V617F mutation of Janus kinase 2 (*JAK2*) is very common in the three classic MPN, occurring in 90-95% of cases of polycythemia vera (PV), and in 40-60% of cases of essential thrombocythemia (ET) and primary myelofibrosis (PMF).[1-4] This led to the inclusion of V617F mutation screening in the diagnostic criteria for MPN.[5] *JAK2* exon 12 mutations occur in rare cases of V617F-negative PV allowing a close to perfect coverage by specific genetic alterations in PV.[6,7] On the other hand, clinicians continue to face challenges during the diagnosis of *JAK2* mutation (*JAK2*^{mut})-negative ET and PMF. Moving the field very close to full coverage, in two parallel seminal discoveries, Klampfl *et al.*[8] and Nangalia *et al.*[9] recently described somatic, recurrent insertions/deletions exclusively affecting exon 9 of the calreticulin (*CALR*) gene. Affecting the same driver pathway, *CALR* mutations were mutually exclusive with *JAK2* V617F or myeloproliferative leukemia virus oncogene gene (*MPL*) mutations. In a plethora of subsequent studies published within 3 months, the initial findings were confirmed and extended focusing on the clinical correlates.[10-13] Several additional studies described low frequencies of *CALR* mutations in different MPN-related diseases, but not in other hematologic diseases.[8,9,14-18]

As a result of the discovery of *CALR* mutations, definite molecular diagnostics have become available for 75-90% of clonal MPN. However, for the introduction of *CALR* mutation screening into routine clinical practice, it is essential to characterize the potential effects of *CALR* mutations on disease phenotype and progression in several independent cohorts. The aim of our study was to apply a complex array of molecular techniques to identify driver mutations. We aimed to confirm previous associations between the presence of acquired genetic alterations and clinical characteristics in a large, independent cohort of patients with MPN. An additional purpose was to systematically analyze the roles of particular *CALR* mutation types and load.

Methods

Subjects

Our study population consisted of 603 patients with *BCR-ABL1*-negative MPN (260 males, 343 females; median age: 60, range: 10-94

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years) diagnosed between 1974 and 2013, as an extension of those published earlier.[19,20] According to World Health Organization 2008 criteria, 215 patients had *JAK2* V617F positive PV, 289 had ET and 99 had PMF. Laboratory parameters and clinical features at the time of diagnosis were collected retrospectively. Coagulation complications and myelofibrotic or acute leukemic transformation were recorded if they were present at diagnosis or occurred during the follow-up. Coagulation complications included venous thrombotic events (deep vein thrombosis, pulmonary embolism, splanchnic or cerebral venous thrombosis), arterial thrombosis (transient ischemic attack, ischemic stroke, acute myocardial infarction, or peripheral arterial vascular complications) and hemorrhagic problems (gastrointestinal bleeding, hemorrhagic stroke, hematuria, severe bleeding during surgery or dental procedures). The median follow-up was 5.7 years (range, 0-40 years). All participants signed informed consent. The study was approved by the Hungarian National Ethics Committee.

Detection of driver somatic mutations

All analyses were performed using genomic DNA isolated from peripheral blood or bone marrow. In a subset of patients, sampling and diagnostic ascertainment occurred within 1 year. In some analyses, these cases were considered separately as the ones best reflecting the patients' condition at diagnosis.

All MPN patients were screened for *JAK2* V617F (c.1849G>T) by allele-specific polymerase chain reaction (PCR).[1] In $JAK2^{mut}$ cases, real-time quantitative PCR was performed to determine the V617F load.[21] The $JAK2^{mut}$ load was calculated as follows: $JAK2^{mut}/(JAK2^{mut}+JAK2^{wild\text{-}type})$.

$JAK2^{mut}$-negative ET and PMF patients were screened for *CALR* mutations by high resolution sizing of fluorescence-labeled PCR products by capillary electrophoresis (fragment analysis).[8] In $CALR^{mut}$ cases, the precise mutation was identified by Sanger-sequencing.[8] To determine the mutant load, the ratio of peak heights was calculated using an analogous formula: $CALR^{mut}/(CALR^{mut}+CALR^{wild\text{-}type})$. Since fragment analysis was a semi-quantitative approach and the final amounts of the PCR products were influenced by the preferential amplification of shorter amplicons, we calculated the load after PCR with 25 cycles, which was a reduced cycling condition compared to the screening condition (35 cycles).

In $JAK2^{mut}$ and $CALR^{mut}$ negative ET and PMF patients, screening for *MPL* S505N and W515 codon mutations was performed by high resolution melting analysis.[22] In cases that were positive in this screening analysis, the exact type of *MPL* mutation was determined by allele-specific PCR and sequencing.[23]

Statistics

Continuous variables are presented as medians with 25th and 75th percentiles. Mann-Whitney or Kruskal-Wallis tests were used to compare continuous variables, while χ^2 or Fisher exact tests were used for dichotomous variables. A log-rank test was performed to compare overall survival between subgroups according to driver mutations. In the case of hematopoietic stem cell transplantation (n=13), the follow-up period was terminated at the date of the transplant. For multivariate analysis, age was considered in a Cox proportional hazards model beside driver mutations. Hazard ratios (HR) and 95% confidence interval (CI) values were computed. The analyses were conducted using the SPSS (version 20.0) software package.

Results

Distribution of different types of driver mutations and comparisons of clinical parameters in subgroups according to driver mutation status

Only *JAK2* V617F positive ($JAK2^{mut}$) PV patients (n=215) were included in this study. In patients with PV, qualitative and quantitative *JAK2* V617F tests, but no *CALR* and *MPL* molecular tests, were performed. For ET, the distribution of driver mutations was as follows: $JAK2^{mut}$: 154/289 (53%), $CALR^{mut}$: 96/289 (33%), MPL^{mut}: 9/289 (3%), triple-negative: 30/289 (11%) cases (Table 1). For PMF, the corresponding figures were: $JAK2^{mut}$: 56/99 (57%), $CALR^{mut}$: 25/99 (25%), MPL^{mut}: 7/99 (7%), triple-negative: 11/99 (11%) cases (Table 2).

The patients' clinical and laboratory parameters were systematically compared according to diagnosis and driver mutation. Given the low number of cases, patients with *MPL* mutation were omitted from all subsequent analyses. Compared to the $JAK2^{mut}$ PV cohort, the $JAK2^{mut}$ ET cohort contained more females ($P<0.001$), had lower hemoglobin levels ($P<0.001$), higher platelet counts ($P<0.001$), less frequent splenomegaly ($P<0.001$) and less frequent myelofi-

Table 1. Clinical and laboratory characteristics of PV and ET patients according to the presence of a driver somatic mutation.

	(A) PV (n=215)	(B) ET, $JAK2^{mut}$ (n=154)	*P* (A) *vs.* (B)	(C) ET $CALR^{mut}$ (n=96)	*P* (C) *vs.* (B)	(D) ET, triple neg. (n=30)
Age, year (25th-75th percentile)	61 (51-70)	61 (51-71)	0.975	53 (37-70)	**0.025**	60 (43-67)
Male/female (%/%)	113/102 (53%/47%)	46/108 (30%/70%)	**<0.001**	39/57 (41%/59%)	0.099	8/22 (27%/73%)
Hemoglobin (g/L, 25th-75th percentile)	183 (173-199)	147 (134-156)	**<0.001**	131 (120-145)	**<0.001**	130 (120-138)
WBC (10^9/L, 25th-75th percentile)	11 (9-13)	10 (8-13)	0.574	9 (8-12)	**0.034**	10 (8-13)
PLT (10^9/L, 25th-75th percentile)	456 (315-619)	778 (651-992)	**<0.001**	981 (767-1389)	**<0.001**	791 (632-1016)
Splenomegaly, n. (%)	96/204 (47%)	37/139 (27%)	**<0.001**	20/74 (27%)	1.00	5/23 (22%)
Venous thrombosis, n. (%)	28/215 (13%)	27/153 (18%)	0.237	6/85 (7%)	**0.03**	2/28 (7%)
MF transformation, n. (%)	28/215 (13%)	9/153 (6%)	**0.034**	13/87 (15%)	**0.034**	1/29 (3%)
AL transformation, n. (%)	16/212 (8%)	4/154 (3%)	0.06	3/88 (3%)	1.00	0/29 (0%)
Cytoreduction, n. (%)	140/160 (87%)	98/123 (80%)	0.107	62/82 (76%)	0.496	17/20 (85%)

ET-patients qualified for triple-negative status (D) if no $JAK2^{mut}$ or $CALR^{mut}$ or MPL^{mut} was present. Clinical and laboratory data apply at the time of first presentation. Cytoreduction was defined positive if hydroxyurea or anagrelide treatment was given for more than 6 months. P values below 0.05 were considered statistically significant and are indicated in bold. AL: acute leukemia; ET: essential thrombocythemia; Hb: hemoglobin concentration; MF: myelofibrotic, PLT: platelet count; PMF: primary myelofibrosis; PV: polycythemia vera; WBC: white blood cell count.

brotic transformation (*P*=0.034) (Table 1). Compared to the *JAK2*^{mut} ET cohort, in the *CALR*^{mut} ET patients we found a tendency toward less pronounced female predominance (*P*=0.099), younger age at diagnosis (*P*=0.025), lower hemoglobin levels (*P*<0.001), lower white blood cell counts (*P*=0.034) and higher platelet counts (*P*<0.001). Coagulation complications (venous and arterial thromboses, plus hemorrhages, taken together) were more frequent in *JAK2*^{mut} ET patients (36%, 55/153) than in *CALR*^{mut} ET patients (18%, 15/85; *P*=0.003). Venous thrombosis was more frequent in *JAK2*^{mut} ET than in *CALR*^{mut} ET (*P*=0.03). Arterial thrombosis occurred in 14% (22/153) of *JAK2*^{mut} and in 9% (8/85) of *CALR*^{mut} ET (*P*=0.3), and hemorrhage in 9% (14/153) of *JAK2*^{mut} and in 5% (4/85) of *CALR*^{mut} ET (*P*=0.3). Myelofibrotic transformation occurred more frequently in the *CALR*^{mut} cohort (*P*=0.03). We did not find any significant differences comparing triple-negative ET patients to other ET subgroups: the sample size (n=30) was, however, small.

Similar analyses in the PMF cohort (Table 2) showed younger age at presentation (*P*=0.002) and higher platelet counts (*P*=0.001) in the *CALR*^{mut} subgroup than in the *JAK2*^{mut} PMF subgroup. Other variables were not different, nor were any of the characteristics of the triple-negative PMF patients except for an increased rate of acute leukemic transformation in triple-negative cases (36% *versus* 9% in *JAK2*^{mut}, *P*=0.038 or *versus* 14% in *CALR*^{mut}, *P*=0.19).

Regarding outcome parameters, overall survival was analyzed, initially by a univariate Kaplan-Meier approach, in patients with ET (Figure 1A) and PMF (Figure 1B) stratified according to different driver mutations. Out of the 289 ET patients, 261 cases had appropriate follow-up information [*JAK2*^{mut} (n=150), *CALR*^{mut} (n=85) and triple-negative (n=26) subgroups]. As shown in Figure 1A, no differences were detected in overall survival by univariate analyses (*P*=0.846).

Among PMF patients (n=87), the subgroups were as follows: *JAK2*^{mut} (n=55), *CALR*^{mut} (n=21) and triple-negative (n=11). In contrast to ET, the overall comparison resulted

Table 2. Clinical and laboratory characteristics of PMF patients according to the presence of a driver somatic mutation.

	(A) PMF, *JAK2*^{mut} (n=56)	(B) PMF, *CALR*^{mut} (n=25)	*P* (A) *vs.* (B)	(D) PMF, Triple neg. (n=11)
Age, year (25th-75th percentile)	68 (57-73)	56 (39-65)	**0.002**	69 (53-79)
Male/female (%/%)	28/28 (50%/50%)	12/13 (48%/52%)	1.00	8/3 (72%/27%)
Hemoglobin (g/L, 25th-75th percentile)	116 (58-183)	112 (97-124)	0.484	95 (79-121)
White cell count (10^9/L, 25th-75th percentile)	12 (8-18)	9 (5-13)	0.147	12 (7-25)
Platelet count (10^9/L, 25th-75th percentile)	250 (132-500)	552 (320-712)	**0.001**	156 (54-452)
Splenomegaly, n. (%)	47/56 (84%)	14/21 (67%)	0.120	9/11 (82%)
Venous thrombosis, n. (%)	9/54 (17%)	1/21 (5%)	0.266	1/11 (9%)
AL-transformation, n. (%)	5/54 (9%)	3/22 (14%)	0.684	4/11 (36%)
Cytoreduction, n. (%)	30/43 (70%)	9/17 (53%)	0.243	6/8 (75%)

For explanation and abbreviations see Table 1.

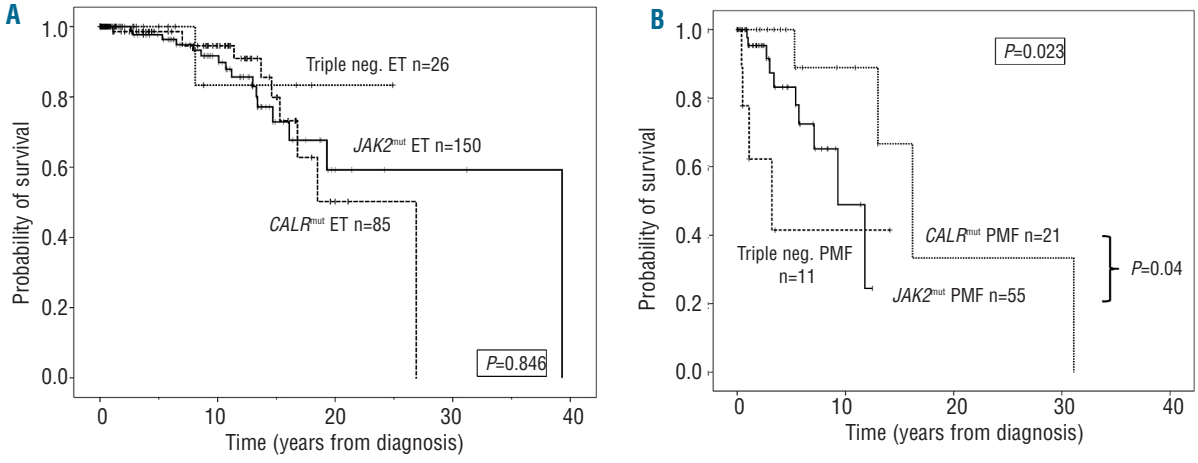

Figure 1. Kaplan-Meier analysis of overall survival in patients with ET (panel A, n=261) and PMF (panel B, n=87) according to the presence of different driver mutations. Patients qualified for triple-negative status if *JAK2*^{mut} and *CALR*^{mut} and *MPL*^{mut} were all absent. For a subset of patients (ET: n=2, PMF: n=11) treated by hematopoietic stem cell transplantation, the follow-up was censored at the date of this intervention. In ET, univariate analyses resulted in an overall *P* value of 0.846 (A). In PMF, the same comparison gave a *P* value of 0.023 (B). Upon pairwise univariate comparisons, the *CALR*^{mut} subgroup showed significantly better survival compared to *JAK2*^{mut} (*P*=0.04) and triple-negative (*P*=0.01) PMF patients while *JAK2*^{mut} patients showed only a tendency towards better overall survival compared to triple-negative patients (*P*=0.076). ET: essential thrombocythemia; PMF: primary myelofibrosis.

in P=0.023 (Figure 1B) with the best overall survival in the *CALR*^{mut} subgroup and the worst in the triple-negative subgroup. This was further confirmed by pairwise univariate comparisons, namely *CALR*^{mut} *versus JAK2*^{mut} (P=0.04) and *CALR*^{mut} *versus* triple-negative (P=0.01). The *JAK2*^{mut} PMF patients showed only a tendency towards having a better overall survival than the triple-negative patients (P=0.076). To further analyze the potential effect of driver mutations on survival, we utilized a Cox proportional hazard model choosing the triple-negative PMF subgroup as the reference. In this model, age was a factor that significantly affected survival (HR 1.082, 95% CI: 1.023-1.144; P=0.006), while the presence of a driver mutation showed an overall tendency (P=0.059). Performing pairwise comparisons, the *CALR*^{mut} subgroup was characterized by a HR=0.131 (95% CI: 0.023-0.739; P=0.021) indicating a significantly better survival than that of triple-negative patients. Comparing the *CALR*^{mut} patients to the *JAK2*^{mut} subgroup, only a tendency towards better survival was observed in the presence of *CALR* exon 9 mutation (HR=0.159, 95% CI: 0.018-1.391; P=0.097).

Subgroup analyses in patients with CALR^{mut} according to type of mutation

Following fluorescence PCR and fragment analyses by capillary electrophoresis, the presence of *CALR* exon 9 mutation was confirmed by Sanger-sequencing. In the entire cohort of 121 *CALR*-positive cases, we found 64 (53%) with a type 1 mutation (52 base pair deletion, c.1092_1143del), 34 (28%) with a type 2 mutation (5 base pair insertion, c.1154_1155insTTGTC) and 23 (19%) with other types of mutations. These other mutations comprised the following types: 3 (c.1095_1140del), 4 (c.1102_1135del), 5 (c.1091_1142del), 19 (c.1110_1140del), 22 (c.1120_1123del), 24 (c.1120_1138del), 29 (c.1135_1152delinsCCTCCTCTTTGTCT), 33 (c.1154_1155insATGTC), 34 (c.1154_delins CTTGTC), 35 (c.1154delinsTTTGTC) and potentially novel variants (n=8). The occurrence of these variants according to diagnosis was as follows: ET: type 1: 48 (50%), type 2: 31 (32%) and other: 17 (18%); PMF: type 1: 16 (64%), type 2: 3 (12%) and other: 6 (24%). Comparing patients with ET and PMF and only considering types 1 and 2, we found a tendency to a different distribution with 48 type 1 (61%) and 31 type 2 (39%) mutations in patients with EF *versus* 16 type 1 (84%) and 3 type 2 (16%) in patients with PMF (P=0.064). An increased frequency of type 2 mutations (*versus* non-type 2 mutations) was observed in ET (32%) compared to PMF (12%, P=0.049, *Online Supplementary Table S1*).

Next, we compared demographic and clinical parameters within patients with *CALR*^{mut} ET according to mutation types. We found a tendency towards older age among type 2 carriers compared to type 1 carriers (median age at diagnosis: 59 years *versus* 51 years, P=0.06) or compared to non-type 2 carriers (median age at diagnosis: 59 years *versus* 51 years, P=0.09). Similarly, platelet count at diagnosis tended to be higher in the subgroup of type 2 mutation carriers than in patients with the type 1 mutation: 1237x10^9/L (25th-75th percentile: 884-1472) *versus* 946 x10^9/L (25th-75th percentile: 764-1280) (P=0.081); or compared to those with non-type 2 mutation: 865x10^9/L (25th-75th percentile: 755-1218) (P=0.041). The frequency of cytoreductive therapy was higher among type 2 *versus* type 1 ET patients (89% *versus* 67% P=0.05). No further differences in any demographic or clinical characteristics (including overall survival) were found upon comparing the two ET subgroups with different types of mutations (*Online Supplementary Table S2*). Similar analyses were not feasible for the PMF cohort because of the low number (n=3) of patients with the type 2 *CALR* mutation.

Subgroup analyses in patients with a positive driver mutation (JAK2^{mut} or CALR^{mut}) according to mutational load

A systematic comparison was performed between MPN subgroups in relation to the quantity of driver mutation (Figure 2). The *JAK2* V617F load was determined by real-time quantitative allele-specific PCR using the TaqMan detection system,[21] while the semi-quantitative estimation of the *CALR* mutation was achieved by high resolution sizing of fluorescence-labeled PCR products.[8] The mutational load in *JAK2* V617F and *CALR* mutation-positive patients was calculated with a similar formula i.e. the quantity of mutant was expressed as a percentage fraction of total gene copies allowing the comparison of loads across MPN subgroups with different mutations. Performing pairwise comparisons, we made the following observations (Figure 2). (i) Neither *JAK2*^{mut}, nor *CALR*^{mut} ET patients without myelofibrotic transformation at the sampling time showed significantly different driver mutational loads comparing the subgroup of patients whose samples were taken within 1 year of diagnosis and the sub-

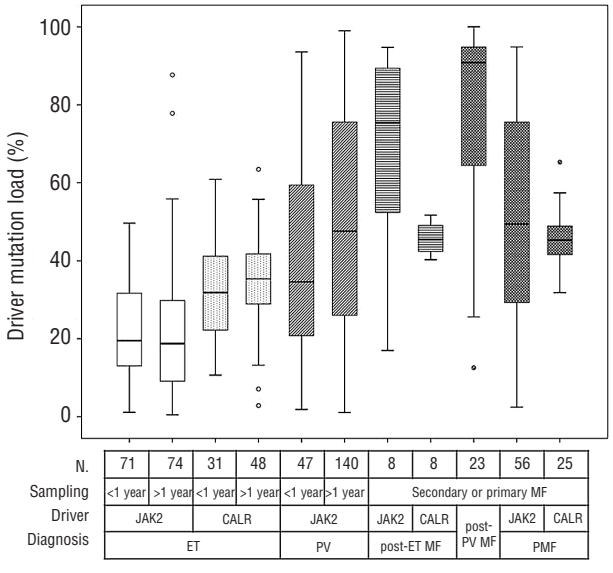

Figure 2. Comparisons of relative quantities of driver mutations (*JAK2* V617F or *CALR*) in different MPN subgroups according to diagnosis and the relation of time of sampling and time of diagnosis. The first row under the x axis shows the number of patients (n) in the respective subgroups. If no primary or secondary myelofibrosis was present at the time of sampling, sampling within 1 year of diagnosis qualified, as seen in the second lines, as "<1 year"; all other situations were allocated into the ">1 year" subgroup. If primary or secondary myelofibrosis was present at the time of sampling, no such subgrouping was performed ("secondary or primary MF"). Quantification of the respective driver mutation was performed by real-time quantitative allele-specific PCR (*JAK2* V617F) or fragment analysis (*CALR* exon 9). Pairwise comparisons were performed with the Mann-Whitney test. ET: essential thrombocythemia; PMF: primary myelofibrosis; MF: myelofibrosis; PV: polycythemia vera.

group of patients whose samples were taken at a later time point. On the other hand, the two PV-subgroups (44 patients with sampling within 1 year of diagnosis and 140 patients with sampling at later time points but without myelofibrotic transformation at this later time point) showed a tendency toward increasing allele burden (P=0.066). (ii) *JAK2* loads increased gradually in parallel with the appearance of more advanced stages of MPN (*JAK2*^{mut} ET *versus* PV: P<0.001, PV *versus* post-PV myelofibrosis: P<0.001). (iii) Within patients with ET, the *CALR* load was significantly higher than the *JAK2* load (P<0.001). (iv) *CALR* load showed a steady, significant, but less steep increase corresponding to the appearance of more advanced MPN stages (*CALR*^{mut} ET *versus* *CALR*^{mut} post-ET MF: P=0.01; *CALR*^{mut} ET *versus* *CALR*^{mut} PMF: P<0.001). (v) In contrast to *JAK2*^{mut} load, the *CALR*^{mut} load only rarely exceeded 50% (in 11 cases between 51-75% and in 1 case above 75%).

To investigate the potential effects of *CALR* mutational load, we divided *CALR*^{mut} ET patients with samples available within 1 year after diagnosis (n=31) into two subgroups according to *CALR*^{mut} load, with the cut-off value being 38.5%, which was the median *CALR* mutational load of all patients with ET in the study (n=20 below the cut off and n=11 above the cut off; Table 3). White blood cell counts (9 *versus* 11x10^9/L, P=0.025) and platelet counts (848 *versus* 1406x10^9/L, P=0.04) were lower among patients with low *CALR*^{mut} load, while hemoglobin concentration was higher in the same comparison (138 *versus* 122 g/dL, P=0.04). Applying different cut-off values to discriminate between groups with lower and higher *CALR*^{mut} burden, white blood cell counts were always lower in subgroups with lower *CALR*^{mut} burden, although the differences shrank between the lower and the higher *CALR*^{mut} burden subgroups applying higher cut-offs. While the differences in hemoglobin levels between patients with lower and higher *CALR*^{mut} load were only detectable above the cut-off value of 38.5% or higher, the differences in platelet counts diminished above this cut-off. We observed higher *CALR*^{mut} burden in type 1 (n=14, 41%) compared to type 2 ET (n=15, 27%, P=0.023). However, this difference may have been influenced by the preferential amplification of the shorter fragment in the presence of the type 1 mutant (52 base pair deletion) during PCR, thus this observation needs to be confirmed with an alternative technique of quantification.

Discussion

We set up sequential application of different molecular techniques to identify the driver mutations in a large cohort of MPN patients, noting that *JAK2* V617F, *CALR* and *MPL* mutations were described as mutually exclusive driver mutations in the first studies.[8,9] Concurrent *JAK2* and *CALR* mutations were reported in only two individuals in subsequent studies.[13,14] Confirming earlier observations in different MPN cohorts,[8-10,13] we found a similar frequency of *CALR* mutations in an independent group of MPN patients, with the frequency of these mutations being high (121/162, 75%) among *JAK2* V617F and *MPL*-negative ET patients, thus leaving only a small cohort of triple-negative patients lacking a disease-causing genetic marker.

In our previous study comparing the clinical characteristics of *JAK2*^{mut} and *JAK2*^{neg} MPN patients (n=328),[19] we already noted a female predominance, older age at presentation, higher hemoglobin values and a higher incidence of coagulation complications (thrombotic and hemorrhagic) in *JAK2* V617F-positive MPN patients *versus* V617F-negative counterparts (including *CALR*^{mut} patients). The identification of the *CALR* mutation allowed a more straight forward comparison of MPN patients with a homogeneous genetic background. Our present data, showing distinct clinical characteristics of *CALR*^{mut} ET subgroups in comparison with *JAK2*^{mut} cohorts, confirmed previous observations regarding younger age, sex distribution with less prominent female abundance (only in the ET subgroup), lower hemoglobin concentration, lower white blood cell counts (not significant in our PMF cohort), and higher platelet counts.[8-12]

Considering venous thrombosis and other coagulation complications, our observations confirmed previous findings describing a lower risk of thrombosis in patients with *CALR*^{mut} ET than in those with *JAK2*^{mut} ET.[8] In their extended cohort, Rumi *et al.*[12] found that the rates of thrombosis at diagnosis in the *CALR*^{mut} and *JAK2*^{mut} groups were 2.8% and 7.1%, respectively (P=0.059), and observed a reduced cumulative incidence of thrombosis (25 *versus* 10 events per 1000 person-years) in *CALR*^{mut} patients (P=0.001). In an independent series of patients with ET, Rotunno *et al.* found that the incidence of thrombosis (in the 2 years preceding diagnosis and during follow-up, combined) was 13.5% in their *CALR*^{mut} cohort compared to 30.1% among

Table 3. Laboratory characteristics of ET patients according to the *CALR* mutational load.

CALR mutational load						
Cut-off	34.6%		38.5%		40.1%	
	Below	Above	Below	Above	Below	Above
Number	17	14	20	11	22	9
Hemoglobin (g/L, 25th-75th percentile)	136 (127-143)	130 (116-151)	138 (130-154)	122 (105-141)	138 (131-147)	120 (100-158)
	P=0.47		**P=0.04**		**P=0.018**	
White cell count (10^9/L, 25th-75th percentile)	8 (6-11)	11 (9-16)	9 (7-11)	11 (9-18)	9 (7-11)	11 (9-18)
	P=0.005		**P=0.025**		**P=0.046**	
Platelet count (10^9/L, 25th-75th percentile)	828 (707-1099)	1319 (849-1669)	847 (706-1210)	1406 (850-1819)	884 (707-1401)	1123 (849-1719)
	P=0.04		**P=0.04**		P=0.20	

In all three pairwise comparisons, the same 31 ET patients with available sampling within 1 year of diagnosis were divided into dichotomous subgroups, according to CALR *mutational load. The following* CALR *mutational load cut-off values were chosen: (i) 34.6%: median* CALR^{mut} *load of all ET patients excluding cases with myelofibrotic-transformation; (ii) 38.5%: median* CALR^{mut} *load of all ET patients; (iii) median* CALR^{mut} *load of all MPN (ET and PMF all included) patients in the present study.* P *values represent pairwise comparisons between dichotomously divided subgroups of patients (Mann-Whitney test).* P *values below 0.05 are considered statistically significant and are indicated in bold. ET: essential thrombocythemia.*

JAK2^{mut} patients (*P*=0.011).

With respect to transformation of ET to myelofibrosis, we observed a more than 2-fold higher transformation rate among *CALR*^{mut} patients (15%) compared to *JAK2*^{mut} patients (6%, *P*=0.034), the rate in the former being similar to that in the cohort of patients with PV (13%). Nangalia *et al.* also found an increased risk of transformation to myelofibrosis (19% in *CALR*^{mut} *versus* 2% in *JAK2*^{mut} patients; *P*=0.03) in a smaller cohort of patients with ET.[9] In a large study, the incidence of myelofibrotic transformation was 7% (95% CI 3-13) in *CALR*^{mut} *versus* 5% (95% CI 3-8) in *JAK2*^{mut} ET *versus* 8% (95% CI 5-12) in *JAK2*^{mut} PV, while the 15-year cumulative incidence of myelofibrotic transformation was 13.4% (CI 95% 5.4-25.2%) in *CALR*^{mut} *versus* 8.4% (CI 95% 3.9-15.3%) in *JAK2*^{mut} ET *versus* 13.6% (CI 95% 7.3-21.9%) in *JAK2*^{mut} PV (*P*=not significant).[12] In our cohort, the median follow-up tended to be longer in the *CALR*^{mut} ET group (8 years in *CALR*^{mut} *versus* 4 years in *JAK2*^{mut}; *P*=0.09), which might have resulted in a higher frequency of myelofibrotic transformation.

Among patients with ET, we did not find differences in overall survival according to the presence of a specific driver mutation. In contrast, Klampfl *et al.* observed better survival in patients with *CALR*^{mut} than in those with *JAK2*^{mut} (*P*=0.04).[8] Interestingly, analyzing a larger ET cohort with substantial overlap with that of Klampfl *et al.*, in univariate analysis, Rumi *et al.* found only a trend (*P*=0.085) towards a better overall survival at 15 years for *CALR*^{mut} patients compared to *JAK2*^{mut} patients, and the trend disappeared when age was considered as a covariate.[12] Similarly, Rotunno *et al.* did not find any difference in overall survival among patients with ET.[11]

As for patients with ET and in previous reports,[8,13] younger age at disease onset and elevated platelet counts were found to characterize our *CALR*^{mut} PMF cohort. Our observations in PMF were in good agreement with previous reports,[8,13] indicating a significant beneficial effect of the presence of *CALR*^{mut}, compared to *JAK2*^{mut}, on overall survival. In our dataset, the differences were further substantiated by pairwise comparisons in a multivariate (with age added as a covariate) Cox model indicating a trend towards better survival in *CALR*^{mut} patients compared to *JAK2*^{mut} ones and a significantly better survival between *CALR*^{mut} and triple-negative PMF patients. In addition, the decreased leukemia-free survival in triple-negative PMF patients reported by Tefferi *et al.*[13] was in line with the increased rate of acute leukemic transformation in our triple-negative PMF cases (Table 2). Recently, a set of gene mutations were described as detrimental in PMF. The presence of any "high molecular risk" mutations affecting *ASXL1*, *EZH2*, *SRSF2*, and *IDH1/2* genes predicted shorter overall survival and increased risk of leukemic transformation. A *CALR* mutation proved to be an independent protective factor for survival in PMF.[24] *ASXL1* mutations, as the most frequent detrimental mutations, were reported to occur with similar frequency in *JAK2*^{mut}, *CALR*^{mut} and triple-negative PMF patients;[13] thus, in our PMF-cohort, the survival benefit observed in the group of *CALR*^{mut} patients was unlikely to result from an uneven distribution of *ASXL1* mutations. The presence of *CALR*^{mut} in PMF may affect prognostication and should be incorporated in the scoring system, as recently suggested.[25,26]

Our data regarding the frequencies of type 1 and type 2 *CALR*^{mut} in ET and in PMF were in good agreement with those of previous cohorts of patients with ET[8-10,12] and PMF.[8,13] The asymmetric distribution of different types of *CALR*^{mut} in ET and PMF, i.e. the relatively lower frequency of type 2 *CALR*^{mut} in PMF, was in line with data from Klampfl *et al.*[8] They also observed a lower (21/105, 20%) relative proportion of type 2 mutant cases among PMF patients than among those with ET (74/195, 38%). The profound differences in the CALR protein structure may explain this asymmetric distribution and may imply slight differences in the pathogenic effects of the respective variants. The observed asymmetric distribution prompted us to systematically analyze clinical and outcome differences in ET patients according to *CALR*^{mut} type, with the key findings being older age at diagnosis, higher platelet counts and more frequent use of cytoreductive therapy in patients with type 2 *CALR*^{mut} compared to type 1 or non-type 2 *CALR*^{mut}. To our knowledge, similar data in ET have not been published yet. In line with our data in ET, very recently Tefferi *et al.*[27] observed distinct characteristics in PMF in the context of *CALR* type 2 mutations and *EZH2*, *IDH* mutations, leukocytosis, and peripheral blast percentage. Univariate survival analysis suggested a less favorable outcome in the presence of type 2 *CALR* mutations.[27] These differences may indicate a distinct pathomechanism related to insertion or deletion type *CALR* mutations.

The systematic analyses of *CALR*^{mut} load in several MPN subgroups indicated higher *CALR*^{mut} load compared to *JAK2*^{mut} load in ET. In contrast to the *JAK2*^{mut} load, the *CALR*^{mut} load rarely exceeded 50%, indicating a decreased tendency of the *CALR*^{mut} locus to undergo mitotic recombination. Uniparental disomy was reported to be less frequent at the *CALR* locus (19p13.3-p13.2) than at the *JAK2* locus (9p24). Using an alternative quantitative PCR technique, we are in the process of addressing the observation that *CALR*^{mut} load may be higher at diagnosis in type 1 *CALR*^{mut} carriers than in type 2 carriers. Our results indicate that the mutational load of *CALR*^{mut} resembled that of *JAK2*^{mut} in that there was a clear trend towards increased load in parallel with the appearance of more severe phenotypes of MPN. Campbell *et al.*[28] suggested that *JAK2* V617F mutated ET, PV and post-ET/PV-myelofibrosis or PMF form a biological continuum, in which the different MPN phenotypes are determined by *JAK2* V617F allele burden modified by other environmental and inherited factors. The increasing *JAK2* V617F allele burden through ET-PV-myelofibrosis phenotypes was later confirmed in several studies,[29-31] and also in a transgenic mouse model.[32] Our study shows, for the first time, that hematologic laboratory parameters (white blood cell count, hemoglobin concentration, platelet count) were directly influenced by *CALR* mutational burden in ET patients with samples available within 1 year of diagnosis. Similarly to *JAK2*^{mut} MPN, *CALR*^{mut} ET and PMF also show overlapping clinical features, suggesting that the common molecular basis represents a biological continuum between *CALR*^{mut} ET and *CALR*^{mut} myelofibrosis, in which the clinical phenotype is modified by the actual *CALR* mutational load.[12]

In summary, analyzing a relatively large, independent cohort of MPN patients, we confirmed recent observations that the presence of *CALR* mutations is responsible for the development of a distinct clinical phenotype in both ET and PMF. These distinctive features manifest as different clinical characteristics at diagnosis, different patterns of complications as well as significant differences in overall survival. Our data supplement and extend previous

observations regarding the distribution of various types of *CALR*^{mut} and associations with different quantities of *CALR*^{mut}. Our study shows, for the first time, that hematologic laboratory parameters are directly influenced by *CALR* mutational burden. The recent discovery of somatic *CALR* mutations not only improves the precision of non-invasive diagnostics of patients with MPN, but may influence prognosis and therapy.

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