

Impact of Calreticulin Mutations on Clinical and Hematological Phenotype and Outcome in Essential Thrombocythemia

Giada Rotunno*, Carmela Mannarelli*, Paola Guglielmelli*[#], Annalisa Pacilli, Alessandro Pancrazzi, Lisa Pieri, Tiziana Fanelli, Alberto Bosi, Alessandro M Vannucchi[#] *on behalf of* the Associazione Italiana per la Ricerca sul Cancro Gruppo Italiano Malattie Mieloproliferative (**AGIMM**) Investigators

Department of Experimental and Clinical Medicine, University of Florence, Florence, Italy

*, equal contribution

Running head: CALR Mutations in Essential Thrombocythemia

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[#] Corresponding Authors:

Alessandro M. Vannucchi, MD

amvannucchi@unifi.it

Paola Guglielmelli, MD PhD

paola.guglielmelli@unifi.it

Both at the

Laboratorio Congiunto-MMPC

Department of Experimental and Clinical Medicine

University of Florence & Az. Ospedaliera-Universitaria Careggi

Largo Brambilla 3, 50134 Florence, Italy.

Telephone and FAX: +39.055.7947.688.

Key points

- *CALR* mutations occur in half of *JAK2* and *MPL* wild type patients with ET and associate with some distinctive phenotypic traits
- Patients with ET harboring *CALR* mutations are at significantly lower risk of thrombosis compared with *JAK2* and *MPL* mutated

Abstract

Mutations in the calreticulin (*CALR*) gene were recently discovered in patients with essential thrombocythemia (ET) lacking the *JAK2V617F* and *MPLW515* mutations, but no information is available on the clinical correlates. In this series, *CALR* mutations were found in 15.5% of 576 WHO-defined ET subjects accounting for 48.9% of *JAK2* and *MPL* wild type. *CALR* mutated patients were preferentially male and showed higher platelet count, lower hemoglobin and leukocyte count compared with *JAK2* and *MPL* mutated patients. Subjects carrying *CALR* mutation had a lower risk of thrombosis than *JAK2* and *MPL* mutated; of interest, their risk was superimposable to patients who were wild type for the above mutations. *CALR* mutation had no impact on survival or transformation to post-ET myelofibrosis. Genotyping for *CALR* mutations represents a novel useful tool for establishing a clonal myeloproliferative disorder in *JAK2* and *MPL* wild type patients with thrombocytosis and may have prognostic and therapeutic relevance.

Introduction

Unlike polycythemia vera, where virtually all the patients harbor *JAK2* mutations (*JAK2V617F* in >95% and *JAK2* exon 12 mutations in about 2-4%), only 50-60% of essential thrombocythemia (ET)¹⁻³ and primary myelofibrosis (PMF)⁴⁻⁶ patients are *JAK2V617F* mutated (*JAK2*⁺). An additional 3-5% of ET and PMF subjects present mutations of *MPL* at codon 515 (*MPL*⁺)⁷⁻⁹. *JAK2* and *MPL* mutations represent major diagnostic criteria in the 2008 WHO classification of chronic myeloproliferative neoplasms (MPN)^{10,11}. All these mutations result in the abnormal activation of the JAK/STAT signaling pathway, that represents an hallmark of MPN and a target for therapy¹². Recently, mutations at exon 9 of *CALR*, the gene encoding calreticulin, an endoplasmic reticulum Ca²⁺ binding chaperone, were discovered in 50-70% of patients with ET and PMF (*CALR*⁺) who were wild type (wt) for *JAK2* and *MPL*^{13,14}. The mechanisms by which *CALR* mutations produce a myeloproliferative phenotype are unknown.

The aim of this study was to describe the prevalence, characteristics and clinical and laboratory features associated with *CALR* mutations in a large population of patients with WHO-defined ET.

Methods

The study involved 576 patients with a diagnosis of ET fulfilling the 2008 WHO criteria who were followed at the Hematology Department, University of Florence. They had a stored sample of granulocyte DNA collected at diagnosis or within 3 years. Patients had provided an informed written consent in accordance with the Declaration of Helsinki for the use of left DNA for investigational purposes. The ethical committee was that of the Azienda ospedaliero-universitaria Careggi in Florence. The *JAK2V617F* and *MPLW515L/K* mutation were assessed by Real Time quantitative

PCR^{15,16} and for *MPL* also by high-resolution melting (HRM) analysis followed by bidirectional Sanger sequencing¹⁷. Mutations in exon 9 of *CALR* were assessed by bidirectional sequencing¹³.

Patients' characteristics reported in Table 1 were obtained at diagnosis. Splenomegaly was defined as a palpable organ below the left costal margin. Major thrombosis and bleeding, at diagnosis and/or in the two preceding years and/or anytime during the follow-up, were recorded when objectively documented¹⁸ and according to standard definitions¹⁹. Microvessel symptoms consisted of erythromelalgia, recurrent episodes of otherwise unexplained blurred vision, tinnitus, paresthesia, headache. Constitutional symptoms included fever, unintentional weight loss, night sweats. Pruritus was recorded when described as a diffuse, recurrent itching exacerbated by water contact. Evolution to post-ET myelofibrosis (PET-MF) and acute leukemia (AL) was diagnosed following the IWG-MRT and WHO criteria, respectively^{11,20}. Patients were treated according to current recommendations²¹; cytoreduction was hydroxyurea in >90% of high-risk patients.

Statistical analysis was performed with SPSS software. Patients' characteristics were compared with the use of chi-square test or Fisher's exact test for categorical variables and t-test or nonparametric test for continuous variables. Significance level was $p < 0.05$ in two-sided tests. Survival estimates were obtained with Kaplan-Meier method; the hazard ratio (HR) was determined using Cox proportional hazards model.

Results and Discussion

In the entire patient series, the median age was 58.1y (range, 13-93); 194 subjects (33.7%) were male. Median follow-up was 71.9 months (2-257); seventy patients (12.1%) had died after a median of 53.9 months (2-249) from diagnosis. Nineteen patients (3.3%) progressed to PET-MF after a

median of 122 months (19-248); transformation to PV was documented in 5 cases (0.9%) and four patients (0.7%) evolved to AL after a median of 117.7 months (56-250). We found 89 subjects (15.5% of total) harboring exon 9 *CALR* mutations. *CALR* mutations were represented by insertions and deletions, as reported^{13,14}. Deletions (60.7%) occurred more frequently than insertions (39.3%); the commonest deletion was del367fs46 (37.0%) and ins385fs47 (71.4%) among insertions. *JAK2V617F* and *MPLW515* mutations occurred in 64.1% (n=369) and 4.3% (n=25) of the patients. *CALR*⁺ patients accounted for 48.9% of *JAK2* and *MPL* wt patients (n=182); 93 subjects (16.1% of total) were wt for the three mutations considered.

We compared hematological and clinical characteristics of the patients who were categorized according to their *JAK2V617F*, *MPLW515* and *CALR* genotype (Table 1). *CALR*⁺ patients were younger than *JAK2*⁺ and no different from *MPL*⁺ and wt; a striking male predominance was found among *CALR*⁺ (59.5%) compared with *JAK2*⁺ (31.7%; p<0.001), *MPL*⁺ (24.0%, p=0.002) and wt (19.4%; p<0.001) patients. Influence of gender on *JAK2V617F* allele burden²², disease class and vascular complications²³ is well documented, and current data add to the understanding of the role of host variations for the expression of the MPN phenotype²⁴.

The leukocyte count, the hemoglobin and the hematocrit level were lower in *CALR*⁺ compared with *JAK2*⁺ (p=0.001, p<0.0001 and p<0.0001, respectively) and similar to *MPL*⁺ and wt subjects; on the other hand, platelet count was higher in *CALR*⁺ than in *JAK2*⁺ and wt subjects (p<0.0001 for both), but comparable to *MPL*⁺ patients who also differed significantly from *JAK2*⁺ (p<0.001). Lactate dehydrogenase level (LDH) was lower in wt compared to patients with any mutation (p<0.001). Constitutional symptoms and pruritus were similarly represented in the different groups; a palpable spleen was less common in wt subjects compared to those harboring any mutation (p<0.01). The

proportion of *CALR*⁺ patients who received cytoreduction was similar to *JAK2*⁺ and *MPL*⁺ and lower than wt (Table 1). Overall, these findings indicate that *CALR*⁺ patients, similar to *MPL*⁺, present a phenotype associated with preferential expansion of the megakaryocytic lineage compared with favored erythropoiesis in *JAK2*⁺ subjects.

Major cardiovascular events occurred in 30.1%, 40.0%, 13.5% and 16.1% of the *JAK2*⁺, *MPL*⁺, *CALR*⁺ and wt subjects; the difference was statistically significant when comparing *CALR*⁺ versus *JAK2*⁺ and *MPL*⁺ subjects ($p=0.01$ for both). On the other hand, microvessel symptoms were more represented among *MPL*⁺ patients ($p<0.01$ compared to the other groups). The thrombosis free survival was significantly longer in *CALR*⁺ and wt subjects compared to *JAK2*⁺ and *MPL*⁺ ($p=0.008$) (Figure 1A). The cumulative incidence of thrombosis at 10 years was 5.12% (95%CI, 1.6-15.2) in *CALR*⁺, 14.54% (95%CI, 10.0-20.8) in *JAK2*⁺, 19.46% (95%CI, 7.6-44.6) in *MPL*⁺, and 8.17% (95%CI, 2.7-23.3) in wt subjects. Taking wt subjects as reference population, the hazard ratio (HR) for thrombosis was 0.74 (95%CI, 0.33-1.00) for *CALR*⁺, 1.78 (95%CI, 1.06-3.18) for *JAK2*⁺ and 1.65 (95%CI, 1.7-3.92) for *MPL*⁺ patients. There was a trend towards more frequent hemorrhages in *MPL*⁺ compared to all other patients. The median survival was not reached in any group, and Kaplan Meier estimates of survival did not show significant differences (Figure 1B). *CALR*⁺ patients were preferentially distributed in the lower risk category of the Thrombosis score, IPSET-thrombosis and IPSET score compared with *JAK2*⁺ (and *MPL*⁺ for IPSET-thrombosis) (Supplemental Table 1). Overall, these data indicate that *CALR*⁺ patients are less prone to thrombotic events compared to *JAK2*⁺ and *MPL*⁺; of note, their risk was similar to subjects lacking any mutations.

Transformation to PET-MF occurred in 19 patients; the HR for PET-MF was similarly increased in *CALR*⁺ (2.36; 95%CI, 0.26-21.8), *JAK2*⁺ (2.21; 95%CI, 0.28-17.8) and *MPL*⁺ (2.50; 95%CI, 0.22-28.5)

patients compared with wt, although possibly due to small number of events did not reach the significance level. Noteworthy, all 5 cases of transformation to PV occurred in the *JAK2*⁺ patients.

With the limitations imposed by its observational nature, that precludes any causal relationships inferences, results of current study identified meaningful associations between the presence of *CALR* mutations and the phenotype of patients with ET. The findings that *CALR* mutated patients are at lower risk of vascular events may have implications for risk stratification and management. Finally, our study underscores the importance of *CALR* genotyping for an accurate diagnosis of patients with thrombocytosis who lack the *JAK2*V617F and *MPL*W515 mutation.

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Authorship Contributions

PG, AMV: designed the study, analyzed the data, and wrote the manuscript; GR, CM, performed molecular analysis, analyzed raw sequencing data; AP, AP, TF contributed to molecular analysis; PG, LP, AB, AMV contributed samples and clinical information. All authors have read the final version of the manuscript and agreed on its content.

Disclosure of Conflict of Interest

There is no conflict of interest to disclose for the work performed in this manuscript.

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Table 1. Laboratory and clinical characteristics of *CALR* mutant patients compared to *JAK2 V617F* or *MPLW515* mutant patients and subjects who were wild type for the above three mutations.

	<i>CALR</i> ⁺	<i>JAK2</i> <i>V617F</i> ⁺	<i>MPL</i> <i>W515</i> ⁺	<i>CALR, JAK2,</i> <i>MPL wt</i>	P value		
					(A) vs (B)	(A) vs (C)	(A) vs (D)
Number of patients (%)	89 (15.5)	369 (64.1)	25 (4.3)	93 (16.1)	-	-	-
Male no-(%)	53 (59.5)	117 (31.7)	6 (24.0)	18 (19.4)	<0.0001	0.002	<0.0001
Age, years	54.7 (13-88)	61 (15-93)	54 (22.89)	53 (15-87)	0.04	0.997	0.519
Leukocyte count (x10 ⁹ /L)	8.1 (3.5-26.0)	8.9 (4.2-35.0)	8.4 (4.5-16.6)	8.3 (4-16.8)	0.001	0.834	0.367
Hemoglobin (g/L)	138 (106-173)	145 (102-173)	136 (110-160)	136 (106-164)	<0.0001	0.315	0.380
Hematocrit (%)	41.2 (35.9-49.4)	43.8 (31.4-53.6)	41.2 (32.8-50)	41.0 (31.3-51.5)	<0.0001	0.887	0.893
Platelet count (x10 ⁹ /L)	866 (504-2348)	726 (455-1881)	898 (607-2000)	697 (482-1659)	<0.0001	0.385	<0.0001
LDH (U/L)	320 (142-725)	288 (102-1178)	365 (254-570)	268 (137-554)	0.307	0.665	<0.01
Splenomegaly, no-(%)	24 (27.0)	91 (24.7)	9 (36.0)	9 (9.7)	0.661	0.416	0.004
Pruritus, no-(%)	5 (5.6)	32 (8.7)	1 (4.0)	9 (9.7)	0.260	0.847	0.228
Constitutional symptoms, no-(%)	1 (1.1)	19 (5.1)	1 (1.2)	6 (6.5)	0.120	0.577	0.078
Major thrombosis, no-(%)	12 (13.5)	111 (30.1)	10 (40.0)	15 (16.1)	0.011	0.012	0.894
Microvessel symptoms, no-(%)	22 (24.7)	101 (27.4)	14 (56.0)	20 (21.5)	0.604	0.003	0.674
Major hemorrhage, no-(%)	4 (4.5)	17 (4.6)	4 (16.0)	3 (3.3)	0.906	0.067	0.587
Progression to PET-MF, no-(%)	4 (4.5)	12 (3.3)	2 (8.0)	1 (1.1)	0.458	0.563	0.128
Progression to PV, no-(%)	0	5 (1.4)	0	0	0.294	-	-
Progression to AL, no-(%)	0	2 (0.5)	1 (4.0)	1 (1.1)	0.507	0.071	0.349
Deceased (n=70; %)	10 (11.2)	49 (13.3)	4 (16.0)	7 (7.5)	0.598	0.515	0.414
Cytoreductive therapy, no-(%)	50 (62.5)	220 (60.8)	19 (76.0)	40 (46.0)	0.440	0.160	0.023

Hematologic and clinical information were collected at diagnosis; information regarding major thrombosis and hemorrhage included events at diagnosis, in the two preceding years and during the follow-up. Cytoreduction means that the patient received cytoreductive drugs (in >90% of cases, hydroxyurea) during the course of disease at the physician discretion, based on conventional criteria. Unless otherwise indicated, values are reported as median (range).

wt= wild type; PET-MF post-essential thrombocythemia myelofibrosis; PV= polycythemia vera; AL= acute leukemia.

Figure legend.

Figure 1. Kaplan Meier estimate of thrombosis-free survival (panel A) and overall survival (panel B) in patients who were categorized according to their mutational status (*JAK2V617⁺*, *MPLW515⁺*, *CALR⁺*, o wild type for the above mutations).

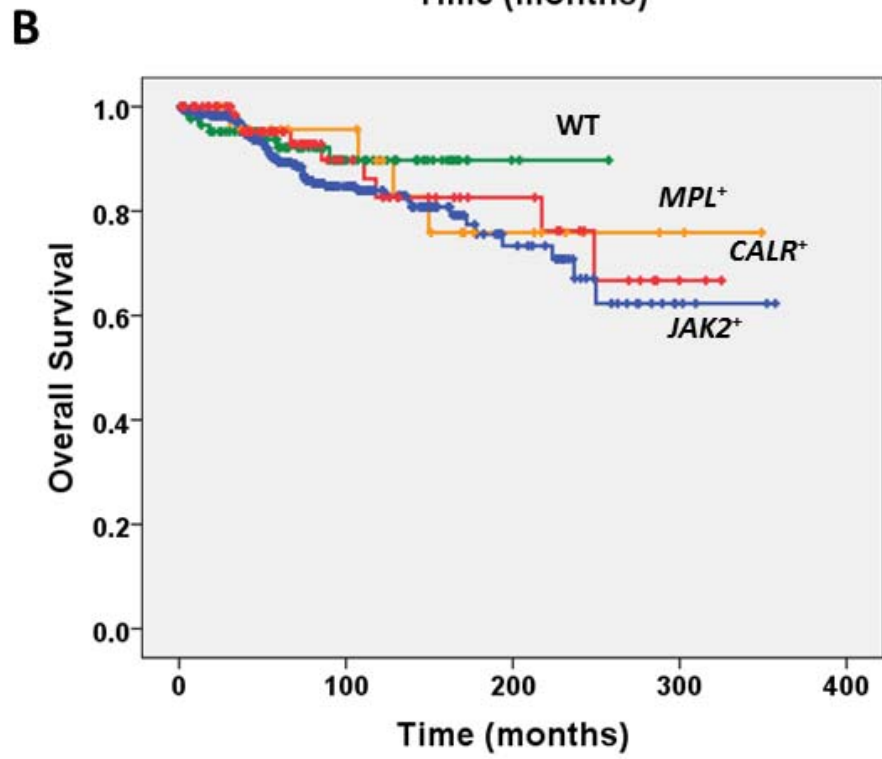
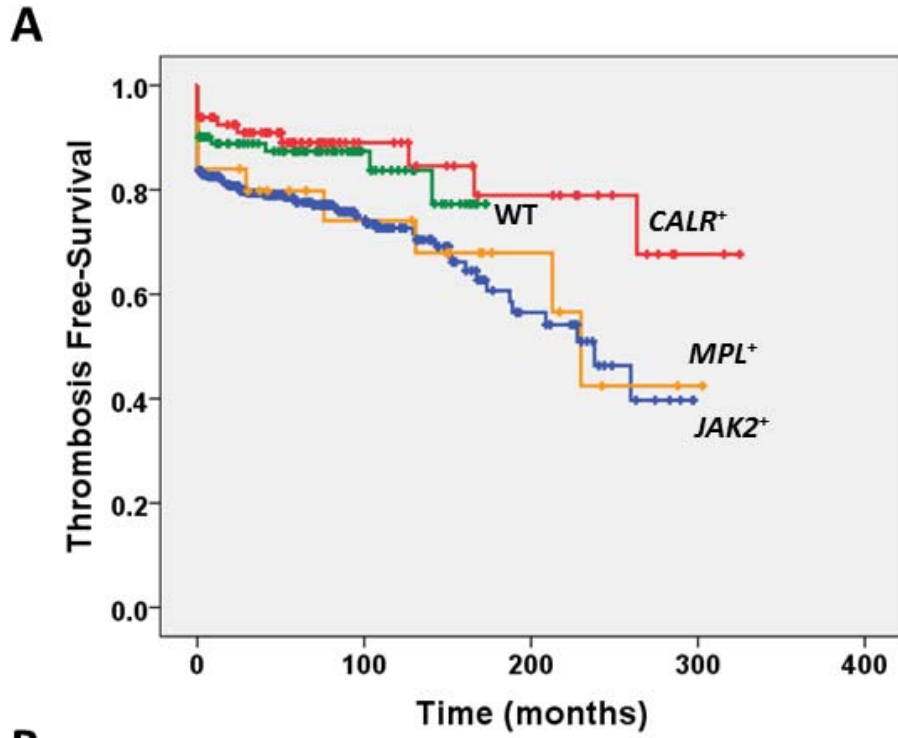


Figure 1