Annals of Hematology (2019) 98:2339–2346
https://doi.org/10.1007/s00277-019-03741-8

ORIGINAL ARTICLE

# CAL2 monoclonal antibody is a rapid and sensitive assay for the detection of calreticulin mutations in essential thrombocythemia patients

Massimiliano Bonifacio[1] · Rachele Montemezzi[1] · Alice Parisi[2] · Giovanna De Matteis[3] · Roberta Bertorelle[4] · Luigi Scaffidi[1] · Cinzia Candiotto[4] · Giuseppe Lippi[3] · Alberto Zamò[5] · Marco Chilosi[6] · Giovanni Pizzolo[1] · Aldo Scarpa[2] · Mauro Krampera[1]

Received: 23 February 2019 / Accepted: 15 June 2019 / Published online: 27 June 2019


## Abstract

Calreticulin (*CALR*) mutations are detected in the majority of *JAK2 wild type* patients with essential thrombocythemia (ET). Unlike $JAK2^{V617F}$ and *MPL* point mutations, *CALR* mutations are highly heterogeneous, with several types of indels being reported so far. CAL2 is a monoclonal antibody specifically recognizing the C-neoterminal peptide derived from all the frameshift mutations of *CALR*. We retrospectively analysed 172 ET patients diagnosed at our Institution from 1980 to 2015. In $JAK2^{V617F}$- and $MPL^{W515K/L}$-*wild type* patients *CALR* mutations were searched on peripheral blood and CAL2 immunostaining was performed on bone marrow. In addition, bone marrow biopsies were histologically reviewed for megakaryocytic features. Thirty-one patients (18%) were *CALR*-mutated. Concordance between molecular and immunohistological detection of *CALR* mutations was near complete, albeit a single patient was found to be positive by molecular tests only. Two patterns were defined in CAL2-positive bone marrow samples, characterized by staining of almost only megakaryocytes (pattern A: 41%) or staining of megakaryocytes and ≥ 2% small non megakaryocytic elements (pattern B: 59%), at least partially being myeloid precursors. Pattern B biopsies had higher cellularity and number of megakaryocytes compared to pattern A samples. In this series, CAL2 allowed rapid and cost-efficient identification of *CALR*-mutated ET patients. The biological significance of different staining pattern should be confirmed in wider and independent series.

**Keywords** Essential thrombocythemia · Calreticulin · CAL2 · Driver mutations · Diagnostic tools

**Electronic supplementary material** The online version of this article (https://doi.org/10.1007/s00277-019-03741-8) contains supplementary material, which is available to authorized users.

✉ Massimiliano Bonifacio
massimiliano.bonifacio@univr.it

[1] Department of Medicine, Section of Hematology, University of Verona, P.le L.A.Scuro 10, Verona 37134, Italy

[2] Department of Diagnostics and Public Health, Section of Pathological Anatomy, University of Verona, Verona, Italy

[3] Department of Diagnostics and Public Health, Section of Clinical Biochemistry, University of Verona, Verona, Italy

[4] Immunology and Molecular Oncology, Veneto Institute of Oncology IOV-IRCCS, Padua, Italy

[5] Department of Oncology, Section of Pathological Anatomy, University of Turin, Turin, Italy

[6] Department of Pathology, Pederzoli Hospital, Peschiera del Garda, Verona, Italy

## Introduction

Essential thrombocythemia (ET) is a myeloproliferative neoplasm (MPN) caused by deregulation of megakaryocytic proliferation. It is typically characterized by long-lasting, indolent course, although vascular events and evolution to more aggressive myeloid neoplasms (myelofibrosis and acute leukaemia) may severely affect the prognosis [1]. Constitutive activation of Janus kinase 2 (*JAK2*) by a point mutation in exon 14 (V617F) represents the most common driver of ET, being detectable in approximately 60% of patients. Point mutations in the myeloproliferative leukaemia virus (*MPL*) gene are present in about 3–5% of ET patients. It has been recently shown that the majority of *JAK2* and *MPL wild type* (wt) ET patients carry a mutation in the calreticulin (*CALR*) gene, encoding for an endoplasmic reticulum protein involved in the calcium metabolism and intracellular protein folding [2, 3]. The significance of this finding has been highlighted in the

2016 revision of World Health Organization (WHO) criteria, which recognize the presence of one of the three driver mutations as a major criterion for diagnosing ET [4]. Strict pathological criteria are also defined for accurate histological differentiation of MPN, particularly for distinguishing ET from early myelofibrosis (MF). A correct molecular and pathological diagnosis of ET has several clinical implications, since ET is associated with significant differences in vascular events according to the driver mutation [5] and different rates of evolution to overt MF according to histological classification [6].

Over 60 different mutations in *CALR* gene have been identified so far, the most common being a deletion of 52 bp (L367 fs*46, or type-1 mutation) and an insertion of 5 bp (K385 fs*47, or type-2 mutation) which altogether represent about 85% of the cases. Unlike the $JAK2^{V617F}$ and $MPL^{W515L/K}$ point mutations, commonly detected by allele-specific polymerase chain reaction (PCR), a full analysis of *CALR* mutations could encompass expensive and time-consuming sequencing techniques. However, all mutations described so far are associated with the synthesis of a C-terminal neopeptide, in which negatively charged amino acids are replaced by positively charged or neutral residuals, which can be detected with immunohistochemistry (IHC) [7, 8]. Although both polyclonal and monoclonal antibodies have been proven effective in detecting *CALR* mutated protein, experience is still limited, and all available studies include both ET and MF cases [7–10]. Moreover, albeit *CALR* mutations are preferentially expressed in megakaryocytes, scant information is available on bone marrow (BM) morphological aspects of *CALR*-mutated MPN.

Therefore, we retrospectively analysed ET patients diagnosed at our Institution from 1980 to 2015, with the aim to compare molecular biology and IHC in detecting *CALR* mutations and investigating specific histomorphological characteristics of BM in *CALR*-mutated patients.

## Methods

### Patients

All patients consecutively diagnosed with ET at our Institution between 1980 and June 2015 were considered for this study. We identified a total of 180 patients, 124 carrying $JAK2^{V617F}$ or $MPL^{W515K/L}$ mutations and 56 with $JAK2^{wt}/MPL^{wt}$. Five patients were excluded from further analysis because clinical and laboratory data at diagnosis were unavailable. Three other patients ought to be excluded because neither BM specimen nor peripheral blood (PB) DNA collected at diagnosis were available. Overall, 172 patients were hence included in this study. The age at diagnosis was defined in individual patients as the age of the first documented and confirmed detection of thrombocytosis in the absence of other causes [4]. Post-ET MF and acute leukaemia were defined according to the International Working Group for MPN Research and Treatment (IGW-MRT) and WHO criteria, respectively [11]. All patients provided written informed consent for participation in this retrospective study, which was reviewed and approved by the local Institutional Review Board.

### Molecular biology

Molecular testing for $JAK2^{V617F}$ mutation became available in 2008. Therefore, 114 patients diagnosed between 1980 and 2007 were tested at the first follow-up visit after introduction of the assay, and 58 patients diagnosed from 2008 onwards were tested at the time of diagnosis. Genomic DNA was extracted from granulocytes isolated from PB after centrifugation with Ficoll gradient (Amersham Biosciences – GE Healthcare, USA) using commercially available extraction reagents (Puregene Blood Kit – Qiagen, Hilden, Germany), and $JAK2^{V617F}$ mutation status was evaluated at the time of PB collection using allele-specific qualitative PCR as previously described [12]. $JAK2^{wt}$ patients were tested for $MPL^{W515K/L}$ exon 10 mutations by qualitative ARMS PCR (home made primers). *CALR* exon 9 mutations were subsequently searched by PCR in $JAK2^{wt}/MPL^{wt}$ patients, using primers described by Klampfl et al. [2] and analysing PCR products by agarose gel electrophoresis. To improve detection sensitivity, samples with discordant results between molecular biology and IHC were re-analysed by capillary electrophoresis sequencer on the ABI Prism 3500DX Genetic Analyser (Applied Biosystems, Foster City, CA, USA) using the same set of primers marked with fluorescent dye (fragment analysis). The same samples were then characterized by Sanger Sequencing. *CALR* exon 9 was bi-directionally sequenced by using the Big Dye Terminator v1.1 Cycle Sequencing Kit on the ABI Prism 3730 Genetic Analyser (Applied Biosystems, Foster City, CA, USA). Sequences were aligned to the *CALR* refseq NM_004343.3 and assigned using HGVS nomenclature.

### Bone marrow morphology and IHC

IHC staining was performed on all available BM samples of $JAK2^{wt}/MPL^{wt}$ patients, regardless of stage of disease at the time of collection. IHC analysis was carried out on 4-μm histological sections of paraffin blocks from formalin-fixed decalcified BM biopsies. Sections underwent antigen retrieval at pH 8.9, 95 °C for 30 min, followed by a 1-h incubation with 1:50 diluted antibody. We used the commercially available monoclonal mouse IgG2a type antibody (CAL2, Optistain, Bioreal) [7], which identifies the neo C-terminal peptide of *CALR*-mutated protein. A polymer system was used as detection system (Bond Polymer Refine Detection, Leica). In

CAL2-positive samples, two patterns of positivity were defined:

- pattern A: positivity in megakaryocytes and in < 2% other (non-MK) cells (out of at least 2500 BM cells);
- pattern B: positivity in megakaryocytes and in ≥ 2% other (non-MK) cells (out of at least 2500 BM cells).

Two samples were also double stained with CAL2 and MNDA (myeloid cell nuclear differentiation antigen, 235A clone, sections underwent antigen retrieval at pH 8.9, 95 °C for 15 min, followed by a 1-h incubation with 1:500 diluted antibody) [13], for assessing cell lineage of small elements. A blind review of all $JAK2^{wt}/MPL^{wt}$ samples was carried out to reclassify patients as ET or prePMF according to the 2016 WHO classification [4], especially evaluating the following parameters: cellularity, myeloid and erythroid hyperplasia, number of megakaryocytes, megakaryocyte morphology (giant, hypersegmented), presence of loose clusters, percentage of CD34+ cells, presence of vessels and degree of fibrosis, considering the highest score of reticulin fibrosis present in at least 30% of BM biopsy as recently suggested by Kvasnicka et al. [14].

### Statistical analysis

The Cohen's kappa test was used to assess concordance between *CALR* exon 9 mutations and IHC detection. Difference in distribution of continuous variables between categories was analysed by either Mann-Whitney or Kruskal-Wallis test, when appropriate. Frequency for categorical variables was compared by $\chi^2$ test. Survival curves were constructed using the Kaplan-Meier approach. Statistical analysis was performed using the software "R" [15].

## Results

### Concordance between molecular biology and IHC in detecting *CALR* mutations

Fifty-one $JAK2^{wt}/MPL^{wt}$ patients were evaluated for *CALR* exon 9 mutations. For 42 of these patients, both frozen DNA and BM biopsy samples were available. PCR and IHC were concordantly positive or negative in 23 and 15 cases, respectively (Cohen's kappa index > 0.80); however, three patients were found to be positive for CAL2 immunostaining only, and one patient by PCR and gel electrophoresis only. To improve the sensitivity, these patients were studied with fragment analysis and Sanger sequencing, and they were all found harbouring a mutation in the *CALR* gene: specifically, of the three patients positive only by CAL2, one had a type-1 mutation at low level and two had atypical indels (c.1153_1154delinsTGTC; p.K385 fs*46 and c.1111_1150delinsTCA; p.E371fs*47, respectively), while the single patient who resulted positive by fragment analysis and negative by CAL2 had a 22 bp type-1-like deletion (c.1121_1142del; p.K374 fs*49).

### Characteristics of patients

Median age at diagnosis was 54 years (range 14–87). Seventy-one patients (41%) were aged 60 or older and ten had a history of previous thrombotic events. Median time from the first evidence of thrombocytosis to BM biopsy was 8.4 months (range 0–175 months).

According to driver mutations, patients were classified as $JAK2^{V617F}$-mutated ($n = 119$: 69%), $MPL^{W515K/L}$-mutated ($n = 2$: 1%), *CALR*-mutated ($n = 31$: 18%) or triple-negative ($n = 20$: 12%).

The median follow-up time for the entire cohort was 9.8 years (range 0.1–35.5 years). The estimated myelofibrosis-free, leukaemia-free and overall survival at 10 years were 95%, 98% and 95%, respectively.

### Comparison of type I and type II *CALR*-mutated patients

*CALR* mutations were detected by exon 9 PCR and/or fragment analysis followed by Sanger sequencing in 27 patients. No differences were found in clinical presentation and outcome among patients with type-1 ($n = 15$: 56%), type-2 ($n = 7$: 26%) or other, uncommon indels ($n = 5$: 18%) (Table 1).

### CAL2 positivity patterns in BM samples

We retrieved a BM sample collected at ET diagnosis in 27 *CALR*-mutated patients (the other having performed BM only at myelofibrotic evolution). All samples fulfilled the 2016 WHO criteria for ET and displayed normal age-adjusted BM cellularity (Supplemental Table 1). In all samples > 90% of megakaryocytes were stained by CAL2, with a range of positivity from strong to weak. In 11 samples, we found that almost only megakaryocytes were stained by CAL2 monoclonal antibody (pattern A), whereas in 16 samples, also small non-megakaryocytic elements were CAL2-positive (pattern B) (Fig. 1). According to our definition, these small elements were ≥ 2% of the total BM cells (median 2%, range 2–5%). Notably, all biopsies performed at fibrotic evolution displayed pattern B. To better understand the nature of these small-sized, non-megakaryocytic elements, a double immunostaining with CAL2 and MNDA in two pattern B BM samples was performed. Some of these cells, morphologically similar to myeloblasts or monoblasts, were stained by both antibodies, thus allowing their recognition as myeloid precursors (Fig. 2), whilst the majority of myeloid cells were stained by MNDA

**Table 1** Characteristics of patients according to the type of *CALR* mutation

| | Type-1 mutations | Type-2 mutations | Other mutations | *p* |
|---|---|---|---|---|
| No. of patients | 15 | 7 | 5 | |
| Median age, years (range) | 49 (28–71) | 52 (42–78) | 55 (33–62) | 0.69 |
| Gender, F (%) | 7 (47) | 5 (71) | 2 (40) | 0.58 |
| Median Hb, g/dl (range) | 13.9 (11.4–16.3) | 12.8 (10.9–15.8) | 12.6 (11.2–15.3) | 0.42 |
| Median Plts, $\times 10^9$/mmc (range) | 626 (461–1583) | 860 (686–1544) | 863 (548–914) | 0.16 |
| Median WBC, $\times 10^9$/mmc (range) | 7.5 (4.4–15.2) | 7.9 (5.6–9.5) | 7.5 (7.3–9.2) | 0.44 |
| Splenomegaly (%) | 3 (20) | 1 (14) | 1 (20) | 1 |
| Thrombosis (%) | 1 (7) | 0 | 0 | 1 |
| Myelofibrotic evolution (%) | 3 (20) | 2 (29) | 0 | 0.67 |
| Leukemic evolution (%) | 0 | 1 (14) | 0 | 0.44 |

but not by CAL2 (negative internal control). To ensure that these elements were not micromegakaryoblasts, we performed in random samples a double staining with CD61 and MNDA, without finding any double positivity (data not shown).

The histological features of BM samples according to the CAL2 pattern were then compared. Pattern B biopsies had higher median cellularity (50% vs 35%, $p = 0.019$), higher median number of megakaryocytes per high power field (15 vs 9, $p = 0.016$) and a trend towards a higher frequency of loose aggregates (87% vs 64%, $p = 0.19$) compared to pattern A samples (Table 2). Pattern B patients also displayed a non-significant trend towards having higher median platelet count at diagnosis ($753 \times 10^9$/L vs $578 \times 10^9$/L, $p = 0.07$) and were associated to a more heterogeneous spectrum of *CALR* mutations, since type-1, type-2 and atypical indels were present in 3, 6 and 4 pattern B patients, respectively, while all pattern A patients harboured a type-1 *CALR* mutation (Table 3). One patient with pattern A and 2 patients with pattern B progressed to post-ET MF or acute leukaemia at 6, 7.8 and 8.1 years after ET diagnosis, respectively.

## Discussion

The 2016 revision of WHO criteria emphasized the crucial role of *JAK2*, *MPL* and *CALR* mutations as drivers of all "classic" MPN. An integrated approach between clinical, histological and molecular data is hence necessary for accurate diagnosis, classification and prognostication of these diseases.

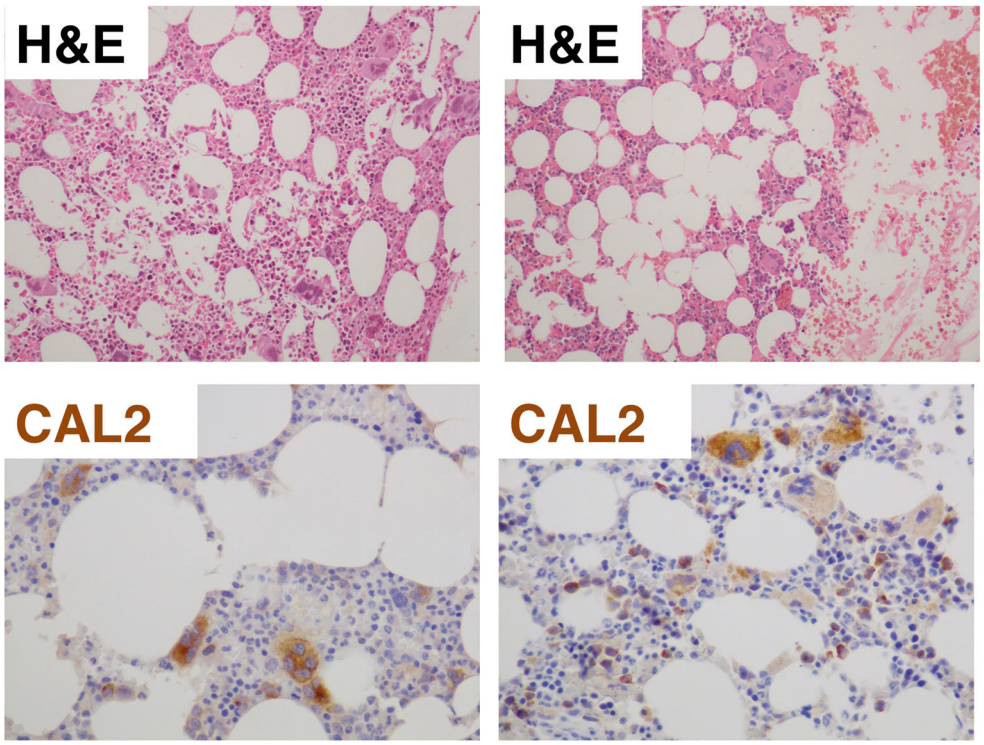


**Fig. 1** Patterns of expression of the CAL2 monoclonal antibody in bone marrow samples at ET diagnosis. In cases defined as pattern A, almost only megakaryocytes were positive for CAL2 staining, and no or less than 10 per HPF small non-MK cells were stained (left panel, H&E × 10, CAL2 × 40), while in pattern B samples, both megakaryocytes and small non-MK cells were stained (right panel, H&E × 10, CAL2 × 40). HPF, high power field, × 40

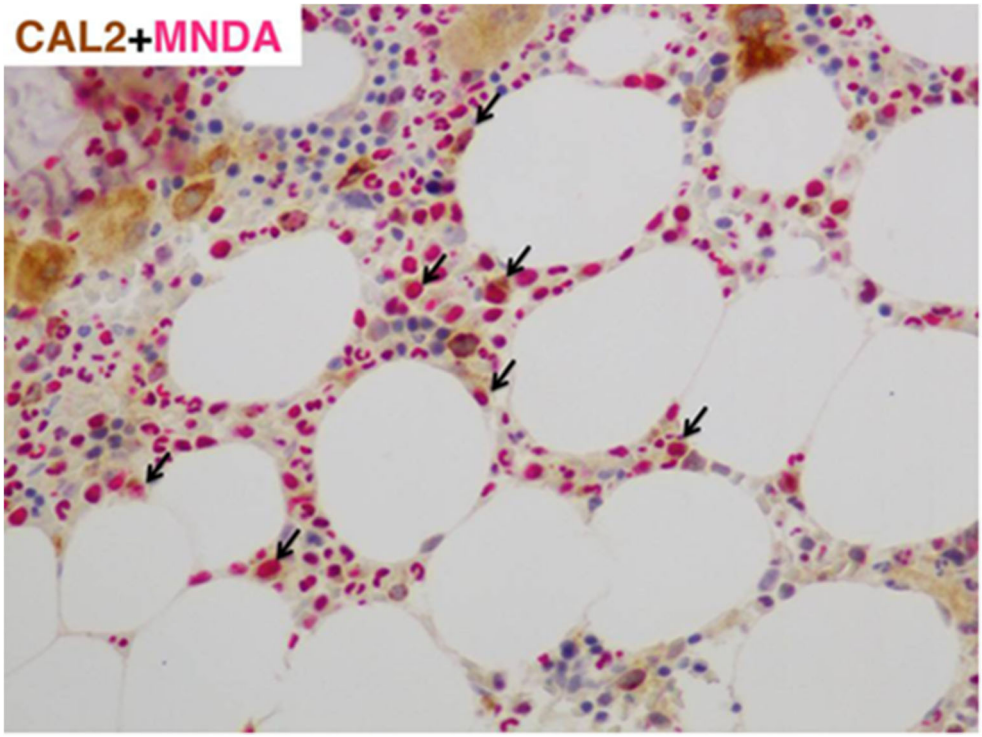


**Fig. 2** Double immunostaining of a pattern B BM sample with CAL2 (brown, cytoplasmatic staining) and MNDA (pink, nuclear staining) monoclonal antibodies. MK are stained with CAL2 only as brown cytoplasmatic positivity, while at least some smaller elements are stained by both antibodies (black arrows), thus supporting their myeloid origin

*JAK2*$^{V617F}$ and *MPL* exon 10 mutations are single nucleotide substitutions, easily identified using specific and reproducible molecular biology techniques. On the contrary, the spectrum of *CALR* mutations is very broad, including more than 60 reported variants [16], so that identification of all possible *CALR* mutations requires expensive, cumbersome and not always available molecular techniques. All *CALR* frameshift mutations result in a novel C-terminal peptide with identical antigenic characteristics regardless of the type of mutation, thus being identifiable with monoclonal antibodies [7, 8]. After the pivotal demonstration by Stein et al. in a research laboratory setting, two recent works showed that IHC staining with the commercially available CAL2 antibody is a feasible, sensitive and highly specific approach for detecting *CALR*-mutated protein in MPNs [9, 10]. Unlike these series, we focused on ET patients, due to preferential association of *CALR* mutations with megakaryocytic lineage and lack of histomorphologic information about megakaryocytes in *CALR*-mutated ET patients.

**Table 2** Characteristics of BM samples according to the CAL2 pattern

| | Pattern A | Pattern B | *p* |
|---|---|---|---|
| No. of patients | 11 | 16 | |
| Median cellularity, % (range) | 35 (25–55) | 50 (30–70) | 0.019 |
| Median MK number per HPF (range) | 9 (5–20) | 15 (5–20) | 0.016 |
| Loose aggregates (%) | 7 (64) | 14 (87) | 0.187 |
| Hypersegmented and giants MK (%) | 8 (73) | 13 (81) | 0.662 |
| Grade I fibrosis (%) | 4/10 (40) | 8/13 (62) | 0.414 |

Although the concordance between molecular biology and IHC was found to be optimal in our study (Cohen's kappa index > 0.80), it did not reach 100% as earlier reported by Stein et al. [7]. In our series, three patients were positive at IHC and negative at *CALR* exon 9 PCR and electrophoresis: to solve this discrepancy, it was necessary to perform exon 9 fragment analysis followed by gene sequencing since it is well known that different genetic screening methods may help in detecting different *CALR* mutations, with targeted NGS being the most sensitive technique [17]. In many large series of MPN patients, gene sequencing always identified new *CALR* mutations not previously described at that time, and the percentage of patients with unclassifiable mutations (neither type-1 nor type-2) was approximately 10–15% of *CALR*-mutated patients [18–20], as in the present work. The significance of the single false negative case at IHC is uncertain, but a similar case was earlier reported by Andrici et al. [9]. Our patient harboured a 22-bp deletion *CALR* mutation which might have led to a defective transcript and therefore to a mutated protein not recognizable by CAL2 staining. Notably, Butylin et al. identified a complex *CALR* mutation variant in one ET case, leading to an alternative C-end of the protein [21].

One of the advantages of molecular biology over IHC is the possibility to differentiate patients with type-1 and type-2 *CALR* mutations. However, the clinical and prognostic significance of this separation is still debated and the published

**Table 3** Clinical characteristics of patients according to the CAL2 pattern on BM samples

| | Pattern A ($n = 11$) | Pattern B ($n = 16$) | $p$ |
|---|---|---|---|
| Gender (M/F) | 6 / 5 | 8 / 8 | 1 |
| Median age, years (range) | 62 (28–72) | 52 (38–78) | 0.399 |
| Median haemoglobin, g/dL (range) | 13.9 (12.8–15.3) | 13.5 (10.9–16.3) | 0.528 |
| Median platelets, $\times 10^9$/mmc (range) | 578 (461–1074) | 753 (477–1544) | 0.069 |
| Median WBC, $\times 10^9$/mmc (range) | 7.3 (4.4–11.7) | 7.7 (6.0–15.2) | 0.561 |
| *CALR* mutation (type-1/type-2) | 10/0 | 3/6 | 0.003 |

information is still controversial. In a clinical study including 216 *CALR*-mutated ET patients, type-2 mutations were associated with higher platelet count, lower risk of thrombosis and clinically indolent course [22]. Conversely, type-2 mutations were found to be associated with more abnormal megakaryocytic features and greater risk of myelofibrotic evolution in a recent histopathological study [23]. These heterogeneous results could be probably explained by the random distribution of patients with prefibrotic MPN in different series. However, the molecular definition of the *CALR* mutation type does not seem currently necessary in clinical practice [24]. Notably, the 2018 revised management recommendations from European LeukemiaNet do not consider the type of *CALR* mutation as a prognostic factor in ET, whilst recognizing the usefulness of this distinction only in myelofibrosis [25].

A second advantage of molecular analysis may be the possibility of quantitative analysis of *CALR*-mutated allele burden. The impact of mutated $JAK2^{V617F}$ allele burden on prognosis of PV and MF patients (and in those with ET, to a lesser extent) has been clearly demonstrated [26]. Although there is still no clear evidence about the usefulness of quantitative determination of *CALR* mutational load, preliminary data showed clonal reduction in patients treated with interferon-alpha [27] and a potential role in monitoring patients after allogeneic stem cell transplantation [28].

Simplicity, rapidity and lower costs compared to molecular techniques are the leading advantages of IHC staining. It also appears reasonable, wherever possible, to evaluate both morphological and IHC features of BM samples in patients with suspected MPN. We identified and defined two patterns of CAL2 antibody expression, the former characterized by almost only megakaryocytic staining (pattern A: 41% of cases) and the latter by staining of megakaryocytes and small non-megakaryocytic elements (pattern B: 59% of cases). The presence of small cells stained by CAL2 antibody has been previously noted by others [7–9]. Unfortunately, their origin remained uncertain, and they have been hypothesised to be small immature megakaryocytes or sporadic myelo-erythroid precursors, with very weak intensity of antibody expression. We showed that the small elements are at least in part of genuine myeloid origin, as demonstrated by staining with MNDA. This is consistent with the work of Nangalia et al., suggesting that *CALR* mutations, although preferentially associated to the expansion of the megakaryocytic lineage, occur in a multipotent progenitor capable of generating also erythroid and myeloid progeny [3].

Our small sample size precluded us to observe striking differences between the two groups. However, pattern B cases had higher median cellularity (anyway always in the range of normal age-adjusted cellularity) and higher number of megakaryocytes. Two patients with pattern B and one patient with pattern A developed leukemic or myelofibrotic evolution. In addition, all three patients whose bone marrow biopsy was only available at the time of fibrotic evolution showed a pattern B. The possibility that the CAL2 antibody pattern of expression could have prognostic significance is intriguing, as well as the possible association of pattern B to a more heterogeneous spectrum of *CALR* mutations (type-1, type-2 and atypical indels). Although our data do not allow making any definitive conclusion, the distinction between the two patterns should allow easy reproducibility of this classification on independent series, which may ultimately validate our hypothesis. Furthermore, evaluation of paired samples of biopsies at the time of ET diagnosis and post-ET MF could be useful in defining if CAL2 pattern of expression could be considered a novel biomarker.

In conclusion, we confirmed that CAL2 staining is a sensitive, specific, rapid and economic technique for detecting *CALR* mutations in patients with suspected ET. Along with histological evaluation of BM characteristics, IHC staining allows to make a definite diagnosis according to the updated 2016 criteria. As proposed originally by Vannucchi [8], our data support the hypothesis of a two-step diagnostic approach to suspected ET, initially based on IHC evaluation of BM and then followed, in CAL2-negative patients showing histological criteria of MPN, by molecular biology search for $JAK2^{V617F}$ and finally for *MPL* mutations.

**Authorship** M.B. and M.C. designed the study; M.B., R.M. and L.S. treated patients, analysed the data and wrote the manuscript; A.P. and A.Z. performed histopathological tests and analysed the data; G.D.M., R.B. and C.C. performed molecular biology tests; G.L., G.P., A.S. and M.K. critically revised the manuscript. All authors have read and approved the final version.

## Compliance with ethical standards

**Conflict of interest** M. Bonifacio declares research funding from Novartis and received speaker bureau and advisory board honorarium from Amgen, Bristol-Myers Squibb, Incyte, Novartis and Pfizer, outside the present study. All the other authors declare no competing financial interests.

**Ethical approval** All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

**Informed consent** Informed consent was obtained from all individual participants included in the study.

## References


1. Passamonti F, Rumi E, Pungolino E, Malabarba L, Bertazzoni P, Valentini M, Orlandi E, Arcaini L, Brusamolino E, Pascutto C, Cazzola M, Morra E, Lazzarino M (2004) Life expectancy and prognostic factors for survival in patients with polycytemia vera and essential thrombocytemia. Am J Med 117(10):755–761. https://doi.org/10.1016/j.amjmed.2004.06.032
2. Klampfl T, Gisslinger H, Harutyunyan AS, Nivarthi H, Rumi E, Milosevic JD, Them NCC, Berg T, Gisslinger B, Pietra D, Chen D, Vladimer GI, Bagienski K, Milanesi C, Casetti IC, Sant'Antonio E, Ferretti V, Elena C, Schischlik F, Cleary C, Six M, Schalling M, Schönegger A, Bock C, Malcovati L, Pascutto C, Superti-Furga G, Cazzola M, Kralovics R (2013) Somatic mutations of calreticulin in myeloproliferative neoplasms. N Engl J Med 369(25):2379–2390. https://doi.org/10.1056/NEJMoa1311347
3. Nangalia J, Massie CE, Baxter EJ, Nice FL, Gundem G, Wedge DC, Avezov E, Li J, Kollmann K, Kent DG, Aziz A, Godfrey AL, Hinton J, Martincorena I, van Loo P, Jones AV, Guglielmelli P, Tarpey P, Harding HP, Fitzpatrick JD, Goudie CT, Ortmann CA, Loughran SJ, Raine K, Jones DR, Butler AP, Teague JW, O'Meara S, McLaren S, Bianchi M, Silber Y, Dimitropoulou D, Bloxham D, Mudie L, Maddison M, Robinson B, Keohane C, Maclean C, Hill K, Orchard K, Tauro S, du MQ, Greaves M, Bowen D, Huntly BJP, Harrison CN, Cross NCP, Ron D, Vannucchi AM, Papaemmanuil E, Campbell PJ, Green AR (2013) Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. N Engl J Med 369(25):2391–2405. https://doi.org/10.1056/NEJMoa1312542
4. Arber DA, Orazi A, Hasserjian R, Thiele J, Borowitz MJ, Le Beau MM et al (2016) The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood 127(20):2391–2405. https://doi.org/10.1182/blood-2016-03-643544
5. Rumi E, Pietra D, Ferretti V, Klampfl T, Harutyunyan AS, Milosevic JD, Them NCC, Berg T, Elena C, Casetti IC, Milanesi C, Sant'Antonio E, Bellini M, Fugazza E, Renna MC, Boveri E, Astori C, Pascutto C, Kralovics R, Cazzola M, on behalf of the Associazione Italiana per la Ricerca sul Cancro Gruppo Italiano Malattie Mieloproliferative Investigators (2014) JAK2 or CALR mutation status defines subtypes of essential thrombocythemia with substantially different clinical course and outcomes. Blood 123(10):1544–1551. https://doi.org/10.1182/blood-2013-11-539098
6. Barbui T, Thiele J, Passamonti F, Rumi E, Boveri E, Ruggeri M, Rodeghiero F, d'Amore ESG, Randi ML, Bertozzi I, Marino F, Vannucchi AM, Antonioli E, Carrai V, Gisslinger H, Buxhofer-Ausch V, Müllauer L, Carobbio A, Gianatti A, Gangat N, Hanson CA, Tefferi A (2011) Survival and disease progression in essential thrombocythemia are significantly influenced by accurate morphologic diagnosis: an international study. J Clin Oncol 29(23):3179–3184. https://doi.org/10.1200/JCO.2010.34.5298
7. Stein H, Bob R, Dürkpo H, Erck C, Kämpfe D, Kvasnicka HM et al (2016) A new monoclonal antibody (CAL2) detects CALRETICULIN mutations in formalin-fixed and paraffin-embedded bone marrow biopsies. Leukemia 30(1):131–135. https://doi.org/10.1038/leu.2015.192
8. Vannucchi AM, Rotunno G, Bartalucci N, Raugei G, Carrai V, Balliu M et al (2014) Calreticulin mutation-specific immunostaining in myeloproliferative neoplasms: pathogenetic insight and diagnostic value. Leukemia 28(9):1811–1818. https://doi.org/10.1038/leu.2014.100
9. Andrici J, Farzin M, Clarkson A, Sioson L, Sheen A, Watson N, Toon CW, Koleth M, Stevenson W, Gill AJ (2016) Mutation specific immunohistochemistry is highly specific for the presence of calreticulin mutations in myeloproliferative neoplasms. Pathology 48(4):319–324. https://doi.org/10.1016/j.pathol.2016.03.002
10. Nomani L, Bodo J, Zhao X, Durkin L, Loghavi S, Hsi ED (2016) CAL2 immunohistochemical staining accurately identifies CALR mutations in myeloproliferative neoplasms. Am J Clin Pathol 146(4):431–438. https://doi.org/10.1093/ajcp/aqw135
11. Barosi G, Mesa RA, Thiele J, Cervantes F, Campbell PJ, Verstovsek S et al (2008) 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 22(2):437–438. https://doi.org/10.1038/sj.leu.2404914
12. Veneri D, Capuzzo E, De Matteis G, Franchini M, Baritono E, Benati M et al (2009) Comparison of JAK2V617F mutation assessment employing different molecular diagnostic techniques. Blood Transfus 7(3):204–209. https://doi.org/10.2450/2009.0070-08
13. Kanellis G, Roncador G, Arribas A, Mollejo M, Montes-Moreno S, Maestre L, Campos-Martin Y, Ríos Gonzalez JL, Martinez-Torrecuadrada JL, Sanchez-Verde L, Pajares R, Cigudosa JC, Martin MC, Piris MA (2009) Identification of MNDA as a new marker for nodal marginal zone lymphoma. Leukemia 23(10):1847–1857. https://doi.org/10.1038/leu.2009.108
14. Kvasnicka HM, Beham-Schmid C, Bob R, Dimhofer S, Hussein K, Kreipe H, Kremer M, Schmitt-Graeff A, Schwarz S, Thiele J, Werner M, Stein H (2016) Problems and pitfalls in grading of bone marrow fibrosis, collagen deposition and osteosclerosis – a consensus-based study. Histopathology 68(6):905–915. https://doi.org/10.1111/his.12871
15. R Core Team (2015) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/. Accessed 25 June 2019
16. Tefferi A, Pardanani A (2015) Myeloproliferative neoplasms. A contemporary review. JAMA Oncol 1(1):97–105. https://doi.org/10.1001/jamaoncol.2015.89
17. Jones AV, Ward D, Lyon M, Leung W, Callaway A, Chase A, Dent CL, White HE, Drexler HG, Nangalia J, Mattocks C, Cross NCP (2015) Evaluation of methods to detect CALR mutations in myeloproliferative neoplasms. Leuk Res 39(1):82–87. https://doi.org/10.1016/j.leukres.2014.11.019
18. Tefferi A, Wassie EA, Guglielmelli P, Gangat N, Belachew AA, Lasho TL, Finke C, Ketterling RP, Hanson CA, Pardanani A, Wolanskyj AP, Maffioli M, Casalone R, Pacilli A, Vannucchi AM, Passamonti F (2014) Type 1 vs type 2 calreticulin mutations in essential thrombocythemia: a collaborative study of 1027 patients. Am J Hematol 89(8):E121–E124. https://doi.org/10.1002/ajh.23743
19. Cabagnols X, Defour JP, Ugo V, Ianotto JC, Mossuz P, Mondet J, Girodon F, Alexandre JH, Mansier O, Viallard JF, Lippert E, Murati

A, Mozziconacci MJ, Saussoy P, Vekemans MC, Knoops L, Pasquier F, Ribrag V, Solary E, Plo I, Constantinescu SN, Casadevall N, Vainchenker W, Marzac C, Bluteau O (2015) Differential association of calreticulin type 1 and type 2 mutations with myelofibrosis and essential thrombocythemia: relevance for disease evolution. Leukemia 29(1):249–252. https://doi.org/10.1038/leu.2014.270

20. Riera L, Osella-Abate S, Benevolo G, Beggiato E, Ferrero S, Pich A, Francia di Celle P (2016) Novel CALR somatic mutations in essential thrombocythemia. Br J Haematol 173(5):797–801. https://doi.org/10.1111/bjh.13638
21. Butylin P, Matyuhina N, Lisina E, Siordia N, Senderova O, Lomaia E, Zaritskey A (2016) Calreticulin mutations variants of ET and PMF patients studied in Almazov Medical Center Russian Federation. Blood 128(22):5499 (ASH annual meeting abstracts)
22. Pietra D, Rumi E, Ferretti VV, Di Buduo CA, Milanesi C, Cavalloni C et al (2016) Differential clinical effects of different mutation subtypes in CALR-mutant myeloproliferative neoplasms. Leukemia 30(2):431–438. https://doi.org/10.1038/leu.2015.277
23. Xu Y, Kwok B, Yung A, Flamholz R, Wu Z, Dabbas B, Reddy P (2015) Clinicopathologic analysis of CALR mutation subtypes in myeloproliferative neoplasms. Blood 126(23):2822 (ASH annual meeting abstracts)
24. Elala YC, Lasho TL, Gangat N, Finke C, Barraco D, Haider M, Abou Hussein AK, Hanson CA, Ketterling RP, Pardanani A, Tefferi A (2016) Calreticulin variant stratified driver mutational status and prognosis in essential thrombocythemia. Am J Hematol 91(5):503–506. https://doi.org/10.1002/ajh.24338
25. Barbui T, Tefferi A, Vannucchi AM, Passamonti F, Silver RT, Hoffman R, Verstovsek S, Mesa R, Kiladjian JJ, Hehlmann R, Reiter A, Cervantes F, Harrison C, Mc Mullin MF, Hasselbalch HC, Koschmieder S, Marchetti M, Bacigalupo A, Finazzi G, Kroeger N, Griesshammer M, Birgegard G, Barosi G (2018) Philadelphia chromosome-negative classical myeloproliferative neoplasms: revised management recommendations from European LeukemiaNet. Leukemia 32(5):1057–1069. https://doi.org/10.1038/s41375-018-0077-1
26. Passamonti F, Rumi E, Pietra D, Elena C, Boveri E, Arcaini L, Roncoroni E, Astori C, Merli M, Boggi S, Pascutto C, Lazzarino M, Cazzola M (2010) A prospective study on 338 patients with polycythemia vera: the impact of JAK2 (V617F) allele burden and leukocytosis on fibrotic or leukemic disease tranformation and vascular complications. Leukemia 24(9):1574–1579. https://doi.org/10.1038/leu.2010.148
27. Cassinat B, Verger E, Kiladjian JJ (2014) Interferon alfa therapy in CALR-mutated essential thrombocythemia. N Engl J Med 371(2):188–189. https://doi.org/10.1056/NEJMc1401255
28. Haslam K, Langabeer SE, Molloy K, McMullin MF, Conneally E (2014) Assessment of CALR mutations in myelofibrosis patients post allogeneic stem cell transplantation. Br J Haematol 166(5):800–802. https://doi.org/10.1111/bjh.12904