Regular Article

MYELOID NEOPLASIA

JAK2 or *CALR* mutation status defines subtypes of essential thrombocythemia with substantially different clinical course and outcomes

Elisa Rumi,[1,2] Daniela Pietra,[1] Virginia Ferretti,[1] Thorsten Klampfl,[3] Ashot S. Harutyunyan,[3] Jelena D. Milosevic,[3] Nicole C. C. Them,[3] Tiina Berg,[3] Chiara Elena,[1] Ilaria C. Casetti,[2] Chiara Milanesi,[1] Emanuela Sant'Antonio,[2] Marta Bellini,[2] Elena Fugazza,[1] Maria C. Renna,[2] Emanuela Boveri,[4] Cesare Astori,[1] Cristiana Pascutto,[1] Robert Kralovics,[3,5] and Mario Cazzola,[1,2] on behalf of the Associazione Italiana per la Ricerca sul Cancro Gruppo Italiano Malattie Mieloproliferative Investigators

[1]Department of Hematology Oncology, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico Policlinico San Matteo, Pavia, Italy; [2]Department of Molecular Medicine, University of Pavia, Pavia, Italy; [3]Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria; [4]Anatomic Pathology Section, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico Policlinico San Matteo, Pavia, Italy; and [5]Department of Internal Medicine I, Division of Hematology and Blood Coagulation, Medical University of Vienna, Vienna, Austria

---

Key Points

- *JAK2* (V617F)-mutated essential thrombocythemia and polycythemia vera are different phenotypes in the evolution of a single neoplasm.
- *CALR*-mutated essential thrombocythemia is a distinct disease entity not only at the molecular level, but also with respect to clinical outcomes.

Patients with essential thrombocythemia may carry *JAK2* (V617F), an *MPL* substitution, or a calreticulin gene (*CALR*) mutation. We studied biologic and clinical features of essential thrombocythemia according to *JAK2* or *CALR* mutation status and in relation to those of polycythemia vera. The mutant allele burden was lower in *JAK2*-mutated than in *CALR*-mutated essential thrombocythemia. Patients with *JAK2* (V617F) were older, had a higher hemoglobin level and white blood cell count, and lower platelet count and serum erythropoietin than those with *CALR* mutation. Hematologic parameters of patients with *JAK2*-mutated essential thrombocythemia or polycythemia vera were related to the mutant allele burden. While no polycythemic transformation was observed in *CALR*-mutated patients, the cumulative risk was 29% at 15 years in those with *JAK2*-mutated essential thrombocythemia. There was no significant difference in myelofibrotic transformation between the 2 subtypes of essential thrombocythemia. Patients with *JAK2*-mutated essential thrombocythemia and those with polycythemia vera had a similar risk of thrombosis, which was twice that of patients with the *CALR* mutation. These observations are consistent with the notion that *JAK2*-mutated essential thrombocythemia and polycythemia vera represent different phenotypes of a single myeloproliferative neoplasm, whereas *CALR*-mutated essential thrombocythemia is a distinct disease entity. (*Blood*. 2014;123(10):1544-1551)

Introduction

In the World Health Organization (WHO) classification of tumors of hematopoietic and lymphoid tissues, Philadelphia-negative myeloproliferative neoplasms (MPNs) include polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF).[1] These disorders have overlapping clinical features and a common molecular basis. In fact, three-quarters of these patients carry the unique *JAK2* (V617F) mutation,[2,3] which is present in about 95% of subjects with PV and in about 60% of those with ET or PMF.[4] Somatic mutations of *JAK2* exon 12 are found in the remaining 5% of patients with PV,[5] whereas mutations of *MPL* exon 10 are present in about 5% of those with ET or PMF.[6] We and others recently found that most patients with ET or PMF with nonmutated *JAK2* and *MPL* carry a somatic mutation of *CALR*, the gene encoding calreticulin.[7,8]

How a single mutation, *JAK2* (V617F), can be associated with different phenotypes has been partly clarified in the past few years.

Campbell et al[9] found that patients with *JAK2* (V617F)-mutated ET had multiple features resembling PV and concluded that *JAK2* (V617F)-mutated ET and PV form a biological continuum in which the degree of erythrocytosis is determined by physiological and genetic modifiers. The *JAK2* (V617F) mutant allele burden is significantly higher in patients with PV than in those with ET,[10] and evidence of *JAK2* (V617F) homozygosity, as a result of mitotic recombination of chromosome 9p, is mainly found in the former.[11,12] Tiedt et al[13] generated *JAK2* (V617F) transgenic mice and studied the relationship between ratio of mutant to wild-type *JAK2* and phenotypic manifestation: they clearly showed that lower ratios were associated with an ET phenotype and higher ratios with a PV phenotype.

The recent identification of somatic mutations in *CALR* that are mutually exclusive with *JAK2* and *MPL* mutations has provided a new powerful tool for studying MPNs. In the present work, we compared

---

There is an Inside *Blood* commentary on this article in this issue.

Table 1. Initial study population including 1235 consecutive patients diagnosed with ET or PV for which at least 1 DNA sample was available*

MPN	JAK2 (V617F) mutated (%)	JAK2 exon 12 mutated (%)	MPL exon 10 mutated (%)	CALR exon 9 mutated (%)	Nonmutated JAK2/MPL/CALR (%)	All genotypes
ET	466 (62)	—	28 (4)	176 (24)	75 (10)	745
PV	468 (96)	22 (4)	—	—	—	490
All patients						1235

*Population included patients followed at the Department of Hematology Oncology, Fondazione IRCCS Policlinico San Matteo and University of Pavia, Italy, between 1980 and 2012.

hematologic and clinical features of patients with *JAK2*-mutated ET with those of patients with *CALR*-mutated ET and related the findings to features observed in patients with PV.

Patients and methods

Study population and definitions

This study was approved by the institutional Ethics Committee (Comitato di Bioetica, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico [IRCCS] Policlinico San Matteo, Pavia, Italy). The procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 2000, and samples were obtained after patients had provided written informed consent.

We identified 1235 consecutive patients diagnosed with ET or PV at the Department of Hematology Oncology, Fondazione IRCCS Policlinico San Matteo, and University of Pavia, Italy, between 1980 and 2012, for which at least 1 DNA sample was available (Table 1). Diagnosis of PV or ET was performed in accordance with the criteria in use at the time of first observation, as previously described.[14] In 2002, we adopted the WHO criteria for diagnosis of MPN[15]; in 2009, we implemented the 2008 revision.[1] Post-PV or post-ET myelofibrosis was diagnosed according to the criteria of the International Working Group of Myelofibrosis Research and Treatment,[16] whereas evolution into acute myeloid leukemia was defined according to the WHO criteria.[1] Thrombotic events were defined as described in detail by the Cytoreductive Therapy in Polycythemia Vera Collaborative Group.[17]

For the assessment of bone marrow fibrosis, paraffin sections were stained with Gomori's silver impregnation technique, and fibrosis was assessed semi-quantitatively following the European consensus guidelines.[18]

In the evaluation of transformation of ET to PV, or polycythemic transformation of ET, we could not routinely use the WHO criteria for diagnosis of PV[1] because bone marrow biopsy was not regularly available during follow-up. We therefore adopted the criteria of the British Committee for Standards in Hematology for diagnosis of PV, described in the supplemental Methods on the *Blood* Web site.[19,20] Basically, according to these latter criteria, the combination of erythrocytosis and *JAK2* mutation allows diagnosis of PV.[20]

Because we excluded from outcome analysis patients with PV carrying a *JAK2* exon 12 mutation (22 subjects) and patients with ET carrying an *MPL* substitution (28 subjects), 1185 of the initial 1235 subjects were eventually studied as detailed in supplemental Table 1. Of these 1185 patients, 674 had been studied in targeted resequencing investigations of our original article on somatic mutations of calreticulin.[7] These 674 cases have been included in the present work with the purpose of analyzing features that were not examined in the original article, including clinical relevance of mutant allele burden, incidence of polycythemic or myelofibrotic transformation, and risk of leukemic evolution. To evaluate the clinical relevance of the mutant allele burden, we also examined an additional cohort of 83 patients with secondary myelofibrosis that was extracted from our database and is reported in supplemental Table 1.

JAK2, MPL, and CALR mutation analysis

Granulocyte *JAK2* (V617F) mutation status and mutant allele burden were assessed using a quantitative polymerase chain reaction–based allelic discrimination assay on a Rotor-Gene 6000 real-time analyzer (Qiagen), as previously described.[10,21]

Patients with PV without *JAK2* (V617F) were studied for *JAK2* exon 12 mutations as previously described.[22] Patients with ET and nonmutated *JAK2* were further evaluated for *MPL* exon 10 mutations using a high-resolution melt assay.[23] Patients with nonmutated *JAK2* and *MPL* were studied for *CALR* exon 9 mutations as previously reported[7] or by Sanger sequencing, as described in the supplemental Methods.

Statistical analysis

Numerical variables have been summarized by their median and range, and categorical variables by count and relative frequency (%) of each category. Comparisons of quantitative variables between groups of patients were carried out by the nonparametric Wilcoxon rank-sum test. The Wilcoxon signed-rank test was applied to compare measures of quantitative variables repeated in different phases of the disease. Association between categorical variables (2-way tables) was tested by the Fisher's exact test. Correlation between numerical variables was tested by the nonparametric Spearman's ρ (ρ) coefficient.

The incidence of polycythemic transformation of ET was estimated with a Poisson model, and comparison of incidence rates was carried out via the likelihood ratio test. The cumulative incidence of polycythemic, myelofibrotic, and leukemic transformation, and that of thrombotic events was estimated with a competing risk approach, considering death for any cause as a competing event.[24] The comparison of cumulative incidence curves in different groups of patients was carried out using the Pepe-Mori test,[25] whereas the effect of quantitative covariates (eg, *JAK2* allele burden at diagnosis) was estimated by applying the Fine-Gray regression model.[26] Overall survival (OS) was estimated using the Kaplan-Meier product limit method, and survival curves were compared by the log-rank test. Multivariate analysis of OS was carried out by Cox regression.

All *P* values were considered statistically significant when <.05 (2-tailed). Statistical analyses were performed using Stata 12.1 (StataCorp LP, College Station, TX) software.

Results

Presenting hematologic and clinical features of JAK2 (V617F)-mutated ET, JAK2 (V617F)-mutated PV, and CALR-mutated ET

We studied 468 patients with PV and 717 with ET. As reported in Table 1, this latter group included 466 patients with *JAK2* mutation, 176 with *CALR* mutation, and 75 with nonmutated *JAK2*, *MPL*, and *CALR*. Overall, 23 types of *CALR* mutation were detected (supplemental Table 2): the 52-bp deletion (mutation type 1) and the 5-bp insertion (mutation type 2) were the most frequent alterations, representing 46% and 38% of all cases, respectively. We found 4 novel deletions and named them type 37, 38, 39, and 40 (supplemental Table 2) in accordance with our previously reported nomenclature.[7] Consistent with the thus far described *CALR* mutations,[7] all the novel deletions caused a change from a negatively to a positively charged C-terminal peptide and loss of the endoplasmic reticulum retention motif KDEL at the C-terminal end.

Table 2. Demographic, hematologic, and clinical features at diagnosis of patients with ET, subdivided according to *JAK2* or *CALR* mutation status, and of patients with PV

| | ET | | PV (C) | P | | |
| | *CALR* mutated (A) | *JAK2* mutated (B) | | (A) vs (B) | (B) vs (C) | (A) vs (C) |
No.	176	466	468			
Sex (male/female)	90/86 (51%/49%)	167/299 (36%/64%)	233/235 (50%/50%)	.001	<.001	.791
Age at onset, years, median (range)	45 (15-83)	50 (15-92)	57 (13-86)	.001	<.001	<.001
Hemoglobin, g/dL, median (range)	13.8 (11.3-17.6)	14.4 (10-17.7)	18.2 (15.0-24.0)	<.001	<.001	<.001
WBC count, ×10⁹/L, median (range)	8.0 (4.0-17.9)	9.0 (4.0-28.0)	10.0 (3.4-55.3)	<.001	<.001	<.001
PLT count, ×10⁹/L, median (range)	883 (500-3000)	700 (456-2148)	464 (109-1472)	<.001	<.001	<.001
Serum erythropoietin, mU/mL, median (range)	9.4 (1.2-27)	4.7 (0-47)	2.7 (0-66)	<.001	<.001	<.001
Splenomegaly, no. (%)	4 (2.3%)	30 (6.4%)	105 (22.4%)	.046	<.001	<.001
Lactate dehydrogenase, mU/mL, median (range)	199 (78-472)	200 (77-540)	217 (104-758)	.83	<.001	.003
Circulating CD34⁺ cells, ×10⁶/L, median (range)	4.1 (0.6-18)	4 (0-15.3)	3.4 (0-261.3)	.50	.037	.039
Thrombosis at diagnosis, no. (%)	5 (2.8%)	33 (7.1%)	49 (10.5%)	.059	.082	.001

Table 2 reports the demographic and clinical characteristics at diagnosis of the patients studied according to their genotype, whereas the main hematologic parameters are summarized in Figure 1. Patients with *CALR*-mutated ET were significantly younger than those with *JAK2*-mutated ET (P =. 001) or PV (P < .001). Compared with patients with *CALR*-mutated ET, those with *JAK2*-mutated ET had higher hemoglobin (Hb) level and white blood cell (WBC) count, and lower platelet (PLT) count and serum erythropoietin (Epo) level (P < .001 in all comparisons). Patients with PV had higher values for Hb level and WBC count, lower values for PLT count and serum Epo, and higher frequency of splenomegaly compared with both *CALR*- and *JAK2*-mutated ET patients (P values shown in Table 2). The incidence of thrombosis at diagnosis was significantly higher in patients with PV than in those with *CALR*-mutated ET (P = .001), but not different between patients with PV and those with *JAK2*-mutated ET (P = .08).
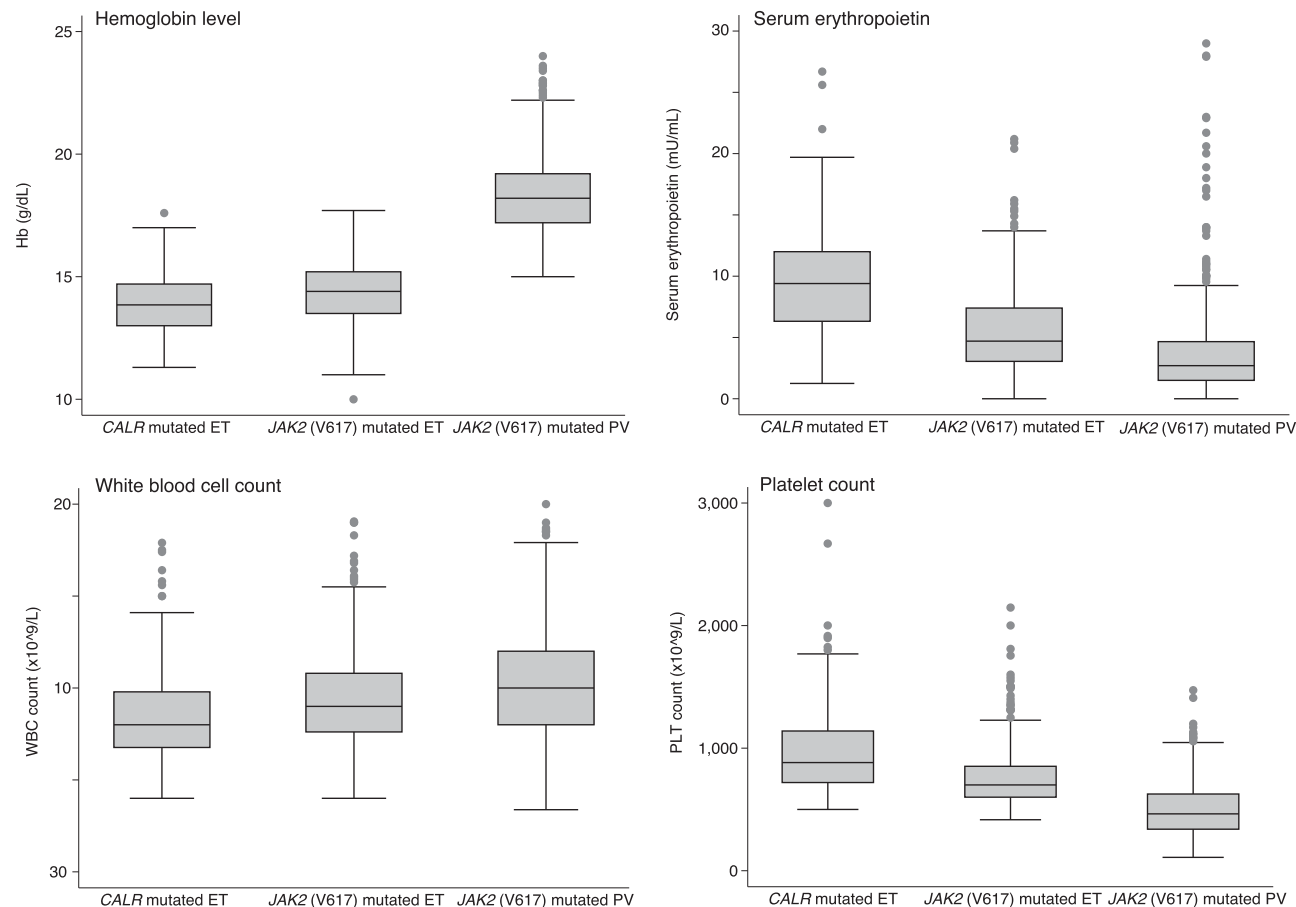
Figure 1. Hematologic parameters in patients with *CALR*-mutated ET, *JAK2* (V617F)-mutated ET, and *JAK2* (V617F)-mutated PV. Data are shown in a box plot depicting the upper and lower adjacent values (highest and lowest horizontal line, respectively), upper and lower quartile with median value (box), and outside values (dots). Of note, patients with *JAK2* (V617F)-mutated ET had markedly lower serum Epo values than those of patients with *CALR* exon 9–mutated ET, despite the fact that the difference in median Hb levels was <1 g/dL.
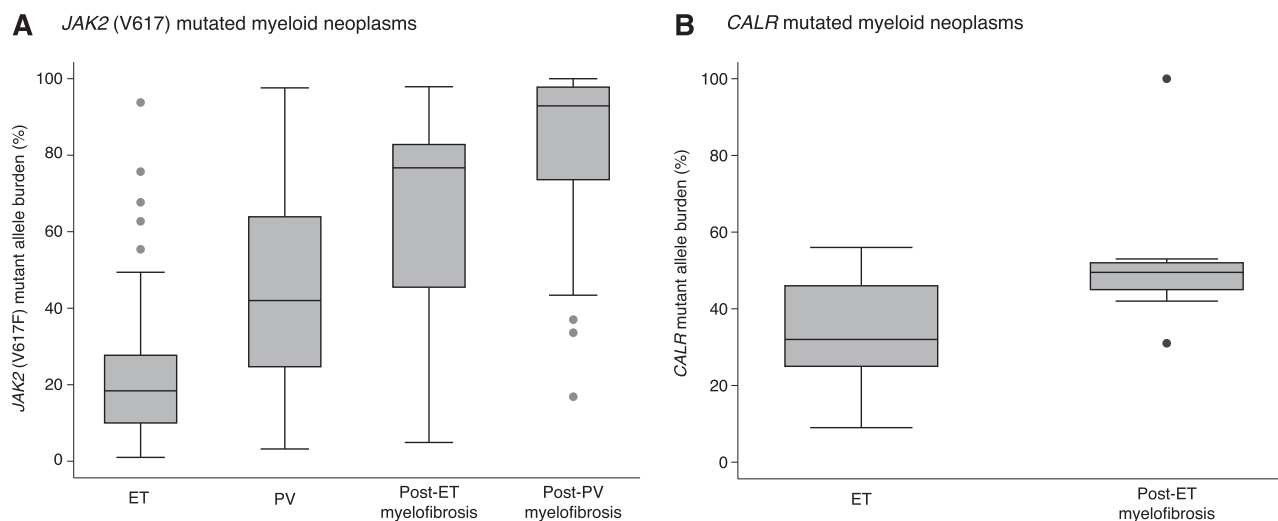
Figure 2. Granulocyte mutant allele burden in *JAK2* (V617F)-mutated and in *CALR*-mutated myeloid neoplasms. Data are shown in a box plot depicting the upper and lower adjacent values (highest and lowest horizontal line, respectively), upper and lower quartile with median value (box), and outside values (dots). (A) This analysis includes 250 patients with ET and 212 patients with PV at presentation, and 18 patients with post-ET myelofibrosis and 55 with post -PV myelofibrosis. In these *JAK2* (V617F)-mutated myeloid neoplasms, progression from the primary disease to secondary myelofibrosis appears to be related to the mutant allele burden. In particular, the proportion of patients with values >50% increases progressively, indicating an increasingly higher proportion of cells that are homozygous for the mutation as a result of copy neutral loss of heterozygosity of chromosome 9p. Most patients with post-ET or post-PV myelofibrosis have values for granulocyte *JAK2* (V617F)-mutant allele burden greater than 75%, consistent with a dominant population of homozygous cells. (B) This analysis includes 38 patients with ET at presentation and 10 patients with post-ET myelofibrosis. Also within these *CALR*-mutated myeloid neoplasms, progression to secondary myelofibrosis appears to be associated with a significant increase in the mutant allele burden. However, only 1 patient with post-ET myelofibrosis had a value consistent with a dominant population of homozygous cells. This might suggest that the higher mutant allele burden in patients with post-ET myelofibrosis most often reflects the progressive expansion of a heterozygous clone that eventually achieves full dominance in the bone marrow.

Granulocyte mutant allele burden in patients with *JAK2* (V617F)-mutated MPNs

The median *JAK2* (V617F) mutant allele burden at presentation was significantly lower in *JAK2*-mutated ET than in PV (18% vs 42%, $P < .001$), as shown in Figure 2A. Patients with myelofibrosis secondary to ET or PV had significantly higher values for *JAK2* (V617F) mutant allele burden than those with the primary MPN (18% vs 77% in ET and post-ET myelofibrosis, respectively, $P < .001$; 42% vs 93% in PV and post-PV myelofibrosis, respectively, $P < .001$) (Figure 2A). A mutant allele burden greater than 50% was observed in 2% of patients with *JAK2*-mutated ET, 41% of those with PV, 72% of those with post-ET myelofibrosis, and 93% of those with post-PV myelofibrosis (Fisher exact test, $P < .001$ for ET vs PV, ET vs post-ET myelofibrosis, and PV vs post-PV myelofibrosis).

Grouping together patients with ET carrying *JAK2* (V617F) and those with PV, significant associations were found between mutant allele burden and hematologic parameters (Hb level, hematocrit, PLT count, and serum Epo) as illustrated in Figure 3. These observations are consistent with the notion that *JAK2*-mutated ET and PV form a biological continuum in which the mutant allele burden acts as a determinant of phenotypic expression.

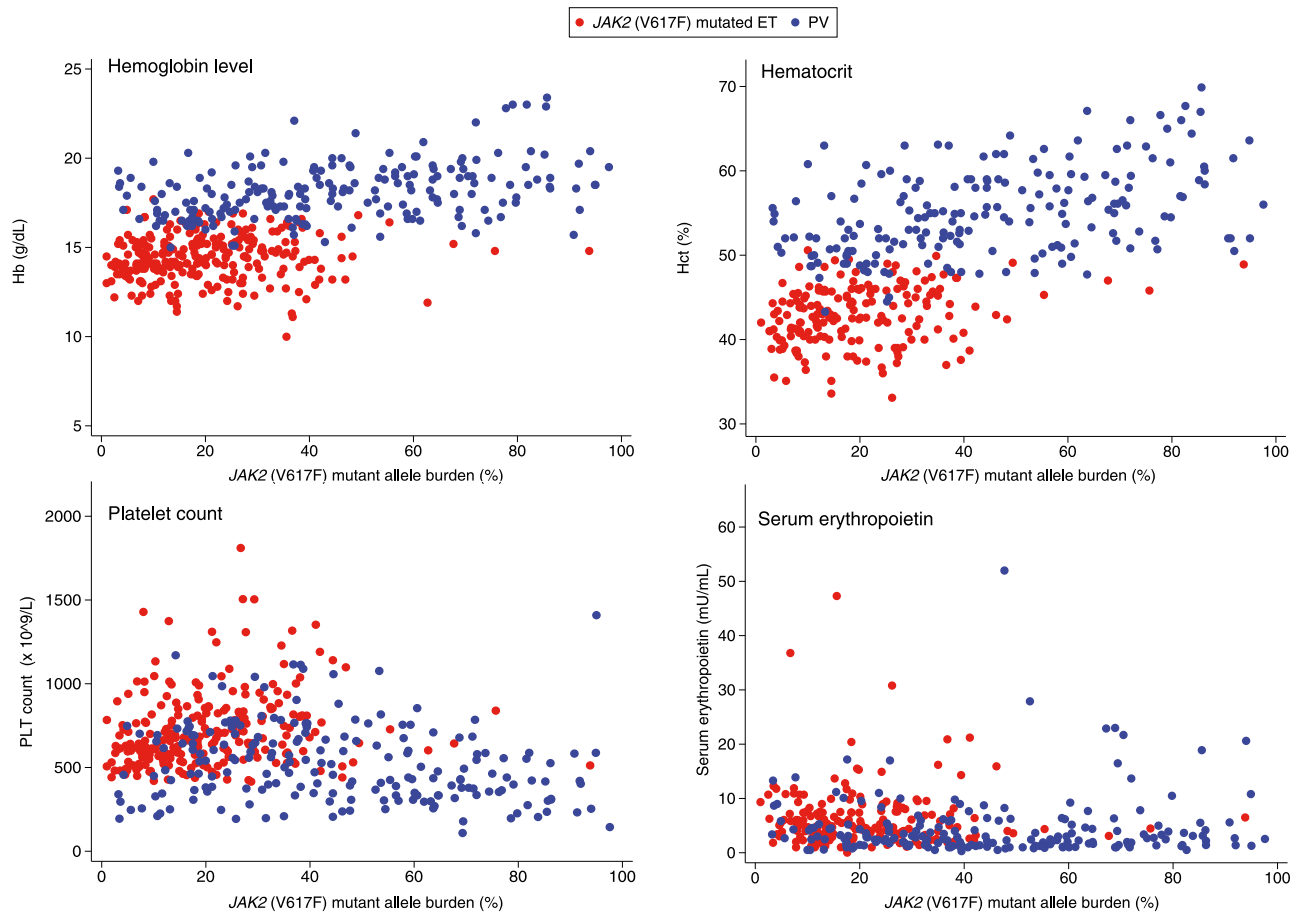## Granulocyte mutant allele burden in patients with *CALR*-mutated MPNs

We assessed the mutant allele burden in 110 of 148 patients with ET who carried a type 1 or type 2 *CALR* mutation (supplemental Table 2) and in 10 patients with post-ET myelofibrosis carrying the same alterations. The median mutant allele burden was 42% in patients with ET vs 50% in those with post-ET myelofibrosis ($P = .018$). We then restricted the analysis to patients with *CALR*-mutated ET evaluated at diagnosis. As shown in Figure 2B, the median mutant allele burden was 32% in patients with ET at clinical onset vs 50% in those with post-ET myelofibrosis ($P = .002$).

To have a relative estimation of the mutant clones, we compared the mutant allele burden at diagnosis in the 2 subtypes of ET. The median mutant allele burden of *CALR*-mutated patients was significantly higher than that of *JAK2*-mutated patients (32% vs 18%, $P < .001$).

Polycythemic transformation of ET and evidence of a prepolycythemic phase mimicking ET in *JAK2* (V617F)-mutated PV

During follow-up, based on the diagnostic criteria of the British Committee for Standards in Hematology,[19,20] polycythemic transformation occurred in 53 patients with *JAK2*-mutated ET (incidence 19 cases per 1000 person-years, 95% confidence interval [CI] 14-25) vs none of the 176 patients with *CALR*-mutated ET (incidence 0 per 1000 person-years, 95% CI 0-3). The median time to progression was 54 (range 4-220) months, and the cumulative incidence is reported in Figure 4A. Progression to PV was significantly associated with higher *JAK2* mutant allele burden at diagnosis (Cox regression, hazard ratio [HR] = 1.04, 95% CI 1.02-1.07, $P = .001$): the median mutant allele burden was 25.5% (range 8.2-75.7) in patients who developed PV vs 17.9% (range 1-93.8) in those who did not ($P = .019$).

To test the hypothesis that PV patients might have a silent prepolycythemic phase mimicking ET, we did an ad hoc search for any complete blood cell (CBC) count that had been performed before diagnosis of PV. Overall, 169 patients had a previous CBC performed at a median time of 22 months (range 1-305) before diagnosis of PV. Although previous values were normal in 27 of these 169 patients (16%), in the remaining subjects there was evidence of erythrocytosis and/or leukocytosis and/or thrombocytosis. In particular, 38 patients had evidence of isolated erythrocytosis and 35 had evidence of isolated thrombocytosis. The median time to PV onset was significantly shorter in patients showing at least 1 abnormality in CBC count than in those with normal CBC count (19 vs 48 months, $P = .004$).

Figure 3. Relationship between granulocyte *JAK2* (V617F)-mutant allele burden and hematologic parameters in patients with ET or PV. The mutant allele burden was directly correlated with Hb level (ρ = 0.53, P < .001) and hematocrit (ρ = 0.61, P < .001), and inversely correlated with PLT count (ρ = −0.18, P < .001) and serum Epo level (ρ = −0.23, P < .001). These correlations suggest that the mutant allele burden is a determinant of the phenotypic features of *JAK2* (V617F)-mutated MPN.

Clinical course of *JAK2* (V617F)-mutated ET, *JAK2* (V617F)-mutated PV, and *CALR*-mutated ET

The median follow-up of the study population was 5.2 years (range 0-32 years).

No difference in OS was observed between patients with *JAK2* (V617F)-mutated ET and those with PV (P = .359). Patients with ET carrying a *CALR* mutation had a better OS than PV patients (95.3% vs 82.5% at 15 years, P = .010), and showed a trend toward a better OS than ET patients carrying *JAK2* (V617F) (95.3% vs 90.4% at 15 years, P = .085). However, after adjusting for age, no significant difference between these subgroups was found (P > .05 in both cases).

The incidence of thrombotic events was 30 (95% CI 23-37) cases per 1000 person-years in patients with PV, 25 (95% CI 19-32) in those with *JAK2*-mutated ET, and 10 (95% CI 5-17) in those with *CALR*-mutated ET. The cumulative incidence in these subgroups is reported in Figure 4B. Patients with a *CALR* mutation had a lower risk of thrombosis than either patients with *JAK2*-mutated ET (P = .001) or those with PV (P < .001), whereas no significant difference was found between *JAK2*-mutated ET and PV (P = .314). These differences in thrombotic risk remained statistically significant after adjusting for age. In fact, when comparing patients with *JAK2*-mutated ET and those with *CALR*-mutated ET, the subdistribution HR was 2.23 (95% CI 1.20-4.12, P = .011). Similarly, when comparing patients with PV and those with *CALR*-mutated ET, the subdistribution HR was 2.34 (95% CI 1.25-4.39, P = .008). Of note, there was no significant difference in the use of cytoreductive therapy between the 2

subtypes of ET. We also evaluated the cumulative incidence of major bleeding using a competitive risk approach and did not find any difference between the subgroups.
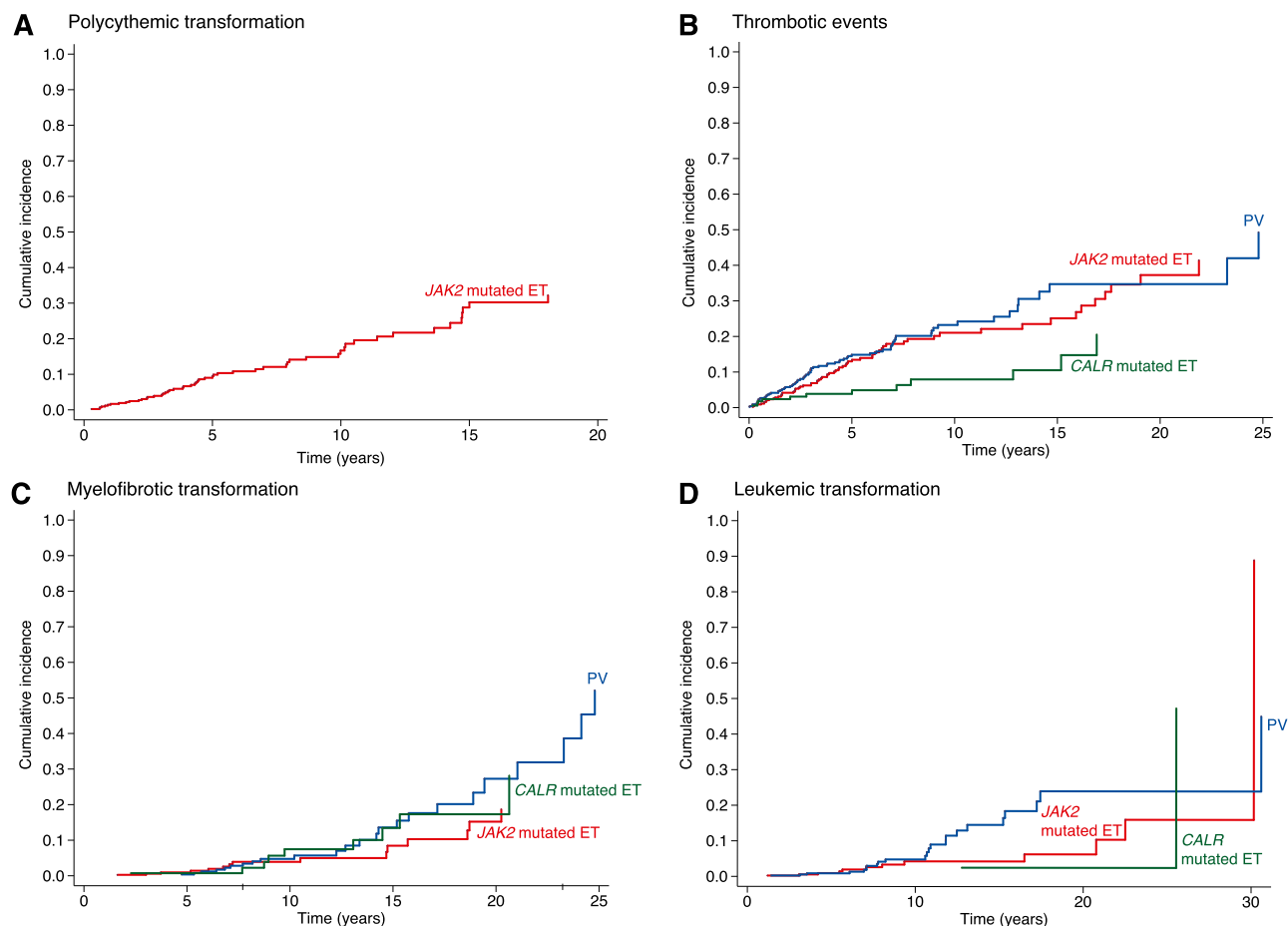
The incidence of myelofibrotic transformation was 8 (95% CI 5-12) cases per 1000 person-years in patients with PV, 5 (95% CI 3-8) in those with *JAK2*-mutated ET, and 7 (95% CI 3-13) in those with *CALR*-mutated ET. The cumulative incidence in these subgroups is reported in Figure 4C; no significant difference in the risk of myelofibrotic transformation was observed.

Leukemic transformation was observed in 23 patients with PV, 12 with *JAK2*-mutated ET, and 2 with *CALR*-mutated ET. All but 1 patient received cytoreductive therapy. The estimated incidence was 7 (95% CI 5-11) cases per 1000 person-years in patients with PV, 4 (95% CI 2-7) in those with *JAK2*-mutated ET, and 2 (95% CI 0-6) in those with *CALR*-mutated ET. The cumulative incidence in these subgroups is reported in Figure 4D; no significant difference was observed after adjusting for age.

Risk classification, IPSET, and *JAK2* or *CALR* mutation status

Age older than 60 years and previous thrombosis are major predictors of vascular complications in ET and PV.[27] We evaluated the distribution of risk groups (high risk: age ≥60 years and/or previous thrombosis; low risk: age <60 years and no previous thrombosis) in patients with ET or PV. The distribution of low and high risk was statistically different (P < .001) among the 4 groups analyzed: the percentage of high-risk patients was 51.3% in PV, 41.6% in

Figure 4. Cumulative incidence of polycythemic transformation, thrombotic events, myelofibrotic transformation, and leukemic transformation in patients with *CALR*- or *JAK2*-mutated ET and in those with PV. The cumulative incidences were estimated with a competing risk approach, considering death for all causes as a competing event.[24] (A) In patients with *JAK2*-mutated ET, the cumulative incidence of polycythemic transformation was 28.6% (95% CI 20.7-37.0) at 15 years. No progression to PV was observed in patients with *CALR*-mutated ET. (B) Patients with *CALR*-mutated ET showed a lower incidence of thrombosis than those with *JAK2*-mutated ET (10.5% vs 25.1% at 15 years, P = .001), or those with PV (10.5% vs 34.7% at 15 years, P < .001). By contrast, patients with *JAK2*-mutated ET and those with PV did not differ in terms of cumulative incidence of thrombosis (P = .314). These differences in risk of thrombosis remained statistically significant even after adjusting for age, as detailed in the text. (C) The 15-year cumulative incidence of myelofibrotic transformation was 13.4% (CI 95% 5.4-25.2) in *CALR*-mutated ET, 8.4% (CI 95% 3.9-15.3) in *JAK2*-mutated ET, and 13.6% (CI 95% 7.3-21.9) in PV, without any significant difference among these 3 subgroups even after adjusting for age. (D) The 15-year cumulative incidence of leukemic transformation was 2.5% (CI 95% 0.2-11.3) in *CALR*-mutated ET, 4.3% (CI 95% 1.9-8.2) in *JAK2*-mutated ET, and 14.6% (CI 95% 8.4-22.3) in PV. Although *CALR*-mutated patients showed a lower risk of leukemic transformation in comparison with both those with *JAK2*-mutated ET (P = .026) and those with PV (P < .001), no significant difference was observed after adjusting for age.

JAK2-mutated ET, 23.3% in *CALR*-mutated ET, and 24% in ET with nonmutated *JAK2*, *CALR*, and *MPL*. When performing a comparison between 2 groups, patients with *CALR*-mutated ET belonged more frequently to the low-risk group than those with *JAK2*-mutated ET (P < .001), and patients with *JAK2*-mutated ET belonged more frequently to the low-risk group than those with PV (P = .003).

The International Prognostic Score for Essential Thrombocythemia (IPSET) model accounts for age (<60 vs ≥60 years), WBC count (<11 vs ≥11 × 10^9/L), and prior thrombosis (no vs yes), and stratifies ET patients into 3 risk categories (low, intermediate, and high) with significantly different survival.[28] The distribution of IPSET categories was statistically different (P < .001) among the 3 groups analyzed: the percentage of high-risk patients was 13.5% in *JAK2*-mutated ET, 3.4% in *CALR*-mutated ET, and 4.1% in ET with nonmutated *JAK2*, *CALR*, and *MPL*. When performing a comparison between 2 groups, both patients with *CALR* mutations and those with nonmutated *JAK2*, *CALR*, and *MPL* belonged more frequently to a lower risk group than those with *JAK*2 mutation (P < .001 and P = .003, respectively).

In a multivariate analysis corrected for IPSET score, we observed no effect of *JAK2* vs *CALR* mutation status on OS of patients with ET

(P = .524). By contrast, using a similar analysis, we found that the *JAK2* mutation maintained its impact on the risk of thrombosis: patients with *JAK2*-mutated ET had higher risk of thrombosis than those with *CALR*-mutated ET (HR 2.12, CI 95% 1.14-3.92, P = .017). This observation confirms the validity of the IPSET-thrombosis model, which includes *JAK2* (V617F) (present vs absent) as a major predictor of the risk of thrombosis in ET.[29]

Discussion

ET is an MPN that involves primarily the megakaryocytic lineage and is mainly characterized by excessive PLT production.[1] The WHO classification has defined as a diagnostic criterion a sustained PLT count ≥450 × 10^9/L, which may be reasonable from a clinical point of view but is likely arbitrary with respect to pathophysiology. In fact, in the initial development of ET, which may be long-lasting, PLT count may be higher with respect to baseline but still in the normal range. ET now has at least 3 biomarkers, ie, *JAK2* (V617F), *MPL* (W515) substitutions, and *CALR* exon 9 mutations. Facing a patient

with an elevated PLT count, the assessment of these biomarkers allows a correct diagnosis in about 90% of cases of ET. Only ~10% of patients with a suspected ET do not carry any of the 3 biomarkers: these individuals may have as-yet-unknown mutant genes or even a non-clonal thrombocytosis.

The findings of this study indicate that the 3 biomarkers have considerable clinical relevance. In particular, while JAK2 (V617F)-mutated ET and PV are different expressions or phases of a single MPN, CALR-mutated ET is a distinct disease entity not only at the molecular level but also at the clinical level.

The concept that JAK2 (V617F)-mutated ET and PV are different expressions of a genotypic/phenotypic continuum, suggested by Campbell et al[9] soon after the identification of the JAK2 mutation, is now supported by a large body of molecular evidence, recently analyzed in a comprehensive review.[30] As illustrated in Figure 3, the hematologic parameters of patients with JAK2 (V617F)-mutated ET or PV form a continuum in which the mutant allele burden behaves as a determinant of phenotypic expression. More importantly, in this study, we found that a significant proportion of patients with JAK2-mutated ET, but none of those with a CALR mutation, progressed to PV during their clinical course. In addition, we were able to show that patients with PV may have an antecedent phase characterized by isolated thrombocytosis, ie, the "thrombocythemic" phase in the natural history of JAK2 (V617F)-mutated MPNs. The final phase of these disorders is clearly secondary myelofibrosis, which occurs in a non-negligible portion of patients.[31] Thus, ET, PV, and myelofibrosis most likely represent different phenotypes in the evolution of JAK2 (V617F)-mutated MPNs.

JAK2 (V617F)-mutated ET and PV have similar clinical outcomes and comparable excess mortality. We previously reported that life expectancy of patients with PV was reduced compared with the general population, whereas that of patients with ET appeared not to be significantly affected by the disease.[14] In a study with a longer follow-up, Wolanskyi et al[32] found that life expectancy of patients with ET was worse than that of the control population, and that ET becomes a more aggressive disease beyond the first decade of diagnosis.[32] In a population-based study, Hultcrantz et al[33] recently confirmed that patients with any MPN subtype have a significantly reduced life expectancy compared with the general population.

Part of the variability in clinical outcomes of patients with ET is likely related to the founding or initiating mutation: JAK2 (V617F), an MPL (W515) substitution, or a CALR exon 9 mutation. In a previous study,[6] we found only minor phenotypic differences between ET patients carrying an MPL (W515) substitution and those carrying JAK2 (V617F). We rather found that acquired copy-neutral loss of heterozygosity of chromosome 1p, involving transition from heterozygosity to homozygosity for the MPL mutation, is a molecular mechanism associated with myelofibrotic transformation. This mechanism is similar to that associated with disease progression in JAK2 (V617F)-mutated MPNs. In fact, acquired copy-neutral loss of heterozygosity of chromosome 9p is responsible for the transition from heterozygosity to homozygosity for the JAK2 (V617F) mutation and in turn for higher JAK2 (V617F) mutant allele burden.[3] The findings of the present study are consistent with previous observations indicating that this transition may be responsible for progression from JAK2 (V617F)-mutated ET to PV and from PV to secondary myelofibrosis. Physiological factors,[9] type of hematopoietic cell involved,[34] and cooperative genetic events[35] may clearly play a role in these processes, but the mutant allele burden represents a major factor, as illustrated by findings reported in Figure 2A.

CALR-mutated ET appears to be substantially different from JAK2 (V617F) ET in terms of biologic, hematologic, and clinical features.

Calreticulin has multiple functions including protein quality control and calcium signaling.[36] The protein resides in the lumen of the endoplasmic reticulum, and its C-terminal domain is responsible for calcium buffering activity, which controls calcium homeostasis. The mechanism by which a mutant calreticulin, characterized by lower calcium-binding activity and devoid of the endoplasmic reticulum retention motif KDEL, determines abnormal proliferation of megakaryocytes and overproduction of PLTs is largely unclear at present. Nonetheless, we found that Janus kinase–signal transducer and activator of transcription signaling is involved in the cytokine-independent growth of mutant CALR-expressing Ba/F3 cells.[7]

Within the patients with CALR-mutated ET studied so far, only a few individuals had mutant allele burdens >75% (ie, a value that unequivocally indicates the existence of at least a subpopulation of cells homozygous for the mutation).[7] In particular, only 1 patient with post-ET myelofibrosis had a value consistent with a fully dominant population of cells homozygous for the mutation (Figure 2B). By contrast, most patients with JAK2-mutated secondary myelofibrosis had very high mutant allele burdens (Figure 2A). Based on values reported in Figure 2A-B, patients with CALR-mutated ET apparently had bigger mutant clones that those with JAK2-mutated ET. In the former patients, the natural history of disease appears to be mainly characterized by the progressive expansion of a heterozygous clone that becomes dominant in the bone marrow and specifically activates megakaryocytes.

At the phenotypic level, CALR-mutated ET was associated with PLT counts greater than 1000×10^9/L in a substantial portion of patients. Despite this, the risk of thrombosis was significantly lower in patients with CALR-mutated ET than in those with JAK2-mutated ET (Figure 4B), even after adjustment for age. These observations are in close agreement with the findings of a recent study in 891 patients with WHO-defined ET,[37] which clearly showed that JAK2 (V617F) represents a strong independent risk factor for thrombosis, whereas a PLT count greater than 1000×10^9/L is associated with a lower thrombotic risk. Absence of JAK2 (V617F) and markedly elevated PLT counts are the main features of CALR-mutated ET. These observations raise questions about the role of PLT count per se in the pathogenesis of vascular complications in ET patients. They reinforce the opinion that granulocyte/PLT activation[38] and increased WBC count[28] may be more relevant to the pathogenesis of thrombotic complications than the PLT count per se.

In conclusion, CALR-mutated ET is an MPN that affects relatively young individuals and is characterized by markedly elevated PLT count but relatively low thrombotic risk.

Acknowledgments

Authorship

Contribution: M.C., E.R., and D.P. conceived this study, collected and analyzed data, and wrote the manuscript; C.E., I.C.C., E.S., C.A., and M.B. collected clinical data; T.K., A.S.H., J.D.M., N.C.C.T., C.M., M.C.R., T.B., and R.K. did molecular investigations; V.F., E.F., and C.P. did statistical analyses; and E.B. studied bone marrow biopsies.

Correspondence: Mario Cazzola, Department of Hematology Oncology, Fondazione IRCCS Policlinico San Matteo, Viale Golgi 19, 27100 Pavia, Italy; e-mail: mario.cazzola@unipv.it.

References

1. Swerdlow SH, Campo E, Harris NL, et al. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. Lyon: IARC; 2008.

2. James C, Ugo V, Le Couédic JP, et al. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. Nature. 2005;434(7037):1144-1148.

3. Kralovics R, Passamonti F, Buser AS, et al. A gain-of-function mutation of JAK2 in myeloproliferative disorders. N Engl J Med. 2005; 352(17):1779-1790.

4. Cross NC. Genetic and epigenetic complexity in myeloproliferative neoplasms. Hematology Am Soc Hematol Educ Program. 2011;2011:208-214.

5. Passamonti F, Elena C, Schnittger S, et al. Molecular and clinical features of the myeloproliferative neoplasm associated with JAK2 exon 12 mutations. Blood. 2011;117(10):2813-2816.

6. Rumi E, Pietra D, Guglielmelli P, et al; Associazione Italiana per la Ricerca sul Cancro Gruppo Italiano Malattie Mieloproliferative. Acquired copy-neutral loss of heterozygosity of chromosome 1p as a molecular event associated with marrow fibrosis in MPL-mutated myeloproliferative neoplasms. Blood. 2013; 121(21):4388-4395.

7. Klampfl T, Gisslinger H, Harutyunyan AS, et al. Somatic mutations of calreticulin in myeloproliferative neoplasms. N Engl J Med. 2013;369(25):2379-2390.

8. Nangalia J, Massie CE, Baxter EJ, et al. Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. N Engl J Med. 2013; 369(25):2391-2405.

9. Campbell PJ, Scott LM, Buck G, et al; United Kingdom Myeloproliferative Disorders Study Group; Medical Research Council Adult Leukaemia Working Party; Australasian Leukaemia and Lymphoma Group. Definition of subtypes of essential thrombocythaemia and relation to polycythaemia vera based on JAK2 V617F mutation status: a prospective study. Lancet. 2005;366(9501):1945-1953.

10. Passamonti F, Rumi E, Pietra D, et al. Relation between JAK2 (V617F) mutation status, granulocyte activation, and constitutive mobilization of CD34+ cells into peripheral blood in myeloproliferative disorders. Blood. 2006; 107(9):3676-3682.

11. Scott LM, Scott MA, Campbell PJ, Green AR. Progenitors homozygous for the V617F mutation occur in most patients with polycythemia vera, but not essential thrombocythemia. Blood. 2006; 108(7):2435-2437.

12. Godfrey AL, Chen E, Pagano F, et al. JAK2V617F homozygosity arises commonly and recurrently in PV and ET, but PV is characterized by expansion of a dominant homozygous subclone. Blood. 2012;120(13):2704-2707.

13. Tiedt R, Hao-Shen H, Sobas MA, et al. Ratio of mutant JAK2-V617F to wild-type Jak2 determines the MPD phenotypes in transgenic mice. Blood. 2008;111(8):3931-3940.

14. Passamonti F, Rumi E, Pungolino E, et al. Life expectancy and prognostic factors for survival in patients with polycythemia vera and essential thrombocythemia. Am J Med. 2004;117(10): 755-761.

15. Vardiman JW, Harris NL, Brunning RD. The World Health Organization (WHO) classification of the myeloid neoplasms. Blood. 2002;100(7): 2292-2302.

16. Barosi G, Mesa RA, Thiele J, et al; International Working Group for Myelofibrosis Research and Treatment (IWG-MRT). Proposed criteria for the diagnosis of post-polycythemia vera and post-essential thrombocythemia myelofibrosis: a consensus statement from the International Working Group for Myelofibrosis Research and Treatment. Leukemia. 2008;22(2):437-438.

17. Marchioli R, Finazzi G, Specchia G, et al; CYTO-PV Collaborative Group. Cardiovascular events and intensity of treatment in polycythemia vera. N Engl J Med. 2013;368(1):22-33.

18. Thiele J, Kvasnicka HM, Facchetti F, Franco V, van der Walt J, Orazi A. European consensus on grading bone marrow fibrosis and assessment of cellularity. Haematologica. 2005;90(8): 1128-1132.

19. McMullin MF, Bareford D, Campbell P, et al; General Haematology Task Force of the British Committee for Standards in Haematology. Guidelines for the diagnosis, investigation and management of polycythaemia/erythrocytosis. Br J Haematol. 2005;130(2):174-195.

20. McMullin MF, Reilly JT, Campbell P, et al; National Cancer Research Institute, Myeloproliferative Disorder Subgroup; British Committee for Standards in Haematology. Amendment to the guideline for diagnosis and investigation of polycythaemia/erythrocytosis. Br J Haematol. 2007;138(6):821-822.

21. Malcovati L, Della Porta MG, Pietra D, et al. Molecular and clinical features of refractory anemia with ringed sideroblasts associated with marked thrombocytosis. Blood. 2009;114(17): 3538-3545.

22. Pietra D, Li S, Brisci A, et al. Somatic mutations of JAK2 exon 12 in patients with JAK2 (V617F)-negative myeloproliferative disorders. Blood. 2008;111(3):1686-1689.

23. Pietra D, Brisci A, Rumi E, et al. Deep sequencing reveals double mutations in cis of MPL exon 10 in myeloproliferative neoplasms. Haematologica. 2011;96(4):607-611.

24. Prentice RL, Kalbfleisch JD, Peterson AV Jr, Flournoy N, Farewell VT, Breslow NE. The analysis of failure times in the presence of competing risks. Biometrics. 1978;34(4):541-554.

25. Pepe MS, Mori M. Kaplan-Meier, marginal or conditional probability curves in summarizing competing risks failure time data? Stat Med. 1993; 12(8):737-751.

26. Fine JP, Gray RJ. A proportional hazards model for the subdistribution of a competing risk. J Am Stat Assoc. 1999;94(446):496-509.

27. Barbui T, Barosi G, Birgegard G, et al; European LeukemiaNet. Philadelphia-negative classical myeloproliferative neoplasms: critical concepts and management recommendations from European LeukemiaNet. J Clin Oncol. 2011;29(6): 761-770.

28. Passamonti F, Thiele J, Girodon F, et al. A prognostic model to predict survival in 867 World Health Organization-defined essential thrombocythemia at diagnosis: a study by the International Working Group on Myelofibrosis Research and Treatment. Blood. 2012;120(6): 1197-1201.

29. Barbui T, Finazzi G, Carobbio A, et al. Development and validation of an International Prognostic Score of thrombosis in World Health Organization-essential thrombocythemia (IPSET-thrombosis). Blood. 2012;120(26): 5128-5133; CME questions 5252.

30. Vainchenker W, Constantinescu SN. JAK/STAT signaling in hematological malignancies. Oncogene. 2013;32(21):2601-2613.

31. Passamonti F, Rumi E, Pietra D, et al. A prospective study of 338 patients with polycythemia vera: the impact of JAK2 (V617F) allele burden and leukocytosis on fibrotic or leukemic disease transformation and vascular complications. Leukemia. 2010;24(9):1574-1579.

32. Wolanskyj AP, Schwager SM, McClure RF, Larson DR, Tefferi A. Essential thrombocythemia beyond the first decade: life expectancy, long-term complication rates, and prognostic factors. Mayo Clin Proc. 2006;81(2):159-166.

33. Hultcrantz M, Kristinsson SY, Andersson TM, et al. Patterns of survival among patients with myeloproliferative neoplasms diagnosed in Sweden from 1973 to 2008: a population-based study. J Clin Oncol. 2012;30(24):2995-3001.

34. Dupont S, Massé A, James C, et al. The JAK2 617V>F mutation triggers erythropoietin hypersensitivity and terminal erythroid amplification in primary cells from patients with polycythemia vera. Blood. 2007;110(3): 1013-1021.

35. Chen E, Beer PA, Godfrey AL, et al. Distinct clinical phenotypes associated with JAK2V617F reflect differential STAT1 signaling. Cancer Cell. 2010;18(5):524-535.

36. Wang WA, Groenendyk J, Michalak M. Calreticulin signaling in health and disease. Int J Biochem Cell Biol. 2012;44(6):842-846.

37. Carobbio A, Thiele J, Passamonti F, et al. Risk factors for arterial and venous thrombosis in WHO-defined essential thrombocythemia: an international study of 891 patients. Blood. 2011; 117(22):5857-5859.

38. Arellano-Rodrigo E, Alvarez-Larrán A, Reverter JC, Villamor N, Colomer D, Cervantes F. Increased platelet and leukocyte activation as contributing mechanisms for thrombosis in essential thrombocythemia and correlation with the JAK2 mutational status. Haematologica. 2006; 91(2):169-175.