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ORIGINAL ARTICLE

Differential clinical effects of different mutation subtypes in *CALR*-mutant myeloproliferative neoplasms

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A quarter of patients with essential thrombocythemia or primary myelofibrosis carry a driver mutation of *CALR*, the calreticulin gene. A 52-bp deletion (type 1) and a 5-bp insertion (type 2 mutation) are the most frequent variants. These indels might differentially impair the calcium binding activity of mutant calreticulin. We studied the relationship between mutation subtype and biological/clinical features of the disease. Thirty-two different types of *CALR* variants were identified in 311 patients. Based on their predicted effect on calreticulin C-terminal, mutations were classified as: (i) type 1-like (65%); (ii) type 2-like (32%); and (iii) other types (3%). Corresponding CALR mutants had significantly different estimated isoelectric points. Patients with type 1 mutation, but not those with type 2, showed abnormal cytosolic calcium signals in cultured megakaryocytes. Type 1-like mutations were mainly associated with a myelofibrosis phenotype and a significantly higher risk of myelofibrotic transformation in essential thrombocythemia. Type 2-like *CALR* mutations were preferentially associated with an essential thrombocythemia phenotype, low risk of thrombosis despite very-high platelet counts and indolent clinical course. Thus, mutation subtype contributes to determining clinical phenotype and outcomes in *CALR*-mutant myeloproliferative neoplasms. *CALR* variants that markedly impair the calcium binding activity of mutant calreticulin are mainly associated with a myelofibrosis phenotype.

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INTRODUCTION

Philadelphia-negative myeloproliferative neoplasms (MPN) include polycythemia vera, essential thrombocythemia (ET) and primary myelofibrosis (PMF).[1] Our understanding of the genetic basis of these disorders began in 2005, when the *JAK2* (V617F) mutation was identified in patients with polycythemia vera, ET or PMF.[2,3] Mutations of *JAK2* exon 12[(refs 4,5)] and *MPL* exon 10[(refs 6,7)] were subsequently detected in subsets of patients, while subclonal driver mutations in other genes were found to be associated with disease progression.[8–11] In 2013, somatic mutations in the gene *CALR*, encoding calreticulin, were detected in most patients with ET or PMF with unmutated *JAK2* and *MPL*.[12,13]

Although polycythemia vera is a condition almost exclusively associated with gain-of-function mutations of *JAK2*, the genetic basis of disease is more heterogeneous in ET and in PMF:[14] *JAK2* (V617F) is present in ~60–65% of cases, *MPL* exon 10 mutations in about 5%, and *CALR* exon 9 indels in ~20–25% of cases.[15–18] We recently found that the remaining 5–10% of cases do not represent a homogeneous disease entity, and that some of them carry novel *MPL* and *JAK2* mutations.[19]

CALR-mutant MPN have distinctive clinical features.[14] Patients with *CALR*-mutant ET have very high platelet counts but a relatively low risk of thrombosis, at least lower than that of patients with *JAK2*-mutant ET.[15] Patients with *CALR*-mutant PMF have an indolent clinical course, and better survival compared with PMF patients carrying *JAK2* (V617F) or an *MPL* exon 10 mutation.[17,18]

More than 50 different indels in *CALR* exon 9 have been found, but a 52-bp deletion (type 1) and a 5-bp insertion (type 2 mutation) are the most common variants.[12,15,18] These indels generate a novel C-terminus of the mutant protein in which the negatively charged amino acids are variably replaced by neutral and positively charged amino acids. The 52 bp deletion eliminates almost all negatively charged amino acids, whereas the 5 bp insertion retains approximately half the negatively charged amino acids.[12] This suggests that impaired Ca^{2+}-binding activity might have a role in cells expressing a mutant calreticulin, and that the different calreticulin mutants may differ considerably in terms of Ca^{2+}-binding activity. Calcium signaling regulates many different cellular processes, and intracellular Ca^{2+} mobilization from endoplasmic reticulum (ER) into the cytoplasm triggers extracellular Ca^{2+} inflow in non-excitable cells,[20] a mechanism termed store-operated Ca^{2+} entry (SOCE).[21] Interestingly, both Ca^{2+} mobilization from intracellular stores and extracellular Ca^{2+} entry regulate human megakaryocyte (Mk) functions.[22]

Recently, Marty *et al.*[23] investigated the effect of the type 1 and type 2 CALR mutant expression by retroviral mouse modeling. CALRdel52 (type 1 mutant) expressing mice rapidly developed marked thrombocytosis and then progressed to a condition similar to myelofibrosis. By contrast, CALRins5 (type 2 mutant) expressing mice had a mild ET phenotype with low propensity to disease progression. These observations in mouse models suggest that the different *CALR* mutations might have different clinical

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effects in humans. In this work, we studied patients with *CALR*-mutant ET or PMF to examine the relationship between mutations subtypes, Mk cytosolic Ca^{2+} signals and clinical outcomes.

PATIENTS AND METHODS

Study population

This study was approved by the institutional Ethics Committee (Comitato di Bioetica, Fondazione 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 1282 consecutive patients (572 males, 710 females; median age 50 years, range 15–92 years) diagnosed with ET or PMF between 1982 and 2014 at Fondazione IRCCS Policlinico San Matteo, Italy, for which at least one DNA sample was available (Table 1). According to the 2008 WHO criteria,[1] adopted for reclassification of patients, 908 out of 1282 subjects had ET (all from the Department of Hematology Oncology, Fondazione IRCCS Policlinico San Matteo, and University of Pavia, Italy) and 374 had PMF (205 from the Department of Hematology Oncology and 169 from the Center for the Study of Myelofibrosis, Fondazione IRCCS Policlinico San Matteo Pavia, Italy). ET patients included the 642 subjects previously reported by Rumi *et al.*,[15] while PMF patients included 358 subjects previously described.[18] Overall, 799 (62%) patients carried *JAK2* (V617F) and 311 (24%) had a *CALR* indel (Table 1). Patients with *MPL* mutation or nonmutated *JAK2*/*MPL*/*CALR* were excluded from subsequent analyses.

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.[24] Post-ET myelofibrosis was diagnosed according to the criteria of the International Working Group of Myelofibrosis Research and Treatment,[25] while evolution into acute myeloid leukemia was defined according to the WHO criteria.[1] Thrombotic events were defined as described in detail elsewhere.[26]

JAK2, *CALR* and *MPL* mutation analysis

Granulocyte *JAK2* (V617F) mutation status and mutant allele burden were assessed using a quantitative PCR (qPCR)-based allelic discrimination assay on a Rotor-Gene 6000 real-time analyzer (Qiagen, Hilden, Germany).[27] *MPL* exon 10 mutations were detected using a high-resolution melt assay or Sanger sequencing,[28] and *CALR* exon 9 mutations were studied as previously described.[15]

In vitro megakaryocyte differentiation from human peripheral blood hematopoietic progenitors

Blood samples were obtained from healthy subjects and patients with MPNs. $CD45^+$ hematopoietic progenitor cells from peripheral blood were separated by immunomagnetic bead selection (Miltenyi Biotec, Bologna, Italy), as previously described.[29] Cells were cultured for 14 days, in Stem Span medium (STEMCELL Technologies Inc, Vancouver, BC, Canada) supplemented with 10 ng/ml TPO, IL-6 and IL-11 at 37 °C in a 5% CO_2 fully humidified atmosphere.

Measurement of intracellular Ca^{2+} concentration

At day 14 of culture, the Mk population was enriched through a bovine serum albumin gradient as previously described,[30] 12-mm glass cover-slips were coated with 100 μg/ml fibrinogen, overnight at 4 °C, 1×10^5 Mks were then harvested and allowed to adhere at 37 °C and 5% CO_2 for 16 h. Then, Mks were loaded with 4 μM fura-2 AM (Molecular Probes Europe BV, Leiden, The Netherlands) in physiological salt solution (NaCl 150 mM, KCl 6 mM, $CaCl_2$ 1.5 mM, $MgCl_2$ 1 mM, glucose 10 mM, HEPES 10 mM, pH 7.4) for additional 30 min at 37 °C and 5% CO_2. After being washed in physiological salt solution, the cover-slip was fixed to the bottom of a Petri dish and the cells were observed using an upright epifluorescence Axiolab microscope (Carl Zeiss, Arese, Italy), equipped with a Zeiss X63 Achroplan objective (water-immersion, 2.0 mm working distance, 0.9 numerical aperture). Mks were excited alternately at 340 and 380 nm, and the emitted light was detected at 510 nm. A first neutral density filter (1 or 0.3 optical density) reduced the overall intensity of the excitation light and a second neutral density filter (0.3 optical density) was coupled to the 380 nm filter to approach the intensity of the 340 nm light. The excitation filters were mounted on a filter wheel (Lambda 10; Sutter Instrument, Novato, CA, USA). Custom software, working in the LINUX environment, was used to drive the camera (Extended-ISIS Camera; Photonic Science, Robertsbridge, UK) and the filter wheel and to measure and plot the fluorescence from rectangular regions of interest each enclosing every Mk present within the analyzed field. Analysis of Ca^{2+} signals was performed according to previously published methods.[22]

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 two groups of patients were carried out by the non-parametric Wilcoxon rank-sum test. The Kruskal–Wallis test was applied when comparing more than two groups. Association between categorical variables (two-way tables) was tested by the Fisher's exact test. Overall survival (OS) was estimated using the Kaplan–Meier product limit method, and hazard ratios were estimated by Cox proportional hazard univariate and multivariate regression models. The cumulative incidence of leukemic transformation, that of thrombotic events and that of fibrotic evolution were estimated with a competing risk approach, considering death for any cause as a competing event. Cumulative incidence curves were estimated with the Kalbfleisch & Prentice method,[31] while the effect of mutation type was estimated by applying the Fine & Gray regression model.[32]

All *P*-values were considered statistically significant when smaller than 0.05 (two-tailed). Statistical analyses were performed using Stata 12.1 (StataCorp LP, College Station, TX, USA) software.

RESULTS

Different types of *CALR* exon 9 mutations and their categorization

Thirty-two different types of *CALR* lesions were identified (Figure 1a): 175 (56%) patients had type 1 mutation, 86 (28%) had type 2, and 50 (16%) carried other indels. The frequency of type 1 mutation was significantly higher in PMF than in ET (75% vs 48%, $P < 0.001$).

As shown in Figure 1a, the observed variants had different predicted effect on three different stretches (here defined as I, II and III) of negatively charged amino acids of the wild-type sequence. *CALR* variants could therefore be categorized as follows: (i) type 1-like mutations ($n = 203$, 65% of all lesions), which predict deletion of stretch II and III, as happens with the L367fs*46 or type 1 mutation; (ii) type 2-like mutations ($n = 98$, 32% of all lesions), which have no impact on the above mentioned stretches, as happens with the K385fs*47 or type 2 mutation; and (iii) other types ($n = 10$, 3% of all lesions), involving deletion of stretch III

Table 1. Initial study population including 1282 patients diagnosed with essential thrombocythemia or primary myelofibrosis at Fondazione IRCCS Policlinico San Matteo, Italy, between 1982 and 2014

Myeloproliferative neoplasm	*JAK2 (V617F) mutated (%)*	*MPL exon 10 mutated (%)*	*CALR exon 9 mutated (%)*	*Nonmutated JAK2/MPL/CALR (%)*	*All genotypes*
Essential thrombocythemia	567 (62%)	36 (4%)	216 (24%)	89 (10%)	908
Primary myelofibrosis	232 (62%)	20 (5%)	95 (26%)	27 (7%)	374
All patients	799 (62%)	56 (5%)	311 (24%)	116 (9%)	1282

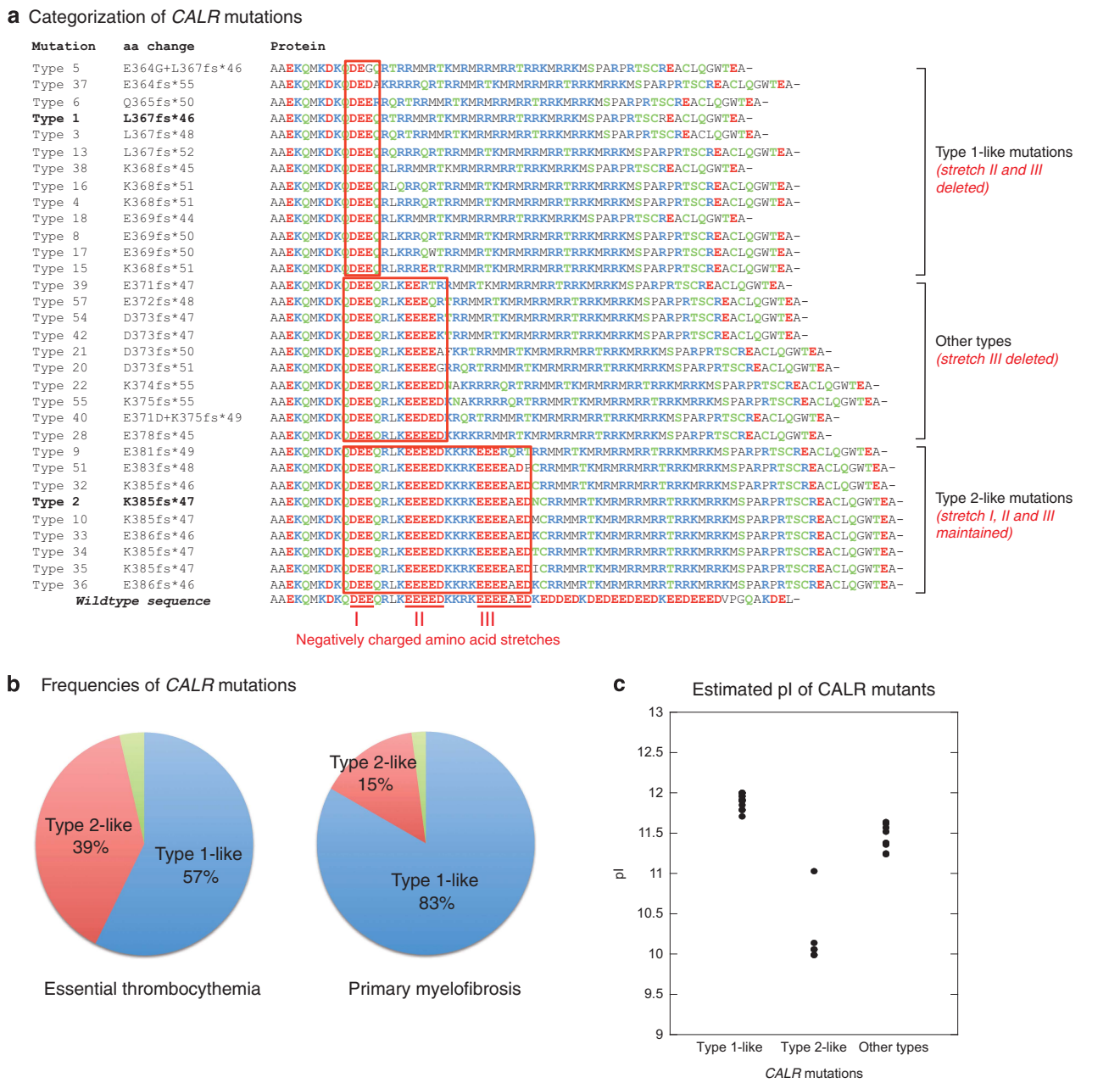

Figure 1. Types and categorization of the 32 different *CALR* mutations found in 311 patients with essential thrombocythemia or primary myelofibrosis. (**a**) Alignment of C-domain in wild-type and mutant CALR proteins. Mutation denomination, as previously defined,[15] is indicated on the left (type 1 and type 2 variants are in bold). The amino acid sequence starts from codon A352: acidic, basic and neutral residues are in red, blue and green, respectively. All the variants involved three different stretches of negatively charged amino acids, here defined as I, II and III, and highlighted in red in the wild-type sequence. Type 1-like mutations predict deletion of stretches II and III (as happens with the L367fs*46 or type 1 mutation), while type 2-like mutations predict conservation of all the three stretches (as happens with the K385fs*47 or type 2 mutation); other types involve deletion of stretches III exclusively. (**b**) Distribution of categorized *CALR* mutations in 216 patients with ET and 95 with PMF (type 1-like mutations in blue, type 2-like in red, and other types in green). The frequency of type 1-like mutations was significantly higher in PMF than in ET (83% and 57%, $P < 0.001$). (**c**) Values for isoelectric point (pI) in mutant CALR peptides starting from codon A352. Estimates were performed using the Scripps Institute's online Protein Calculator v.3.3 (http://protcalc.sourceforge.net). Mutants had higher pI values (9.99–12.00) than wild-type sequence (4.11). The pI values were significantly different in the three categories ($P < 0.001$), and were significantly higher in type 1-like than in type 2-like mutants ($P < 0.001$).

exclusively. The frequency of type 1-like mutations was significantly higher in PMF than in ET (83% vs 57%, $P < 0.001$, Figure 1b).

Estimated isoelectric point of CALR mutants

The isoelectric point (pI) is the pH at which a protein carries no net electrical charge, and its value depends on the aminoacid composition. We therefore assessed the pI of mutant CALR peptides starting from codon A352 using the Scripps Institute's online Protein Calculator (http://protcalc.sourceforge.net). As shown in Figure 1c, markedly higher pI values (ranging from 9.99 to 12.00) were estimated in CALR mutants compared with the wild-type sequence, whose estimated pI is 4.11. Moreover, pI

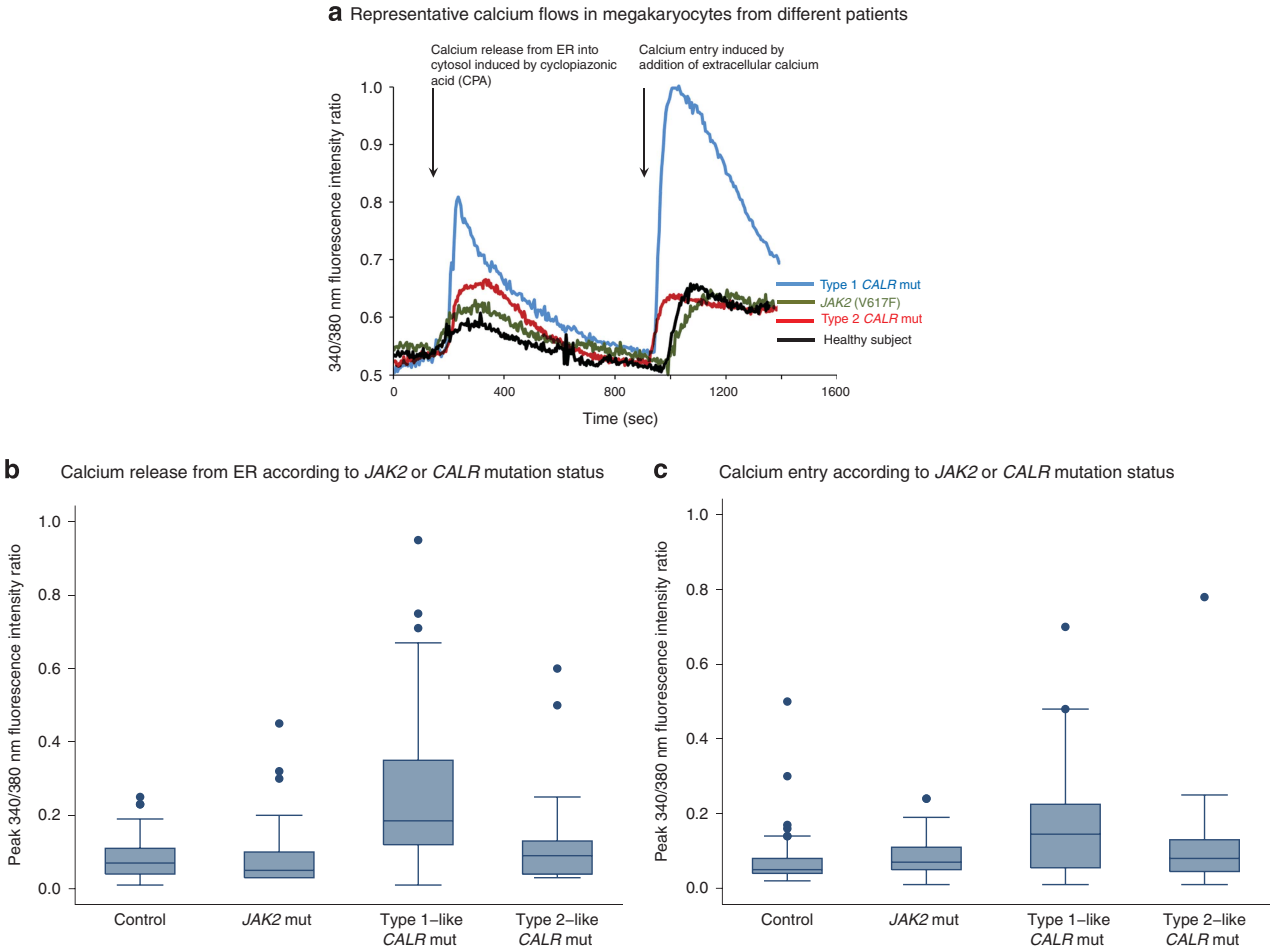

Figure 2. Fluorescence-based measurements of Ca^{2+} flows in cultured megakaryocytes. (**a**) Representative examples of fluorescence-based measurements of Ca^{2+} flows in cultured megakaryocytes from a healthy subject and from patients with a myeloproliferative neoplasm carrying different driver mutations. Intracellular Ca^{2+} pools were depleted, in absence of extracellular Ca^{2+}, by cyclopiazonic acid, an inhibitor of the sarco-endoplasmic reticulum Ca^{2+}-ATPase. This treatment evoked a transient rise in intracellular Ca^{2+} concentration because of the emptying of Ca^{2+} stores (Ca^{2+} release); thereafter, Ca^{2+} levels dropped to the baseline. When extracellular Ca^{2+} concentration was restored to 1.5 mM, this drove a second increase in intracellular Ca^{2+} levels due to activated influx through plasma membrane channels (Ca^{2+} entry). (**b**) Measurements of calcium release in cultured megakaryocytes. Sequential fluorescence-based measurements of Ca^{2+} flow in cultured megakaryocytes from 16 subjects were performed: four healthy subjects as controls, four *JAK2* (V617F)-mutated patients (two ET and two PMF), four type 1-like *CALR*-mutated cases (two ET and two PMF, all carrying the type 1 mutation), and four type 2-like *CALR*-mutated patients (two ET and two PMF, all carrying the type 2 mutation). Overall, each molecular group consisted of 60 measurements, and in each patient at least eight megakaryocytes were analyzed. Data are shown in a box plot depicting the upper and lower values (lowest and highest horizontal line, respectively), lower and upper quartile with median value (box), and outside values (dots). Patients with type 1-like *CALR* mutation had higher values than both normal controls and patients with either *JAK2* or type 2-like *CALR* mutation ($P < 0.001$). (**c**) Measurements of calcium entry in cultured megakaryocytes from patients of **b**. Patients with type 1-like *CALR* mutation had higher values than normal controls and patients with either *JAK2* or type 2-like *CALR* mutation ($P < 0.001$).

values were significantly different in three groups of categorized mutations, and were significantly higher in type 1-like than in type 2-like mutants ($P < 0.001$).

Cytosolic Ca^{2+} signals in cultured megakaryocytes from patients with myeloproliferative neoplasms

CALR mutants may affect both ER Ca^{2+} levels and SOCE, this latter likely being the most important pathway for Ca^{2+} entry in Mks.[22] To evaluate cytoplasmic Ca^{2+} mobilization in human Mks from MPN patients carrying *JAK2* (V617F), type 1 or type 2 *CALR* mutation, we exposed cells to cyclopiazonic acid (10 μM).[22] Cyclopiazonic acid specifically blocks the sarco-ER Ca^{2+} ATPase activity, thereby preventing Ca^{2+} sequestration into the stores and leading to their depletion.[33] This maneuver is routinely employed to assess ER Ca^{2+} levels and, consequently, to activate SOCE in non-excitable cells.[33] As illustrated in Figure 2a, sequential fluorescence measurements showed that cyclopiazonic acid-mediated store depletion, in the absence of extracellular Ca^{2+}, induced a transient increase in cytosolic Ca^{2+} concentration because of passive emptying of ER Ca^{2+} stores (calcium release). Thereafter, Ca^{2+} levels dropped to the baseline as the plasma membrane extruded Ca^{2+} from the cytosol. At this stage, ER Ca^{2+} stores are fully depleted. Therefore, the subsequent restoration of extracellular Ca^{2+} induced a second increase in cytosolic Ca^{2+} concentration due to SOCE activation (calcium entry).

Mks from patients with type 1 *CALR* mutation displayed the greatest cytosolic Ca^{2+} oscillations (defined as peak fluorescence ratio) in terms of both Ca^{2+} release from ER (Figure 2b) and Ca^{2+} entry (Figure 2c). Although there was considerable variability, peak

values for both Ca^{2+} release and Ca^{2+} entry were significantly higher in patients with type 1 *CALR* mutation than in patients with *JAK2* (V617F) or type 2 *CALR* mutation, or in healthy subjects ($P < 0.001$ in all comparisons).

Relationship between driver somatic mutation and clinical phenotype

Tables 2 and 3 report clinical and laboratory features at diagnosis in ET and PMF patients, respectively, stratified according to their driver mutation.

Compared with those carrying *JAK2* (V617F) or type 1-like *CALR* mutation, ET patients with type 2-like *CALR* mutation were younger, and had higher platelet count but lower incidence of thrombosis at diagnosis. Within PMF patients, no significant differences were observed between patients with type 1-like *CALR* and those with type 2-like *CALR* mutation (Table 3). Compared with patients carrying *JAK2* (V617F), those with type 1-like *CALR* mutation were younger and had higher values for circulating CD34-positive cells.

In PMF patients, there was no significant difference between the three genetic categories with respect to the International Prognostic Score System risk.

Impact of driver mutation on clinical outcome

The median follow-up of the study cohort was 4.7 years (range 0–31 years).

In ET patients, death occurred in 25 out of 567 patients with *JAK2* (V617F) (4.4%), 2 out of 124 with a type 1-like *CALR* mutation (1.6%) and 2 out of 84 of those carrying a type 2-like *CALR* indel (2.4%). The OS at 10 years was 98.6% (95% confidence interval (CI), 90.7–99.8) and 98% (95% CI, 86.6–99.7) in patients with a type 1-like or type 2-like *CALR* mutation, respectively, and 94% (95% CI, 90–96.4) in those with the *JAK2* (V617F), with no significant difference between groups.

In PMF patients, death occurred in 12 out of 79 patients with type 1-like *CALR* mutation (15.2%), 4 out of 14 with type 2-like *CALR* mutation (28.6%), and 58 out of 232 with *JAK2* (V617F) (25%). Median OS was 17.7 years in type 1-like *CALR*-mutated, 15.5 years in type 2-like *CALR*-mutated and 12.8 years in *JAK2*-mutated patients, with a survival rate at 10 years of 85.7% (95% CI, 71.7–93.1), 84.4% (95% CI, 50.4–95.9) and 58.6% (48.3–67.6), respectively. Patients carrying a type 1-like *CALR* mutation had a better OS compared with those carrying *JAK2* (V617F) (*JAK2* vs type 1-like: hazards ratio (HR) 2.4, 95% CI 1.3–4.6, $P = 0.007$), while there was no difference between patients with type 1-like and those

Table 2. Main clinical and hematological features of patients with essential thrombocythemia stratified according to their driver mutation

	JAK2 (V617)	*Type 1-like CALR mutation*	*Type 2-like CALR mutation*	*Comparisons (P-value)*		
				JAK2 vs type 1-like CALR mut	*JAK2 vs type 2-like CALR mut*	*Type 1-like vs type 2-like CALR mut*
Patient no.	567	124	84			
Age at onset, years, median (range)	50 (15–92)	45 (15–88)	40 (19–91)	0.094	< 0.001	0.049
Hemoglobin, g/dl, median (range)	14.3 (10–17.7)	13.8 (10.7–17.6)	13.8 (9.2–16.5)	0.002	< 0.001	0.411
WBC count, $x10^9$/l, median (range)	9.2 (3.8–62.2)	7.9 (4–17.5)	8.1 (4.3–17.9)	< 0.001	< 0.001	0.945
PLT count, $x10^9$/l, median (range)	700 (456–2148)	832 (502–3000)	982 (500–2670)	< 0.001	< 0.001	0.027
Patients with thrombosis at diagnosis, no. (%)	48 (8%)	7 (6%)	0	0.293	0.006	0.027

Abbreviations: PLT, platelet; WBC, white blood cell.

Table 3. Main clinical and hematological features of patients with primary myelofibrosis stratified according to their driver mutation

	JAK2 (V617F)	*Type 1-like CALR mutation*	*Type 2-like CALR mutation*	*Comparisons (P-value)*		
				JAK2 vs type 1-like CALR mut	*JAK2 vs type 2-like CALR mut*	*Type 1-like vs type 2-like CALR mut*
Patient no.	232	79	14			
Age at onset, years, median (range)	60 (18–86)	47 (27–75)	54 (24–76)	< 0.001	0.139	0.422
Hemoglobin, g/dl, median (range)	12.2 (5.5–17.9)	11.8 (7.1–15.7)	12.3 (7.1–15.9)	0.316	0.952	0.375
WBC count, $x10^9$/l, median (range)	9.7 (1.6–54)	7.6 (2.2–27)	8.6 (5.4–13.5)	0.008	0.444	0.257
PLT count, $x10^9$/l, median (range)	350 (38–1963)	492 (89–1679)	745 (46–1463)	< 0.001	0.012	0.288
Circulating CD34+ cells, $x10^6$/l, median (range)[a]	16.1 (0.8–1190.2)	34.1 (0.2–1902)	25.2 (1.7–974.7)	0.030	0.496	0.924
IPSS risk group, no. (%)				0.115	0.558	0.647
Low	87 (43.5%)	42 (60%)	7 (53.8%)			
Intermediate 1	58 (29%)	13 (18.6%)	3 (23.1%)			
Intermediate 2	34 (17%)	10 (14.3%)	3 (23.1%)			
High	21 (10.5%)	5 (7.1%)	0			

Abbreviations: IPSS, International Prognostic Score System; PLT, platelet; WBC, white blood cell. [a]Data were available for 114 patients carrying *JAK2* (V617F), 41 with type 1-like *CALR* mutation, and 6 with type 2-like *CALR* mutation.

with type 2-like *CALR* mutation (type 2-like vs type 1-like: HR 1.4, 95% CI 0.4–4.4 $P = 0.562$), and between patients with *JAK2* (V617F) and those with type 2-like *CALR* mutation (*JAK2* vs type 2-like: HR 1.7, 95% CI 0.6-4.8, $P = 0.294$). These results were confirmed after adjusting for International Prognostic Score System, with a better OS for patients with type 1-like *CALR* mutation compared with those with *JAK2* (V617F) (*JAK2* vs type 1-like: HR 3.4, 95% CI 1.4–8.3, $P = 0.009$), and no difference between patients with type 1-like and those with type 2-like *CALR* mutation (type 2-like vs type 1-like: HR 2.3, 95% CI 0.6–8.3, $P = 0.213$), as well as between patients with type 2-like *CALR* mutation and those with *JAK2* (V617F) (*JAK2* vs type 2-like: HR 1.5, 95% CI 0.5–4.5, $P = 0.491$).

Leukemic transformation was observed in 16 patients with ET and in 46 with PMF. In ET, the 10-year cumulative incidence of leukemic transformation was 0% in patients with type 1-like *CALR* mutation, 0% in those with type 2-like *CALR* mutation, and 5.4% (95% CI, 3.2–8.4) in those with *JAK2* (V617F), with no significant difference among the three molecular subgroups. In PMF, the 10-year cumulative incidence of leukemic transformation was 19.2% (95% CI, 12.2–27.5) in patients with *JAK2* (V617F), 12.2% (95% CI, 4.2–24.8) in those with type 1-like *CALR* mutation, and 7.1% (95% CI, 0.5–27.5) in those with type 2-like *CALR* mutation; there was no significant difference between these subgroups.

In ET patients, we evaluated the impact of driver mutation on the risk of thrombotic events and myelofibrotic transformation. The 10-year cumulative incidence of thrombotic events was 14.9% (95% CI, 7.2–25.2) in patients with type 1-like *CALR* mutation, 4.3% (95% CI, 0.7–13.4) in those with type 2-like *CALR* mutation, and 20.6% (95% CI, 15.8–25.7) in those with *JAK2* (V617F) (Figure 3a). Patients carrying *JAK2* (V617F) had a higher risk of thrombosis compared with those with type 2-like *CALR* mutation (subdistribution hazard ratio (SHR) 5.3, 95% CI, 1.7–16.8, $P = 0.004$).

The 10-year cumulative incidence of myelofibrotic transformation was 12.9% (95% CI, 4.9–24.8) in ET patients with type 1-like *CALR* mutation, 0% in those with type 2-like *CALR* mutation and 3.3% (95% CI, 1.5–6.4) in those carrying *JAK2* (V617F) (Figure 3b). ET patients with type 1-like *CALR* mutation showed a higher risk of myelofibrotic transformation compared with either those with type 2-like *CALR* mutation (SHR 7.8, 95% CI 1.8–34.4, $P = 0.007$) or those carrying *JAK2* (V617F) (SHR 5.4, 95% CI 2.5–11.6, $P < 0.001$), while no difference was observed between patients with *JAK2* mutant ET and those with type 2-like *CALR* mutant ET (SHR 1.4, 95% CI 0.3–6.3, $P = 0.627$).

DISCUSSION

Somatic mutations of *CALR* exon 9 are found almost exclusively in patients with myeloid neoplasms associated with thrombocytosis, that is, ET, PMF and refractory anemia with ring sideroblasts associated with marked thrombocytosis.[12] This observation and the finding that *CALR*-mutant MPNs are characterized by a gene signature associated with activated JAK2 signaling[34] strongly suggests that the mutant calreticulin activates the JAK-STAT pathway through the thrombopoietin receptor, thereby leading to excessive platelet production. As shown in Tables 2 and 3, we found only minor differences in platelet counts between patients with ET or PMF carrying different *CALR* mutation subtypes, suggesting that there should be no major differences between these subtypes in terms of abnormal MPL-JAK-STAT signaling.

Calreticulin binds Ca^{2+} ions in the ER, rendering these ions inactive. In our initial work on *CALR* mutation, we already suggested that loss of calcium binding activity of mutant calreticulin and higher levels of cytoplasmic calcium might contribute to the pathogenesis of MPN.[12] A bioinformatic analysis of *CALR* mutations has indeed shown that only +1 frameshift mutations (that is, those observed in MPN patients) exhibit a positively charged C-terminus.[35] Interestingly, type 1 *CALR* mutation and more generally exon 9 deletions create mutant proteins with more altered calcium binding activity.[35,36] Since cytoplasmic Ca^{2+} levels regulate human Mk[22] and platelet

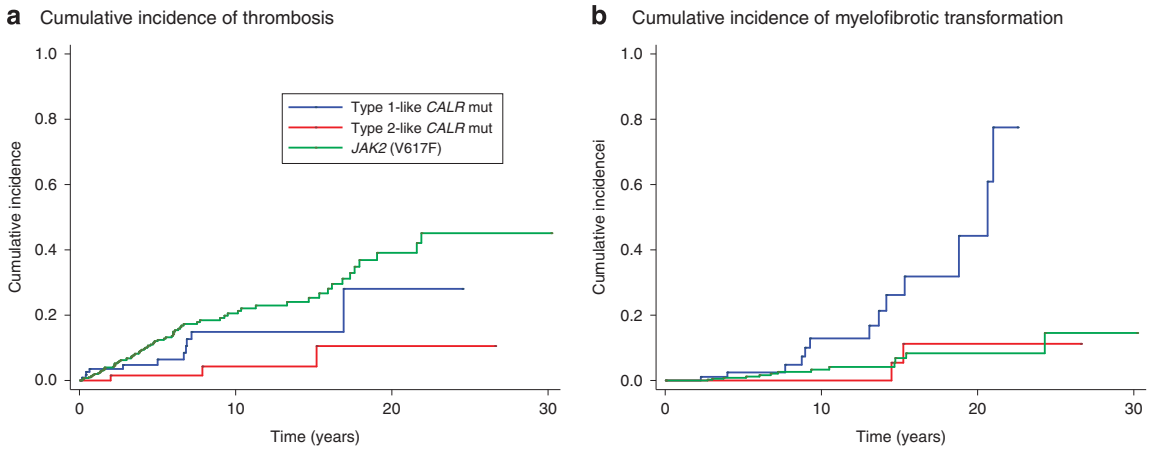

Figure 3. Cumulative incidence of thrombosis and myelofibrotic transformation in patients with essential thrombocythemia carrying *JAK2* (V617F), type 1-like *CALR* mutation, or type 2-like *CALR* mutation. (**a**) The 10-year cumulative incidence of thrombotic events was 20.6% (95% CI, 15.8–25.7) in patients with *JAK2* (V617F), 14.9% (95% CI, 7.2–25.2) in those with type 1-like *CALR* mutation and 4.3% (95% CI, 0.7–13.4) in those with type 2-like *CALR* mutation. ET patients carrying *JAK2* (V617F) had a higher risk of thrombosis compared with those carrying type 2-like *CALR* mutation (SHR 5.3, 95% CI, 1.7–16.8, $P = 0.004$), while there was no significant difference compared with those carrying type 1-like *CALR* mutation (SHR 1.6, 95% CI, 0.9–3.1, $P = 0.135$). Patients with type 2-like *CALR* mutation showed a trend towards a lower risk of thrombosis compared with those carrying type 1-like *CALR* mutation (SHR 0.3, 95% CI 0.1–1.1, $P = 0.067$). To compare the estimates of this study with those of our previous work,[15] we calculated also values at 15 years. The 15-year cumulative incidence of thrombotic events was 25.3% (95% CI, 19.3–31.7) in patients with *JAK2* (V617F), 14.9% (95% CI, 7.2–25.2) in those with type 1-like *CALR* mutation, and 4.3% (95% CI, 0.7–13.4) in those with type 2-like *CALR* mutation. (**b**) The 10-year cumulative incidence of myelofibrotic transformation was 12.9% (95% CI, 4.9–24.8) in patients with type 1-like *CALR* mutation, 3.3% (95% CI, 1.5–6.4) in those carrying *JAK2* (V617F), and 0% in those carrying type 2-like *CALR* mutation. ET patients carrying type 1-like *CALR* mutation showed a higher risk of myelofibrotic transformation compared with both those carrying type 2-like *CALR* mutation (SHR 7.8, 95% CI 1.8–34.4, $P = 0.007$) and those with *JAK2* (V617F) (SHR 5.4, 95% CI 2.5–11.6, $P < 0.001$), while no difference was observed between *JAK2* mutated and type 2-like *CALR* mutated patients (SHR 1.4, 95% CI 0.3–6.3, $P = 0.627$). The 15-year cumulative incidence of myelofibrotic transformation was 26.2% (95% CI, 11.8–43.2) in patients with type 1-like *CALR* mutation, 6.9% (95% CI, 3.2–12.5) in those carrying *JAK2* (V617F) and 5.4% (95% CI, 0.4–22.0) in those carrying type 2-like *CALR* mutation.

function,[37] we reasoned that different *CALR* mutants might differentially contribute to the pathogenesis of MPN.

We first categorized the different *CALR* indels into three subgroups based on their predicted effect on three different stretches of negatively charged amino acids of the wild-type sequence (Figure 1). We then estimated the pI value of each mutant CALR peptide starting from codon A352 to validate our categorization of *CALR* mutants. As shown in Figure 1c, pI values were significantly different between the three groups of *CALR* mutation, while they were homogeneous within each group, thus supporting our categorization of *CALR* mutants.

The three categories showed significantly different frequencies in patients with ET compared with patients with PMF. In particular, the high proportion of PMF patients with type 1-like *CALR* mutation suggests that the MPN associated with this driver mutation is very likely to develop bone marrow fibrosis. By contrast, the MPN associated with type 2-like *CALR* mutation is less likely to undergo myelofibrotic transformation, and indeed only 15% of patients with *CALR*-mutant myelofibrosis carried this mutation subtype.

The two major subtypes of mutant calreticulin had different effects on calcium flows in cultured Mks (Figure 2). More specifically, the largest cytosolic Ca^{2+} mobilization and SOCE were found in Mks from patients with type 1 *CALR* mutation, that is, a mutation subtype in which the negatively charged amino acids of C-terminal region of calreticulin are almost completely replaced by both neutral and positively charged amino acids (Figure 1a). Accordingly, type 1 CALR mutants are also those with the highest estimated pIs (Figure 1c). The larger ER-dependent Ca^{2+} release observed in Mks from these patients is fully consistent with the impairment of the ER Ca^{2+}-storage ability caused by the loss of the ER Ca^{2+}-binding residues. In the presence of a Ca^{2+}-mobilizing stimulus, such as cyclopiazonic acid, Mks derived from type 1 *CALR* mutated patients release a significantly higher amount of Ca^{2+} as compared with cells obtained from patients with *JAK2* (V617F) or type 2 *CALR* mutation (Figure 2b).

The higher Ca^{2+} release associated to type 1 mutation was associated with an increase in SOCE amplitude. Whether the higher SOCE magnitude could be due to the higher ER Ca^{2+} mobilization or to the end tail modifications of calreticulin mutants still remains to be understood.[38] In principle, a larger ER Ca^{2+} depletion should lead to a larger SOCE activation. However, it is known that partial dissociation between Ca^{2+} mobilization and SOCE activation may occur.[39] Given that type 1, but not type 2 mutation results in the loss of stretches II and III at the C-terminus of calreticulin, which might be involved in SOCE development,[40] the differences observed between the two mutants could be because of their different ability to interact with SOCE machinery. Therefore, our hypothesis is that del52 may result in a lesser inhibition of the SOCE amplitude, thus resulting in the second higher peak of cytosolic Ca^{2+}.

CALR mutation subtype had a profound impact on clinical outcomes of patients with ET, as illustrated in Figure 3. Despite the fact that these individuals had the highest values for platelet count (Table 2), patients with type 2-like *CALR* mutation had the lowest risk of thrombosis, significantly lower than that of patients carrying *JAK2* (V617F) (Figure 3a). This observation reinforces the opinion that *JAK2* (V617F) represents the most thrombogenic driver mutation in MPN, and that the platelet count does not represent *per se* a predictive factor for thrombosis.[41]

We previously reported that the cumulative incidence of myelofibrotic transformation was not significantly different between *JAK2*-mutant and *CALR*-mutant ET.[15] This is true if the whole population of *CALR*-mutant patients is considered. However, by taking into account *CALR* variant subtypes, we have now found a significantly higher risk of myelofibrotic transformation in ET patients carrying type 1-like than in those carrying type 2-like *CALR* mutation. Since type 1-like *CALR* mutation is much more frequent in patients with PMF than in those with ET (Figure 1b), these observations support a unifying model in which *CALR*-mutant ET and *CALR*-mutant PMF represent different phenotypes/stages in the evolution of a *CALR*-mutant MPN.[14] The initial phenotype is necessarily ET (isolated thrombocytosis), since overproduction of platelets is faster than deposition of reticulin fibers in the bone marrow. Development of bone marrow fibrosis occurs with time, more actively in patients with type 1-like than in those with type 2-like *CALR* mutation, and this leads to myelofibrosis. This process has been clearly demonstrated in retroviral mouse models, in which CALR mutants were found to be able and sufficient to induce a thrombocytosis progressing to myelofibrosis, thus mimicking the natural history of MPN patients:[23] in particular, progression to a myelofibrosis was much more common in CALRdel52- than in CALRins5-expressing mice. Whether this difference in risk of myelofibrotic transformation is related to different degrees of abnormal cytosolic Ca^{2+} flows in Mks remains to be demonstrated, but our findings strongly support this notion. In the minority of ET patients with type 2-like *CALR* mutation who progress to myelofibrosis, this transformation is likely related to the occurrence of cooperating subclonal mutations.[42]

With respect to patients with PMF, we did not find any difference in terms of OS between patients carrying type 1-like and those carrying type 2-like *CALR* mutation. Tefferi *et al.*[43] previously reported survival data on 440 patients with PMF stratified by their *JAK2* and *CALR* mutation status. *CALR* mutations were sub-stratified into type 1/type 1-like and type 2/type 2-like, based on the helical propensity of their mutant CALR. Compared with *JAK2*-mutated cases, OS was longer in patients with type 1/type 1-like but not in those with type 2/type 2-like *CALR* mutation. Although this study suggests a better prognosis for patients with type 1 *CALR* mutation, Cabagnols *et al*[44] found that type 1 *CALR* mutation is associated with a shorter survival in PMF. All these studies—including ours—have analyzed relatively small patient populations, and larger studies that take into account also subclonal mutations are needed to define the prognostic significance of mutation subtypes in *CALR*-mutant PMF.

In conclusion, this study shows differential clinical effects of mutation subtypes in *CALR*-mutant MPNs. Type 2-like *CALR* mutations are mainly associated with an ET phenotype, low risk of thrombosis and indolent clinical course, while type 1-like mutations are mainly associated with a myelofibrosis phenotype and a high risk of progression from ET to myelofibrosis. The different clinical outcomes should be taken into account in clinical-decision making.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

MC, DP and ERu conceived this study, collected and analyzed data and wrote the manuscript; CADB, VA, FM and AB performed megakaryocyte studies; CC, ESA, ICC, MB, ERo, CA, VR and GB collected clinical data; CM and MCR did molecular investigations; VVF and EF did statistical analyses; EB studied bone marrow biopsies.

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