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LETTER TO THE EDITOR

Droplet digital PCR assay for quantifying of CALR mutant allelic burden in myeloproliferative neoplasms

Luisa Anelli[1] · Antonella Zagaria[1] · Nicoletta Coccaro[1] · Giuseppina Tota[1] · Angela Minervini[1] · Paola Casieri[1] · Luciana Impera[1] · Crescenzio Francesco Minervini[1] · Claudia Brunetti[1] · Alessandra Ricco[1] · Paola Orsini[1] · Cosimo Cumbo[1] · Giorgina Specchia[1] · Francesco Albano[1]

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Dear Editor,

Calreticulin (*CALR*) gene mutations (*CALR*^{mut}) have recently been discovered in about 20–35 % of patients affected by essential thrombocythemia (ET) and primary myelofibrosis (PMF) [1, 2]. Several molecular assays have been developed to detect the most frequent *CALR*^{mut} (type 1 consisting of a 52-bp deletion, and type 2 of a 5-bp insertion) [3, 4]. All these techniques are useful for identifying *CALR*^{mut} at the diagnosis, but they are not suitable for minimal residual disease (MRD) monitoring, since the maximum sensitivity is 1 %. The droplet digital PCR (ddPCR) technology is a third-generation PCR method that started to be used in hematological malignancies [5–7]. We describe a ddPCR assay with a sensitivity of 0.01 % developed for the absolute quantification of *CALR* type 1 and 2 mutations and analyze a cohort of 57 *JAK2V617F*-negative myeloproliferative neoplasm patients. ddPCR experiments were performed using the QX-200 instrument (BioRad) and specific primers and probes were designed for both type 1 and type 2 mutations (see Supplementary Files). *CALR*^{mut} load in each sample was expressed as fractional abundance (FA, mutant allele/mutant allele + wild-type allele). The *CALR*^{mut} allelic burden resulted heterogeneous in both ET (min.13.8 %–max. 51 %) and PMF (min. 34.5 %–max. 51.3 %) patients. We show that the median *CALR*^{mut} allelic burden at diagnosis was significantly higher in PMF patients as compared to ET case (47.9 vs 43.8 %, $p = 0.008$) whereas no significant difference was observed between the type 1 and 2 mutations (Fig. 1a). Moreover, no relationship between the gene mutation type and the *CALR*^{mut} amount was observed within each group of ET and PMF patients. In our ET series, there were 14 (29.7 %) patients with a very low FA, <30 %; this group was not statistically different in terms of hemoglobin, white blood cells and platelet counts, age, sex, thrombosis and/or hemorrhage, and *CALR*^{mut} type compared to those with FA >30 %. The PMF group included ten patients, too few for any type of statistical considerations.

Sequential evaluations by ddPCR experiments were performed in three patients to monitor the *CALR*^{mut} load during treatment. *CALR*^{mut} load at diagnosis was 15.8 and 48 % in two ET patients. The former patient was treated with interferon-α (IFN-α) and after 5 years from diagnosis the FA was 7.7 %. The latter was also treated with IFN-α and after 2 years from diagnosis, the *CALR*^{mut} load was 14.7 %. Both patients had stable disease and a well-controlled platelet count. A 44-year-old man at PMF diagnosis showed a FA of 49.7 %; 8 years later, we observed a leukemic transformation. At the time of the AML evolution, the *CALR*^{mut} load was 0 %; this finding was also confirmed by PCR qualitative analysis. The patient underwent induction chemotherapy, achieving complete remission, then allogeneic bone marrow transplantation (ABMT) from a HLA-matched related donor. Two months later, the FA observed by ddPCR analysis was 0.01 %; 7 months after, the ABMT and AML relapsed and at this time, the *CALR*^{mut} load was 13.5 % (Fig. 1b, c).

Although the importance of the CALR allelic burden determination has not yet been defined at the disease onset, the utility of and need for a sensitive method, like our ddPCR assay, are unquestionable for the purposes of MRD monitoring [8–10].

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✉ Francesco Albano
francesco.albano@uniba.it

[1] Department of Emergency and Organ Transplantation (D.E.T.O.), Hematology Section, University of Bari, P.zza G. Cesare, 11, 70124 Bari, Italy

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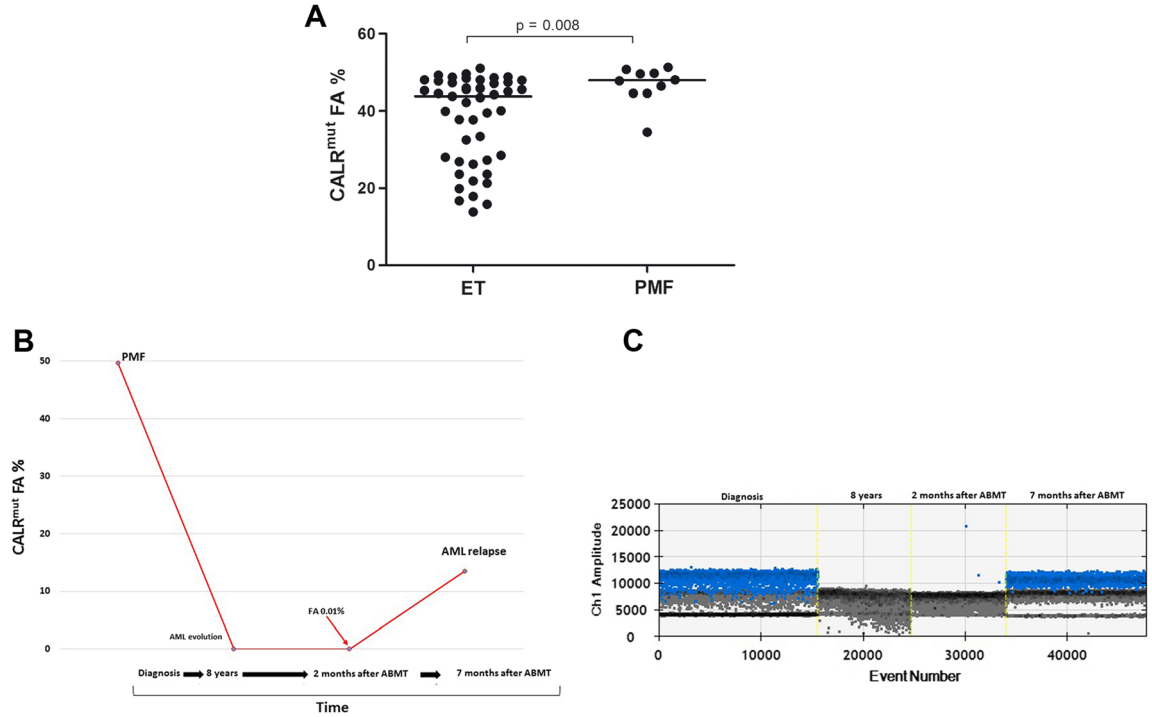

Fig. 1 **a** Distribution of *CALR* type 1- and 2-mutated copies determined by ddPCR and expressed in FA in 57 MPN patients. The median of the $CALR^{mut}$ allelic burden at diagnosis resulted to be 43.8 % in ET patients and 47.9 % in PMF, showing a statistically significant difference ($p = 0.008$). Each *dot* represents a patient. The *lines* indicate the median for each group. **b** Assessment of *CALR* mutation load by ddPCR in a PMF patient receiving ABMT. *CALR* mutation was not revealed at the time of AML evolution, whereas a low positivity (FA 0.01 %) was detected 2 months after ABMT and five months before hematological relapse. **c** 1-D plot showing each droplet corresponding to *CALR* mutations plotted on the graph of fluorescence intensity versus droplet number. All positive droplets are indicated in *blue*, whereas negative droplets are indicated in *gray*

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in this study were in accordance with the local Ethical Committee (Comitato Etico Indipendente Locale, Azienda Ospedaliera "Ospedale Policlinico Consorziale" di Bari, Regione Puglia) 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.

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