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## **JAK2V617F mutation persists in blasts and mature cells of transformed- JAK2V617F-positive-myeloproliferative neoplasia: a European Leukemia Net (ENL) study.**

Ciro Roberto Rinaldi, Paola Rinaldi, Marica Gemei, Francesco Grimaldi, Giorgia Battipaglia, Luigi del Vecchio, Bruno Martino, Giorgina Specchia, Anna Candoni, Luigi Gugliotta, et al.

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Keywords:	JAK2V617F mutation, secondary leukemia, myeloproliferative neoplasia



***JAK2V617F* mutation persists in blasts and mature cells of transformed-*JAK2V617F*-positive-myeloproliferative neoplasia: a European Leukemia Net (ENL) study.**

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**Running Title:** *JAK2V617F* mutation in secondary acute myeloid leukemia

**Keywords:** *JAK2V617F* mutation, secondary leukemia, myeloproliferative neoplasia

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This article is dedicated to the memory of Prof. Bruno ROTOLI

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3 **To the Editor.**  
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6 **Transformation to acute myeloid leukemia (AML) is a known complication of**  
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8 **myeloproliferative neoplasia (MPN). Recent studies reported the high incidence (53%) of**  
9  
10 **JAK2 negative blasts from transformed *JAK2V617F*-MPN. We collected, by cell sorting,**  
11  
12 **blast cells and mature cells (GRA) from total bone marrow (BM) of 34 patients newly**  
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14 **diagnosed of secondary AML. At MPN diagnosis (PMF n = 18; PV n = 9; ET n = 7), *JAK2***  
15  
16 **was mutated in 22 of 34 patients. Twenty of 22 *JAK2V617F*-MPN (91%) maintained the**  
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18 **mutation in blasts and GRA after leukemic switch, while in 2 of 22 patients the selected**  
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20 **compartments lost the mutation. Surprisingly we also found the first case of *JAK2V617F*-**  
21  
22 **AML from a wild type (WT)-MPN. In contrast to the previous study, we conclude that**  
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24 ***JAK2V617F*-MPN yields rarely (9%) a *JAK2WT*-AML and any *JAK2*-status**  
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26 **modification/persistence involves always the entire BM during leukemic transformation.**  
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33 Myeloproliferative neoplasia (MPN) are a heterogeneous group of diseases characterized by the  
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35 hyperproliferation of myeloid precursors in the bone marrow (BM) and/or in extra-medullary  
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37 sites leading to elevated number of non-lymphoid cells and/or platelets in the peripheral blood[1].  
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39 The discovery of an acquired somatic mutation in the *JAK2* gene resulting in a valine-to-  
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41 phenylalanine substitution at position 617 (*JAK2V617F*) has substantially modified the molecular  
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43 knowledge's behind these syndromes[2-5]. By a sensitive allele-specific polymerase chain  
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45 reaction (ASO-PCR) assay[6], it has been well established that around 97% of patients affected  
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47 by polycythemia vera (PV), 60-70% of patients with essential thrombocythemia (ET), and 60%  
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49 of primary myelofibrosis (PMF) are positive for the *JAK2V617F* mutation. Transformation to  
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51 acute myeloid leukemia (AML) is a known complication of MPN but the role of *JAK2V617F*  
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53 mutation is still undefined [7-14]. In 2006, Campbell analyzed 4 patients with secondary AML  
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3 describing a possible model for the development of a *JAK2*WT-AML in a patient with  
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5 *JAK2*V617F-MPN[10] and more recently, Theocharides et al. reported that in up to 53% of the  
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7 patients who developed secondary AML from a *JAK2*-mutated MPN the mutation was no longer  
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9 detectable; however the results, in a smaller cohort of patients (n=27), obtained with DNA  
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11 extracted from cells scraped or laser-capture-microdissected from bone marrow (BM) or  
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13 peripheral blood (PB) smears, were confirmed in fresh samples only in some cases with no-  
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15 uniform cell sources (PB granulocytes, BM-blasts, PB-blasts) [15]. In this study, we collected, by  
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17 cell sorting, blast cells and mature myeloid cells (granulocytes, GRA) from whole BM aspirates  
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19 of 34 newly diagnosed patients with AML secondary to MPN (18 derived from PMF; 9 from PV  
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21 and 7 from ET) and analyzed the *JAK2* status before and after leukemic transformation in  
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23 selected cell compartments. In contrast to the previous studies, we conclude that a *JAK2*V617F-  
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25 positive-MPN yields rarely (9%) a *JAK2*V617F-negative-AML. At the time of MPN diagnosis,  
26  
27 *JAK2*V617F was detectable in 22 of 34 patients (65%) (10 of 18 PMF; 9 of 9 PV and 3 of 7 ET)  
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29 (Table 1). Information on treatment during the chronic phase was available in 25 of 34 patients.  
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31 Twenty patients had received cytoreductive treatment with hydroxyurea (HuOH), 2 patients with  
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33 HuOH associated to busulphan, 1 patient with IFN-alpha, 1 patient with busulphan plus  
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35 anagrelide, 1 patient HuOH plus clorambucil (Table 1). Four PV-untreated-patients had a  
36  
37 spontaneous transformation to AML. No cytogenetic abnormalities were detected during the  
38  
39 chronic phase of the disease in 30/34 evaluated patients. No *MPL* or *JAK2*-exone 12 mutations  
40  
41 were present at this stage (data not shown). Median time to AML progression (TTP) was 5.09  
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43 years (yrs) (range 0.38 - 27.81). A significant difference (p= 0.02) in TTP was found grouping  
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45 patients according to *JAK2* status during the MPN phase [*JAK2*WT-MPN n=12, TTP median  
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47 15.10 yrs (0.38-16.32); *JAK2* mutated-MPN n=22, TTP median 4.07 yrs (0.67-27.81)].  
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49 Cytogenetic data were available in 23 of 34 patients during AML transformation: 8 patients  
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3 showed additional abnormalities involving chromosomes 1, 5, 7, 8, 9, 12, 14, 17 and 20 (Table 2)  
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5 while normal karyotype was confirmed in 14 patients. No other AML-associated mutations  
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7 (FLT3, NPM, CEBPA, RUNX1) were detectable at this stage. To evaluate the modification in the  
8  
9 *JAK2* status before and during leukemic transformation we performed ASO-PCR and (QRT)-  
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11 PCR assay on total BM of the MPN phase and sorted cell populations from AML phase. No  
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13 *JAK2V617F* mutation was detectable in sorted CD3+ cells (Table 2). In our cohort of patients we  
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15 found that *JAK2V617F* mutation was still present at the blast transformation in both  
16  
17 compartments: CD34+ cells (blasts) and CD15+ cells (GRA) in 20 of 22 *JAK2* mutated MPN  
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19 (91%) (Table 2). Two of 22 patients (9%) developed *JAK2V617F* negative AML starting from a  
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21 mutated PV with a mean TTP of 5.14 yrs. Interestingly, the WT status was confirmed in blast  
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23 cells but also in GRA (Table 2). Surprisingly we found a case of *JAK2V617F* mutated AML  
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25 transforming from a WT-PMF. The negativity for the mutation at MPN stage was confirmed in  
26  
27 two different laboratories using 3 different PCR assays. Also in this case the *JAK2V617F*  
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29 positivity in the AML phase occurred in both GRA and blast compartments with an allele burden  
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31 ratio of 42% and 41% respectively. The remaining 11 *JAK2WT*-MPN maintained the same *JAK2*  
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33 status during blast crisis. To determine whether leukemic transformation could modify the *JAK2*-  
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35 mutational burden we performed (QRT)-PCR assay. For patients with persisting *JAK2*-mutated  
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37 disease (n=20), no differences (p= 0.3) in the allele burden were found comparing MNCs from  
38  
39 chronic phase (allelic ratio, median  $\pm$ SD: 57%  $\pm$ 1.75) with MNCs of leukemic transformations  
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41 (allelic ratio, median  $\pm$ SD: 56%  $\pm$ 1.75). No differences in the allele burden (p= 0.4) were also  
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43 found comparing GRA (allelic ratio, median  $\pm$ SD: 56% $\pm$ 2.5) with blasts (allelic ratio, median  
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45  $\pm$ SD: 56% $\pm$ 1.5) in AML phase. These results contrast with previous studies in which the  
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47 *JAK2V617F* mutation levels decreased during leukemic evolution [19, 20] (data summarized in  
48  
49 Table 2). At the time of the study, 6 of 34 patients were eligible for intensive chemotherapy: 4 of  
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3 6 patients achieved complete remission after induction treatment: 3 *JAK2*mutated-AML from  
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5 *JAK2*mutated-MPN (2 ET and 1 PV); 1 *JAK2*WT-AML from *JAK2*mutated-PMF; 2 *JAK2*WT-  
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7 AML from *JAK2*WT-MPNs obtained partial remission. Twenty-three patients underwent  
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9 palliative treatment while five deceased before starting cytotoxic treatment. One patient,  
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11 *JAK2*mutated-AML from *JAK2*mutated-ET (patient 9), underwent allogeneic stem cell  
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13 transplantation from sibling donor and achieved a stable remission. Remarkably, the *JAK2*V617F  
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15 mutation was no longer detectable by quantitative PCR.  
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21 In conclusion, these results contrast with the previous study in which the *JAK2* mutation was  
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23 lost in 53% of blasts during leukemia transformation. In our work, the loss of *JAK2*V617F  
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25 mutation during AML progression is a rare event (9%). One possible explanation may relate to  
26  
27 the different approach used by Theocharides et al [15] who first isolated DNA from cells  
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29 collected using laser-dissection or brushing from stored BM or PB smears and then confirmed the  
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31 results in half of patients in fresh isolated cells. It can be possible, in theory, that a poor quality-  
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33 DNA could not be efficiently amplified by using mutation-specific, single-base mismatched  
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35 primers while amplification of wild-type sequence could have been favored. Furthermore in  
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37 several cases laser dissection could have collected a non-representative part of the leukemic clone  
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39 considering the non-homogenous distribution in BM or PB smears implying an underestimation  
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41 of the *JAK2* mutated blasts which could have been diluted in a majority of WT cells and not  
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43 detected by conventional PCR. For those cases in which the results were confirmed in fresh cells  
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45 the source of the cells was often represented by PB-CD34+ cells collected using non comparable  
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47 techniques (FACS or MACS), sometimes mixing PB and BM blasts but no additional  
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49 information about number and quality of the cells were provided. When the type of MPN studied  
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51 is taken into account one aspect seems of interest: no mutated-PMF patients (n=10), who  
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3 represent the majority of our mutated cases, lost the *JAK2V617F* mutation during leukemia.  
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5 PMF patients are much more likely to transform than ET and PV, which represent a more benign  
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7 diseases also more frequently *JAK2*-mutated, but surprisingly in this view the only 2 patients  
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9 changing the *JAK2* status after transformation were PV. This finding is in accordance with  
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11 recently published data [17] that demonstrated how *JAK2*-mutant AML, but not *JAK2*-WT AML,  
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13 usually arises from a preceding myelofibrotic phase. This allow to think that probably the  
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15 mechanisms of leukemic transformation are different in PMF comparing with ET and PV and  
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17 that the *JAK2V617F* status of AML secondary to PMF may play a different role respect to the  
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19 *JAK2V617F* status of AML secondary to ET and PV. One PMF patient developed a secondary  
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21 mutated-AML from a WT disease. Both ASO-PCR and real time assays excluded the presence  
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23 of *JAK2V617* mutation during the chronic phase of the MPN in both BM cells and in peripheral  
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25 GRA, reinforcing strongly the hypothesis that the mutation could have been acquired during  
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27 leukemia switch as it has been reported to occur in 3-4% of *de novo* AML [18]. However, the  
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29 possibility that a very low burden of *JAK2* mutated allele during the chronic phase may have  
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31 gone undetected cannot be ruled out. One additional finding resulting from this study is that  
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33 presence of the *JAK2V617F* mutation during the MPN phase seems to be associated with an  
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35 acceleration to leukemic transformation compared to WT disease, although the incidence of AML  
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37 in the two groups was comparable. Additional studies in larger patient series and multivariate  
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39 analysis are needed before a prognostic role of *JAK2V617F* mutation regarding time to leukemia  
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41 transformation can be definitely assessed. However the persistence of the *JAK2V617F* mutation  
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43 in the actually largest patient series provides a new intriguing scenery that  
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45 opens again the discussion.  
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## PATIENTS AND METHODS

**Patients.** We performed the molecular analysis on 34 BM aspirates obtained by patients newly diagnosed with secondary AML (18 derived from PMF; 9 from PV and 7 from ET). Informed consent for the study from all patients was obtained in accordance with the Declaration of Helsinki. The study was conducted according to the guidelines of Italian local ethics committee. The diagnosis of MPN was made according to WHO[16]. MPN-secondary-AML was diagnosed according to WHO criteria using 20% blasts in BM as cut off. Patient's characteristics are summarized in Table 1.

**Cell Sorting and DNA preparation.** Mononucleated cells (MNCs) were isolated by Ficoll centrifugation. After erythrocyte lyses, 10 million BM (or PB) cells were labelled by incubation with the antibodies CD15-APC, CD3-FITC, CD45-PerCP, CD34-Pe (BD Biosciences, San Jose, CA), analyzed and sorted with a fluorescence-activated cell sorter FACS Aria (BD Biosciences) using FACS Diva software (BD Biosciences), according to manufacturer's recommendations. Leukemic blasts were collected using side scatter according to CD45 and CD34 expression. Mature cells (GRA) and T-cell were sorted by FACS using CD15 and CD3 antibodies, respectively. Leukemic blasts and GRA were  $\geq 98\%$  pure by morphological criteria and FACS re-analysis. Genomic DNA was isolated using QIAmp DNA Blood Kit (Qiagen).

**ASO-PCR and QRT-PCR.** The *JAK2V617F* mutation status was determined by ASO-PCR as previously described [2] with a sensitivity of 1%. Measurement of *JAK2V617F* allele burden was performed by a quantitative real time (QRT)-PCR assay, using 80 ng DNA. PCR amplification and detection were performed on an ABI Prism 7300 analyzer (Applied Biosystem) using the following cycling conditions: 10 minutes at 95°C followed by 50 cycles of 15 s at 95°C and 60 s

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3 at 60°C. QRT-PCR assay was performed with specific forward primers [*JAK2*-wild type (WT):  
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5 5'-GCGCGGTTTTAAATTATGGAGTATGTG-3'; *JAK2V617F*: 5'-  
6  
7 GCGCGGTTTTAAATTATGGAGTATGTT-3'), common reverse primers (5'-  
8  
9 GCGGTGATCCTGAAACTGAATTTTC-3'). FAM-labeled MGB probe sequence was: 5'-  
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11 TGGAGACGAGAGTAAGTAAACTACAGGCT-3'. All samples were analyzed in triplicate  
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13 and the amount of *JAK2V617F* allele was calculated by comparison with cloned *JAK2* plasmids.  
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15 The mean of triplicate  $\Delta$ CT determinations ( $C_T^{JAK2V617F} - C_T^{JAK2WT}$ ) was used to calculate the  
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17 percentage of mutated allele. Positive and negative controls were included in each assay; inter-  
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19 and intra-assay variation was 3% and 5%, respectively[5]. To compare continuous variables  
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21 among the groups we used the Mann-Whitney *U* test and Student's t-Test.  
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31  
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3 **Authorship and disclose**  
4

5  
6 Contribution: C.R.R. performed research, analyzed data, and wrote the paper; P.R., F.G.and G.B.  
7  
8 performed research and analyzed data; B.M., G.S., A.C., L.G. provided patients; M.G., L.D.V.  
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10 performed the FACS sorting; A.V. performed real time PCR; T.B. and F.P. designed the research  
11  
12 and wrote the paper. The authors have no conflict of interest to disclose.  
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For Peer Review

Table 1. Summary of patient characteristics: MPN diagnosis and previous treatment

<i>patient</i>	<i>gender</i>	<i>age</i>	<i>diagnosis</i>	<i>JAK2</i>	<i>cytoreductive treatment</i>	<i>TTP (yrs)</i>
1	M	72	PMF	WT	HuOH	16.42
2	M	67	ET	WT	BU+HuOH	15.43
3	F	67	PMF	WT	BU+HuOH	20.3
4	M	35	PMF	WT	-	6.01
5	F	32	PMF	WT	HuOH	5.09
6	M	68	PMF	MUT	HuOH	2.01
7	F	70	ET	MUT	HuOH	4.71
8	F	88	PMF	MUT	HuOH	27.81
9	M	66	PV	MUT	HuOH	4.99
10	M	45	ET	MUT	IFN	2.64
11	F	57	PV	MUT	HuOH	4.71
12	F	59	PMF	MUT	-	1.00
13	M	55	PV	MUT	None	5.98
14	F	74	PV	MUT	None	7.51
15	F	77	PMF	WT	Clor+HuOH	16.32
16	M	74	PMF	WT	HuOH	4.13
17	F	81	ET	MUT	-	1.00
18	M	58	PMF	WT	HuOH	0.38
19	F	68	PMF	MUT	HuOH	13.16
20	F	65	PV	MUT	None	1.02
21	F	60	PMF	MUT	HuOH	5.96
22	M	49	PMF	MUT	HuOH	1.01
23	F	71	PMF	MUT	HuOH	4.07
24	F	66	ET	WT	HuOH	15.11
25	F	70	PMF	MUT	-	11.09
26	F	77	ET	WT	HuOH	9.03
27	F	71	PV	MUT	HuOH	10.16
28	F	75	PV	MUT	HuOH	1.08
29	M	81	PMF	MUT	HuOH	2.29
30	M	83	PV	MUT	None	5.30
31	F	76	PV	MUT	HuOH	11.32
32	M	65	PMF	MUT	HuOH	0.67
33	M	64	PMF	WT	-	8.13
34	F	67	ET	WT	BU+ANA	1.09

M, male; F, female; PMF, primary myelofibrosis, ET, essential thrombocytemia; PV, polycythemia vera; HuOH, hydroxiurea; BU, busulphan; IFN, interferon; ANA, anagrelide; Clor, clorambucil; (-), treatment information's not available; TTP, time to progression; yrs, years

Table 2. Summary of patient characteristics: molecular analysis in AML secondary to MPN

patient	MPN		AML						
	diagnosis	JAK2 MNCs (AB %±SD)	source	% BLAST	cytogenetic	JAK2 MNCs (AB %±SD)	JAK2 BLAST (AB %±SD)	JAK2 CD15+ Cells (AB %±SD)	JAK2 CD3+ Cells
1	PMF	WT	BM	80	ND	WT	WT	WT	WT
2	ET	WT	BM	90	COMPLEX	WT	WT	WT	WT
3	PMF	WT	BM	95	DEL(5)+TRI(8)+TRI(1)	WT	WT	WT	WT
4	PMF	WT	BM	70	DEL(7)+DEL(9)+TRI(14)	WT	WT	WT	WT
5	PMF	WT	BM	70	ND	MUT (42±2.85)	MUT (41±1.75)	MUT (42±2)	WT
6	PMF	MUT (58±1.75)	BM	76	46,XY	MUT (57±2.5)	MUT (58±2)	MUT (57±2.25)	WT
7	ET	MUT (55±1.51)	BM	40	46, XX	MUT (54±1.5)	MUT (55±2)	MUT (55±1.25)	WT
8	PMF	MUT (57±2.00)	BM	83	46, XX	MUT (56±1.75)	MUT (58±2.5)	MUT (57±0.75)	WT
9	PV	MUT (62±2.13)	BM	71	46, XY	WT	WT	WT	WT
10	ET	MUT (32±0.95)	BM	72	46, XY	MUT (30±2.05)	MUT (32±1.5)	MUT (35±1.5)	WT
11	PV	MUT (60±1)	BM	40	46, XX	MUT (61±1.05)	MUT (60±1)	MUT (59±1.5)	WT
12	PMF	MUT (56±1.2)	PB	59	MONO(7), TRI(1)	MUT (57±2)	MUT (56±2.5)	MUT (56±2)	WT
13	PV	MUT (59±2)	BM	78	ND	MUT (55±1.75)	MUT (60±1.5)	MUT (59±1.75)	WT
14	PV	MUT (81±3)	BM	55	ND	MUT (76±2)	MUT (71±2.75)	MUT (69±2.2)	WT
15	PMF	WT	BM	90	ND	WT	WT	WT	WT
16	PMF	WT	BM	62	ND	WT	WT	WT	WT
17	ET	MUT (26±1.95)	BM	27	ND	MUT (21±3.5)	MUT (30±2.76)	MUT (23±2.7)	WT
18	PMF	WT	BM	69	46, XY	WT	WT	WT	WT
19	PMF	MUT (57±1.75)	BM	60	COMPLEX	MUT (57±2.5)	MUT (58±2)	MUT (58±2)	WT
20	PV	MUT (56±1.15)	BM	37	46, XX	MUT (55±2.5)	MUT (56±2)	MUT (55±2)	WT
21	PMF	MUT (57±1.6)	BM	85	DEL(20)	MUT (55±1.67)	MUT (55±1.25)	MUT (56±1.5)	WT
22	PMF	MUT (56±2.02)	BM	60	ND	MUT (56±0.75)	MUT (55±1)	MUT (56±1.2)	WT
23	PMF	MUT (58±3.05)	BM	100	46, XX	MUT (58±0.5)	MUT (57±1)	MUT (57±1.97)	WT
24	ET	WT	BM	100	46, XX	WT	WT	WT	WT
25	PMF	MUT (52±3.5)	PB	47	46, XX	MUT (50±1.5)	MUT (50±2)	MUT (51±2.3)	WT
26	ET	WT	BM	24	TRI(8)	WT	WT	WT	WT
27	PV	MUT (71±2)	BM	78	46, XX	MUT (69±2)	MUT (65±1.5)	MUT (68±2.3)	WT
28	PV	MUT (59±1.5)	BM	90	46, XX	MUT (58±1.75)	MUT (58±1.5)	MUT (59±1.5)	WT
29	PMF	MUT (61±2)	BM	53	ND	MUT (60±1.15)	MUT (60±1.5)	MUT (59±1.75)	WT
30	PV	MUT (49±3)	BM	72	ND	WT	WT	WT	WT
31	PV	MUT (58±3)	BM	22	ND	MUT (58±2)	MUT (59±2)	MUT (59±0.95)	WT
32	PMF	MUT (56±2)	BM	66	46, XY	MUT (57±2.65)	MUT (55±2)	MUT (55±2.13)	WT
33	PMF	WT	BM	60	46, XY	WT	WT	WT	WT
34	ET	WT	BM	77	t(9;12); TRI(8); ISO(17)	WT	WT	WT	WT

MPN, myeloproliferative neoplasia; BM, bone marrow; PB, peripheral blood; WT, wild type; MUT, V617F mutated; AB, allele burden; SD, standard deviation; MNCs, mononucleated cells; ND, not done; DEL(x), deletion; TRI(x), trisomy of chromosome x; ISO(x), isochromosome.