# Impact of genotype on leukemic transformation in polycythaemia vera and essential thrombocythaemia

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Impact of genotype on leukaemic transformation in polycythaemia vera and essential thrombocythaemia

Running title: acute leukaemia in myeloproliferative neoplasms

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The authors have no relevant conflict of interest to declare regarding this article

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Abstract

The influence of driver mutations on leukemic transformation was analysed in 1747 patients with polycythaemia vera or essential thrombocythaemia. With a median follow-up of 7.2 years, 349 patients died and 62 progressed to acute leukaemia or myelodysplastic syndrome. Taken death as a competing risk, CALR genotype was associated with a lower risk of transformation (SHR: 0.13, 95%CI: 0.2-0.9, p=0.039), whereas JAK2V617F showed borderline significant higher risk (SHR: 2.05, 95%CI: 0.9-4.6, p=0.09). Myelofibrotic transformation increased leukemic risk, except in CALR-mutated patients. Next generation sequencing of 51 genes at time of transformation showed additional mutations (median number: 3; range: 1-5) in 25 out of 29 (86%) assessable cases. Mutations (median: 1; range: 1-3) were detected in 67% of paired samples from the chronic phase. Leukaemia appeared in a JAK2V617F negative clone in 17 (58%) cases, eleven of them being previously JAK2V617F-positive. JAK2V617F-mutated leukaemia was significantly associated with complex karyotype and acquisition of TP53 mutations, whereas EZH2 and RUNX1 mutations were more frequent in JAK2V617F-negative leukaemia. Survival was longer in JAK2V617F-unmutated leukaemia (343 versus 95 days, p=0.003). In conclusion, CALR genotype is associated with a lower risk of leukemic transformation. Leukaemia arising in a JAK2V617F-negative clone is TP53 independent and shows better survival.
Patients with myeloproliferative neoplasms (MPN) who develop acute myeloid leukaemia (AML) have poor prognosis, with a median survival of 3 to 6 months (Tam et al, 2008; Kennedy et al, 2013). In polycythaemia vera (PV) or essential thrombocythaemia (ET), the frequency of AML transformation ranges from 2% to 5% of patients (Kiladjian et al, 2011; Tefferi et al, 2013; Tefferi et al, 2014a). Currently, the identification of risk factors predicting for leukemic transformation, as well as the prognostic stratification and treatment strategy following disease transformation, constitute unmet clinical needs.

Advanced age, leucocytosis, cytogenetic abnormalities and exposure to leukemogenic agents are well-established risk factors for transformation into AML in patients with PV and ET (Finazzi et al, 2005; Gangat et al, 2007a; Gangat et al, 2007b; Passamonti et al, 2008; Kiladjian et al, 2011; Hernández-Boluda et al, 2012; Tefferi et al, 2013). Although, the prognostic value of genotype (\textit{JAK2}, \textit{CALR}, \textit{MPL} or triple negative) has been widely studied in patients with myelofibrosis (MF)(Guglielmelli et al, 2011; Vannucchi et al, 2013; Tefferi et al, 2014a; Tefferi et al, 2014b; Tefferi et al, 2014c; Rumi et al, 2014a), few studies have addressed this subject in patients with PV or ET (Rotunno et al, 2014; Rumi et al, 2014b; Palandri et al, 2015; Lundberg et al, 2014).

Notably, \textit{JAK2}V617F-mutated MPNs can develop AML in the \textit{JAK2}V617F-positive or in the \textit{JAK2}V617F-negative clone, with this suggesting the existence of different pathways of disease transformation (Campbell et al, 2006). However, the spectrum of molecular abnormalities and the clinical outcome according to the \textit{JAK2}V617F mutational status of the leukemic clone are not well characterised.

The objective of the present study was to analyse the influence of the genotype on the risk of AML/MDS transformation in patients with PV and ET. In addition, we aimed to
determine if the JAK2V617F mutational status of the leukemic clone is related with a
different transformation pathway and outcome.

Patients and methods

Clinical data

The medical charts of patients consecutively diagnosed with PV and ET in 5 institutions in
Spain from 1973 to 2015 were reviewed. In every case, the diagnosis of PV and ET was
reassessed using the criteria of the World Health Organization (Tefferi et al, 2007). The
indication of cytoreductive therapy was decided according to the criterion of the attending
haematologist on the basis of the clinical guidelines and prevailing recommendations at that
time. Informed consent for the scientific use of the patients’ clinicohaematological data and
biological samples was obtained in accordance with the requirements of the local ethics
committees.

Molecular analysis

Targeted NGS was performed on DNA extracted from mononuclear cells at time of
acute transformation of the disease. Targeted amplicon libraries were constructed using the
Human Myeloid Neoplasms GeneRead DNAmseq Targeted Panel V2 (Qiagen, Hilden,
Germany), a commercially available panel covering the full exonic regions of 50 commonly
mutated genes in myeloid malignancies and were sequenced using either MiSeq or NextSeq
(Illumina, San Diego, CA, USA). Genes tested in the panel are as follows: ABL1, ASXL1,
ATRX, BCOR, BCORL1, CBL, CBLB, DAXX, DNMT3A, EED, ETV6, EZH2, FLT3,
GATA1, GNAS, IDH1, IDH2, IKFZ1, JAK1, JAK2, JAK3, KAT6A, KIT, KRAS, MLL, MPL, NF1, NPM1, NRAS, PHF6, PRPF40B, PTPN11, RAD21, RB1, RUNX1, SETBP1, SF1, SF3A1, SF3B1, SH2B3, SMC1A, SMC3, STAG2, SUZ12, TET2, TP53, U2AF1, U2AF2, WT1, ZRSR2. Secondary analysis was performed using MiSeq Reporter Software 2.4 (Illumina) and variants were annotated using Illumina VariantStudio v2.2. Nonsynonymous coding and splice site variants were considered when variant allele frequency (VAF) was greater than 2% unless previously reported as benign or germline polymorphisms.

Relevant genetic regions non-properly covered by the gene panel used were sequenced on a 454 GS Junior (Roche, Basel, Switzerland) including SRSF2 (exon 1) and ASXL1 (exon 12). Additionally, all patients were screened for CALR, NPM1 and FLT3 mutations using fragment analysis on an ABI3500DX (Applied Biosystems, Foster City, CA, USA). Those variants not previously described in databases or literature, were sought in constitutive DNA from paired samples when the allele frequency suggested potential germline origin using Sanger sequencing in an ABI3500DX. Mutations with a variant allele frequency (VAF) <10% were confirmed by sequencing on a 454 GS Junior or by allele-specific oligonucleotide PCR on a 7500 Fast Real-Time PCR System (Applied Biosystems).

All confirmed somatic mutations were then sought and quantified in the first available paired sample from the moment of diagnosis or chronic phase of the disease using a 454 GS Junior. See Supplemental Methods for detail.

Statistics

Overall survival curves were drawn by the method of Kaplan and Meier. The cumulative incidence of AML/MDS transformation was calculated by taking death as a competing risk. Multivariate analyses of factors predicting the hematologic transformation were done within the framework of competing risks by the method of Fine & Gray (Fine and
Gray, 1999). Variables evaluated for their potential prognostic significance were age (> 65 vs. ≤ 65 years), sex, haematological values at diagnosis, presence of cytogenetic abnormalities, cytoreductive therapy, and genotype. Regarding cytoreductive therapy, each drug was explored individually and also the exposure to any leukemogenic agent (radioactive phosphorus, busulfan, melfalan, pipobroman). All the statistical analyses were performed with Stata, version 11 (www.stata.com). Curves of the cumulative incidence of AML/MDS and the competing risk of dying without AML/MDS were drawn by the “stcompet” Stata module developed by Coviello (Coviello and Boggess, 2004).

Results

Characteristics of the patients

A total of 1747 patients diagnosed with PV (n=896) or ET (n=851) were included in the study. The main clinical and laboratory characteristics of the patients at MPN diagnosis are shown in table I. **MPL-mutated patients were significantly older at ET diagnosis (median 73 years in comparison with 65 years, 53 years and 57 years for JAK2V617F, CALR and TN genotypes, respectively p<0.001).** During the observation period, 215 (12%) patients received at least one leukemogenic agent including radioactive phosphorus (n=119), busulfan (n=89), melfalan (n=15), or pipobroman (n=1). In 132 (8%) patients, sequential treatment with hydroxyurea plus a leukemogenic agent was administered. There were no significant differences in the proportion of patients with PV and ET who received any leukemogenic agent or sequential therapy. However, patients with PV received more frequently busulfan, whereas ET patients were more commonly treated with radioactive phosphorus. **Sixty patients received interferon (see supplemental material for details).**
Follow-up was longer in ET patients due to the inclusion of a higher proportion of ET patients diagnosed before year 2000.

Survival, time to AML/MDS transformation and risk factors

With a median follow-up of 7.2 years (range: 0.1-42), 349 patients died, resulting in a projected median survival for the overall series of 22 years (95%CI: 19.7-24.5). In ET, median survival was not reached in CALR-mutated patients, in comparison with 21, 18 and 11 years for those with JAK2, TN and MPL genotypes, respectively (p<0.0001, Figure S1).

Overall, 54 patients developed AML and 8 patients MDS. At 10 years, the cumulative incidence of progression to AML/MDS was 3.3% (95%CI: 1.9-4.4). The incidence rate of AML/MDS was slightly higher in ET than in PV but the difference was not statistically significant. There were no statistical significant differences in AML/MDS progression according to year of diagnosis (see supplemental material).

Progression to AML/MDS in PV and ET is shown in Figure S2. Twenty four patients with ET, all JAK2V617-positive, evolved into PV, and one of them subsequently developed AML. A total of 144 patients progressed to MF (67 post-ET and 77 post-PV), with 14 of them evolving into AML/MDS afterwards.

After a median time of 8.8 years (range: 1.2-28.9) from PV diagnosis, 26 patients progressed to AML/MDS (24 AML, 2 MDS), with a cumulative incidence of 2.5% at 10 years. On multivariate analysis, the only factors independently associated with a higher risk of myeloid transformation were leucocytosis >10 x 10^9/L at PV diagnosis, exposure to leukemogenic agents, and abnormal cytogenetics (table II and table S1).

In ET, a total of 36 patients developed AML/MDS (30 AML, 6 MDS) after a median follow-up of 9.5 years (range: 2.8-27.6), resulting in a cumulative incidence of 3.8% at 10 years. In multivariate analysis, factors associated with a higher incidence of AML/MDS were
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male sex, exposure to leukemogenic agents, prior evolution to MF, and genotype, with the latter computed as one variable with four categories (table III). Age at diagnosis was not associated with a higher risk of AML/MDS transformation and when included in the multivariate model it did not affect the prognostic value of genotype (data not shown). The cumulative incidence of AML/MDS according to genotype is shown in figure 1. When each genotype was singled out and compared to the remaining, CALR mutation showed the lowest risk of acute transformation, with only one patient developing AML after 25 years of follow-up (SHR: 0.13, 95%CI: 0.2-0.9, p=0.039). JAK2V617F mutation was associated with a borderline significance (SHR: 2.05, 95%CI: 0.9-4.6, p=0.09), while MPL and TN lacked any prognostic significance. With regard to post-ET MF, 11 out of 67 (16%) patients with this evolution pattern progressed to AML/MDS (Figure S3). According to the genotype of post-ET MF, the proportion of patients progressing to AML/MDS for the JAK2V617F, CALR, MPL and TN genotypes was 5 out of 28 (18%), 1 out of 20 (5%), 1 out of 3 (33%) and 3 out of 4 (75%), respectively.

Molecular studies at time of transformation and in paired samples from the chronic phase

Twenty-nine patients were studied by targeted NGS at the time of transformation (27 AML, 2 MDS). Somatic mutations other than JAK2, CALR or MPL were found in 25 out of 29 patients (86%) (Figure 2 and table S2). Median number of mutations was 3 (range: 1-5). Five (17%) patients had one, 7 (24%) two, 9 (31%) three, and 4 (14%) four or more additional mutations. The frequencies of mutated genes were: TP53 38%, TET2 28%, RUNXI 17%, DNMT3A 17%, IDH1/2 17%, ASXL1 17%, EZH2 14%, SRSF2 10%, SETBP1 10%, and <5% for CBL, PTPN11, SH2B3, SF3B1, NOTCH1, ETV6, NF1, ZRSR2, NRAS, KRAS, NPM and FLT3. The mutational profile of each patient at the time of transformation is shown in figure
2. The majority of mutated genes were equally distributed among patients with post-PV and post-ET AML, with the exception of EZH2, which appeared more frequently mutated in patients with TN ET.

Paired samples from the chronic phase were available in 20 out of 29 transformed cases. These samples were collected at PV or ET diagnosis in 11 cases (55%) or during follow-up in the remaining 9 patients after a median time of 9 years (Range: 5-17). The variant allele frequency among pre- and post-transformation samples is shown in **figure 3**. As can be seen, all mutations, except for JAK2V617F, were stable or increased at transformation. Mutations were detectable in the chronic phase in 14 cases (67% of the total cohort and 74% of patients with mutations at transformation). Median number of mutations in the chronic phase was 1 (range: 1-3). The majority of mutations in the leukemic phase were also detected in the chronic phase (67%, 100%, 80% and 100% for TET2, ASXL1, DNMT3A, and SRSF2, respectively) whereas 43% and 25% of patients with mutations in TP53 and RUNX1 carried the same mutations in the chronic phase, usually at subclonal levels. In one case, the TP53 mutant load was 73%, but this sample corresponded to a late chronic phase that was collected just one year before leukemic transformation.

**Clinical and molecular characteristics according to the JAK2V617F mutational status of the leukemic clone**

The original diagnosis of the 29 assessable cases was JAK2V617F-mutated PV (n=14), JAK2V617F-mutated ET (n=9), MPL-mutated ET (n=1), and TN ET (n=5). Among the 23 JAK2-mutated patients who developed AML, in 11 (48%) of them the leukemic evolution took place in a JAK2V617F-negative clone. Overall, leukaemia was categorised as
JAK2V617F mutated or un-mutated in 12 (41%) and 17 (59%) patients, respectively. All patients had received cytoreduction with hydroxyurea before transformation. There were no significant differences among the two study groups in the proportion of patients receiving leukemogenic agents.

In comparison with JAK2V617F-unmutated, patients with JAK2V617F-mutated leukaemia showed significantly higher frequency of complex karyotypes (table IV). TP53 mutations were highly prevalent in JAK2V617F-mutated leukaemia whereas only one JAK2V617F-unmutated leukaemia was associated with TP53 mutations (p< 0.0001, table IV). Mutations in RUNX1, EZH2 and DNMT3A genes were more prevalent in JAK2V617F-unmutated leukaemia although the differences were not significant (Table S3). In 2 out of 3 patients with mutations in SRSF2 there was an expansion of a RUNX1-mutated leukemic clone (p=0.079 for the association), with four out of five RUNX1 mutations appearing in patients who had a JAK2V617F-negative clone arising from a previous JAK2V617F-positive MPN (Table S3). The JAK2V617F mutational status of the leukemic clone was significantly associated with survival (Median survival 95 and 343 days for JAK2V617F mutated and un-mutated, respectively, p=0.003, figure S4).
Discussion

The results of the present study demonstrate the significant impact of the genotype on the risk of leukemic transformation in patients with ET. Thus, patients with CALR-mutated ET had a much lower risk of transformation to acute leukaemia than the other molecular subtypes. Moreover, the incidence of AML in CALR-mutated ET was minimal even after MF transformation. Limited evidence has previously been published on the protective effect of the CALR-mutation regarding the risk of acute transformation in ET patients. In this sense, in a series of 717 ET patients, only 2 among the 176 CALR-mutated cases progressed to AML (Rumi et al, 2014b). By contrast, 12 cases of AML transformation were seen among 466 JAK2V617F-mutated patients, resulting in a cumulative incidence of AML of 2.5% and 4.3% for CALR and JAK2V617F genotypes, respectively (Rumi et al, 2014b). In our series, even more pronounced differences in AML risk were observed, with only 1 case of AML out of 117 CALR-mutated ET patients as compared to 19 cases of AML/MDS out of 403 JAK2V617F-mutated ET cases.

Lundberg et al detected somatic mutations in additional non-driver genes in 18% of ET patients with the CALR genotype, a figure clearly lower than the 32% and 41% reported in JAK2V617F-mutated ET and PV patients, respectively (Lundberg et al, 2014). In addition, CALR mutations are usually initial events whereas JAK2V617F mutations may be initial or late events (Kralovics et al, 2006; Nangalia et al, 2013; Lundberg et al, 2014; Ortmann et al, 2015). This difference in the frequency and timing of somatic mutations could explain the lower rate of AML/MDS transformation observed in patients with CALR genotype. In fact, in our cohort, 48% of AML cases evolving from patients with JAK2V617F-mutated PV or ET appeared in a JAK2V617F-negative clone, illustrating the important role of pre-JAK2V617F somatic mutations in leukemic transformation. To clarify this aspect it would be advisable to carry out NGS studies in our whole series of patients. Unfortunately, in the
present study available samples were restricted only to patients who progressed to AML/MDS.

Rampal et al, in a study performed mainly in patients with post-MF AML, showed that somatic TP53 mutations were common in JAK2V617F-mutated post-MPN AML (Rampal et al, 2014). In addition, in a murine model, expression of JAK2V617F combined with loss of TP53 leads to fully penetrant AML (Rampal et al, 2014). In the same line, Lundberg et al found the loss of heterozygosity in TP53 to be associated with AML transformation (Lundberg et al, 2014). In our study, restricted to patients with post-PV and post-ET AML, we observed a significant association between the acquisition of mutations in TP53 gene and the development of AML in a JAK2V617F-mutated clone. Noteworthy, these patients frequently showed a complex karyotype and a dismal outcome (median survival of 3 months only). In contrast, AML developing from a JAK2V617F-negative clone displayed fewer cytogenetic abnormalities, very low proportion of TP53 mutations and higher rate of mutations in RUNX, EZH2, and DNMT3A genes, suggesting that this type of leukaemia is mostly driven by a TP53-independent pathway. In addition, survival probability of JAK2V617F-negative leukaemia resembles that of elderly patients with de novo AML.

The analysis of paired samples by NGS showed that non-driver MPN mutations were already present in the chronic phase in most cases, with some important exceptions. Thus, while mutations in TET2, ASXL1, DNMT3A or SRSF2 genes were often detectable years before AML transformation, those in TP53 or RUNXI genes were clearly associated with disease progression, since they were undetectable or present at subclonal levels in the early stages of MPN. Moreover, variant allele frequency confirmed the expansion of clones harbouring non-driver MPN mutations at time of acute transformation, with this supporting their contributory role in the pathogenesis of disease progression.
Our findings not only provide further insights into the mechanisms involved in MPN progression but, in addition, they might have potential implications for clinical practice. In general, the prognosis of post-MPN AML is poor (Tam et al, 2008; Kennedy et al, 2013). Advanced patients’ age and the unfavourable results with standard intensive chemotherapy make that most patients are usually offered palliative treatment only. However, our data suggest that the molecular profile of the leukemic clone could be useful to guide on treatment decisions. Thus, patients with AML appearing in a JAK2V617F-negative clone seem to have a more favourable disease profile and their treatment approach could be similar to that of de novo AML. On the other hand, patients with PV and ET in whom additional somatic mutations are detected at diagnosis or during follow-up could be candidates for molecular surveillance in order to document the expansion of clones with increased leukemogenic potential. Another important clinical implication of molecular NGS assessment would be to avoid the exposure to leukemogenic agents in those patients with non-driver MPN mutations in order to decrease the propensity to acute leukaemia.

In conclusion, our results indicate that CALR mutation is associated with a low risk of leukemic transformation in ET patients. Non-MPN driver mutations play a key role in leukemic transformation and can be detected by NGS techniques at MPN diagnosis in most cases. Finally, the JAK2V617F mutational status of the leukemic clone reflects different pathways of disease transformation that are associated with distinct clinical features and prognosis.
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Authorship

AAL designed the study, collected the data, performed the statistical analysis, analysed and interpreted the results and wrote the paper. AS and CFR collected the data, performed the molecular studies and wrote the paper. AP: performed the statistical analysis, analysed and interpreted the results and wrote the paper. EAR, AA, MG, FFM, JM, BN collected the data and approved the final version. LC: performed the molecular studies and approved the final version. DC: collected the data, performed the molecular studies and wrote the paper. FC: interpreted the results and wrote the paper. CB, BB and JCHB: designed the study, interpreted the results and wrote the paper.

Conflict of interest

The authors have no relevant conflict of interest to declare regarding this article.

Supplementary information is available at British Journal of Haematology website.
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Table I: Main clinico-haematological characteristics at diagnosis in 1747 patients with polycythaemia vera and essential thrombocythaemia

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<td>Age, years*</td>
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<td>66 (10-94)</td>
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<td>10.6 (2.9-42)</td>
<td>8.8 (3.9-26)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Platelet count, x10^9/L*</td>
<td>639 (110-2814)</td>
<td>505 (110-1756)</td>
<td>759 (451-2814)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Cytogenetic abnormalities, n/total available (%)</td>
<td>43/883 (5)</td>
<td>29/369 (8)</td>
<td>14/514 (3)</td>
<td>0.001</td>
</tr>
<tr>
<td>Therapy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydroxyurea, n (%)</td>
<td>1325 (76)</td>
<td>737 (82)</td>
<td>588 (71)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Busulfan, n (%)</td>
<td>89 (5)</td>
<td>69 (8)</td>
<td>20 (2)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Radioactive phosphorus, n (%)</td>
<td>119 (7)</td>
<td>36 (4)</td>
<td>83 (10)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Genotype, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JAK2**</td>
<td>1191 (84)</td>
<td>788 (95)</td>
<td>403 (64)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CALR</td>
<td>117 (8)</td>
<td>-</td>
<td>117 (19)</td>
<td></td>
</tr>
<tr>
<td>MPL</td>
<td>16 (1)</td>
<td>-</td>
<td>16 (2.5)</td>
<td></td>
</tr>
<tr>
<td>Triple negative</td>
<td>92 (6.5)</td>
<td>-</td>
<td>92 (15)</td>
<td></td>
</tr>
</tbody>
</table>

*Median (range). ¶Not available in 69 and 223 patients with PV and ET, respectively.
**V617F or exon 12 mutation out of 827 assessed patients. ns, not significant
Table II: Multivariate analysis of risk factors for AML/MDS transformation in 896 patients with PV taking death as a competing event

<table>
<thead>
<tr>
<th>Feature</th>
<th>SHR  (95%CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age &gt; 65 years</td>
<td>1.5 (0.7-3.4)</td>
<td>0.28</td>
</tr>
<tr>
<td>Male sex</td>
<td>1.5 (0.7-3.5)</td>
<td>0.28</td>
</tr>
<tr>
<td>WBC &gt; 10x10⁹/L</td>
<td>2.7 (1.01-7.4)</td>
<td>0.047</td>
</tr>
<tr>
<td>Exposure to leukemogenic agents</td>
<td>2.9 (1.3-6.9)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

SHR: subdistribution hazard ratio.
Table III: Multivariate analysis of risk factors for AML/MDS transformation in patients with ET taking death as a competing event

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>SHR</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex</td>
<td>3.08 (1.5-6.4)</td>
<td>0.003</td>
</tr>
<tr>
<td>Exposure to leukemogenic agents</td>
<td>3.57 (1.5-8.2)</td>
<td>0.003</td>
</tr>
<tr>
<td>MF transformation</td>
<td>3.5 (1.3-2.4)</td>
<td>0.001</td>
</tr>
<tr>
<td>Genotype*</td>
<td>1.75 (1.3-2.4)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

TN: triple negative. *Genotype was coded as 0=CALR, 1=JAK2V617F, 2=MPL and 3=TN. SHR: subdistribution hazard ratio
Table IV: Clinical and biological characteristics at time of AML/MDS transformation according to JAK2V617F mutational status of the leukemic clone in 29 patients with PV or ET

<table>
<thead>
<tr>
<th></th>
<th>JAK2V617F-positive N=12</th>
<th>JAK2V617F-negative N=17</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years*</td>
<td>66 (50-83)</td>
<td>69 (40-85)</td>
<td>ns</td>
</tr>
<tr>
<td>Male sex, n</td>
<td>2 (17)</td>
<td>10 (59)</td>
<td>0.05</td>
</tr>
<tr>
<td>WBC count, x10⁹/L*</td>
<td>16.9 (7.2-48.9)</td>
<td>7.5 (0.8-40)</td>
<td>0.07</td>
</tr>
<tr>
<td>Peripheral blasts, %</td>
<td>30</td>
<td>19</td>
<td>ns</td>
</tr>
<tr>
<td>Complex karyotype, n/total available (%)</td>
<td>7/8 (88)</td>
<td>5/17 (29)</td>
<td>0.01</td>
</tr>
<tr>
<td>TP53 mutation, n (%)</td>
<td>10/12 (83)</td>
<td>1/17 (6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Median time to AML/MDS, years</td>
<td>9</td>
<td>9.3</td>
<td>ns</td>
</tr>
<tr>
<td>Median survival from AML diagnosis, days</td>
<td>95</td>
<td>343</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Abbreviation: ns, not significant, *Median (range)
Legends for the figures

**Figure 1:** cumulative incidence of progression to AML/MDS in ET according to mutational status. Curves were drawn from the multivariate model after adjustment for exposure to leukemogenic agents and progression to MF. (p <0.0001 for the comparison).

**Figure 2:** somatic mutations at time of transformation in 29 patients with post-PV or post-ET AML/MDS. In the central panel, each column corresponds to an individual case. Coloured squares denote mutated genes. The right part of the figure shows the distribution of mutated genes according to original diagnosis. **Cases 9 and 20 developed MDS before AML. Cases 14 and 25 progressed to MDS without posterior AML. The remaining cases progressed directly from the chronic phase to AML.**

**Figure 3:** variant allele frequency of the most frequent mutated genes in 20 cases with paired samples from the chronic and AML/MDS phases. Blue circles correspond to mutant load in the chronic phase. Red circles denote mutant load at time of transformation.
Figure 1

57x56mm (300 x 300 DPI)
Figure 2

177x156mm (300 x 300 DPI)
Figure 3

65x15mm (300 x 300 DPI)
Supplemental Data

1. Supplemental methods
   1.1. Clinical data
   1.2. DNA extraction
   1.3. Library preparation and targeted amplicon analysis by NGS of LAM-phase samples
   1.4. NGS analysis
   1.5. Validation of somatic mutations and chronic-phase sample examination
   1.6. Complementary genetic analysis
   1.7. Statistics

2. Supplemental clinical data

3. Supplemental tables

4. Supplemental figures
1.1. Clinical data

In all patients the main clinicohaematological data at presentation of PV or ET were collected, including age, sex, spleen size measured by palpation, haemoglobin (Hb) level, white blood cell (WBC) counts, platelet counts, cytogenetic abnormalities, and genotype. For the purpose of the present study, four genotype categories were considered based on the mutational profile in the MPN-driver genes: JAK2-mutated (V617F or exon 12 mutations), CALR-mutated, MPL-mutated, and triple negative (TN). Genotype studies were performed in the hospital where the patient was controlled. All cytoreductive therapies administered during the patient’s follow-up were registered, as well as the hematologic progression to MF, AML, MDS or evolution from ET to PV.

1.2. Genomic DNA extraction

Peripheral blood or bone marrow samples were used for this study. Peripheral blood samples were centrifuged on a density gradient (Biocoll Separating Solution, 1.077g/ml; Biochrom AG, Berlin, Germany) for PBMC separation. Then, granulocytes were obtained from remaining material by sedimentation on a 2% dextran solution. Bone marrow samples were directly processed to total leucocytes separation by dextran sedimentation. Genomic DNA extracts were prepared with an automated procedure using the GenoM-48 (GenoVision AS, Oslo, Norway) or manually using the QIAGEN QIAamp (QIAgen, Valencia, CA, USA) as described by the manufacturers.

1.3. Library preparation and targeted amplicon sequencing (NGS) of LAM-phase samples

The concentration of dsDNA of each sample was measured by Quant-iT PicoGreen dsDNA or Qubit High Sensitivity assay kit (Invitrogen, Eugene, OR, USA). Sequence enrichment was performed using a targeted, multiplexed amplicon-based approach (Human Myeloid Neoplasms GeneRead DNaseq Targeted Panel v2; QIagen, Hilden, Germany) starting from 40 ng of genomic dsDNA. This panel targets the following 50 genes, with a coverage of the 98.1% over the CDS region (according to the manufacturer datasheet specifications): ABL1, ASXL1, ATRX, BCOR, BCORL1, CBL, CBLB, DAXX, DNM3A, EED, ETV6, EZH2, FLT3, GATA1, GNAS, IDH1, IDH2, IKZF1, JAK1, JAK2, JAK3, KAT6A, KIT, KRAS, MLL, MPL, NF1, NPM1, NRAS, PHF6, PRPF40B, PTPN11, RAD21, RB1, RUNX1, SETBP1, SF1, SF3A1, SF3B1, SH2B3, SMC1A, SMC3, STAG2, SUZ12, TET2, TP53, U2AF1, U2AF2, WT1, ZRSR2.

After PCR clean-up and quantification, the NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) was used to construct indexed libraries following the manufacturer’s instructions. Length and quantity of dsDNA libraries were assessed by Agilent DNA 1000 Kit using 2100 Bioanalyzer system (Agilent Technologies, Palo Alto, CA, USA) and KAPA kit (KAPA Biosystems, Woburn, MA, USA) and ultimately normalized and pooled for sequencing. Libraries sequencing was performed either on MiSeq or NextSeq platforms (Illumina, San Diego,
CA, USA) with Reagent v3 Kit and High Output v2 Kit, respectively, using 2x150bp paired-end reads.

1.4. NGS analysis

Secondary analysis comprising demultiplexing, FASTQ file generation, alignment to reference genome (hg19, GRCh37) and variant calling was performed using MiSeq Reporter Software v2.4 or the cloud version in BaseSpace (Illumina) for NextSeq data. Per default TruSeq Amplicon workflow was launched selecting the Somatic Variant Caller, which allows the identification of variants with an allele frequency above 1%. Further details of algorithms used by the software are available at the Illumina website http://support.illumina.com/sequencing/sequencing_software/miseq_reporter/documentation.html. Resulting VCF files were then annotated and filtered using Illumina VariantStudio v2.2. Filters applied were: per default pass filter (excluding variants showing low quality and strand bias), minimum 30 reads, minimum 2% variant allele frequency (VAF) and population frequency of the variant under 1%. Non-synonymous and splice site variants were considered and checked using Integrative Genome Viewer (IGV) v2.3. All variants were classified according to genomic databases (dbSNP, COSMIC, ExAC, 1000 Genomes, ClinVar) and literature and known polymorphisms were not considered.

1.5. Validation of somatic mutations and chronic-phase sample examination

All variants observed in a VAF lower than 10% were confirmed by deep sequencing using a 454 GS Junior (Roche, Basel, Switzerland). Processed and quality-filtered reads were analysed using the GS Amplicon Variant Analyser software version 2.5p1 (Roche). In addition JAK2 p.V617F and IDH1 p.R132H mutations were confirmed by allele-specific oligonucleotide PCR on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

Unknown variants found in a VAF of 40-60%, therefore suggesting potential germline origin, were analysed in constitutional DNA (obtained from CD3+ T cells, buccal swabs or saliva) of a paired sample by Sanger sequencing on an ABI3500DX (Applied Biosystems).

Confirmed somatic mutations were then assessed and quantified in paired DNA from granulocytes obtained at time of diagnosis or chronic phase of the disease before the acute transformation by deep sequencing on a 454 GS Junior.

1.6. Complementary genetic analysis.

Since medium-large deletions and repetitive genomic regions are still challenging for current NGS methodology, all patients were screened for CALR, NPM1 and FLT3 mutations using fragment analysis on an ABI3500DX. Moreover, the Human Myeloid Neoplasms GeneRead DNAseq Targeted Panel we used for the NGS, in the current available version (v2) does not target two relevant genomic regions in MPNs: the SRSF2 gene (exon 1) and a 169 bp-long CG rich region of ASXL1 (from His631 to
Cys687, NP_056153), which corresponds to the region with higher mutation rates of ASXL1. These two regions were therefore sequenced using the 454 GS Junior with the following primers: SRSF2-ex1A forward 5’-CGGGCGCCACTCAG-3’, reverse 5’CAGCTCGCGGGCCTC3’, SRSF2-ex1B forward 5’-TCGGCGACGTGTACATCC-3’, reverse 5’GCCGCGGACCTTTGTGAG3’, ASXL1-R634 forward 5’-GGACCCTCAGACATTAAA3’, reverse 5’-TGGGTATGCTCCCCATTTAG-3’.

1.7 Statistics

The framework of competing risks was made by the method of Fine & Gray which allows relating a covariate to the cumulative incidence of a specific event type using a model that is similar to the Cox model in that covariates act to multiply the baseline subdistribution hazard in a time-independent manner. Interpretation of the subdistribution hazard ratio (SHR) is similar to that of the Cox model.

2. Supplemental clinical data

Treatment with interferon: sixty patients received interferon. Forty one such patients received other types of cytoreduction including hydroxyurea n=39, busulfan n= 2, and radioactive phosphorus n=6. There were 2 cases of AML transformation among patients treated with interferon, both of them being also exposed to leukemogenic agents; one received hydroxyurea and melfalan and the other hydroxyurea and radioactive phosphorus.

A total of 805 patients were diagnosed before 2005 with 263 of them (33%) having no available genotype, whereas 942 patients were diagnosed in 2005 or thereafter and only 66 (7%) of them lacked genotype (p<0.001). The incidence of AML was similar according to year of diagnosis (before or after 2005). The cumulative incidence of AML, taking death as a competing event, did not change in 2005 and thereafter as compared with the previous period both in ET (SHR 1.90, 95% CI: 0.90-3.97, p=0.09) and PV (SHR: 1.09, 95%CI: 0.42-2.54, p=0.83 for PV).
3. **Supplemental tables**

<table>
<thead>
<tr>
<th>Table S1: Multivariate analysis of risk factors for AML/MDS transformation in 369 patients with PV and available cytogenetics</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Feature</strong></td>
</tr>
<tr>
<td>----------------------------------------------------------------</td>
</tr>
<tr>
<td>Age &gt; 65 years</td>
</tr>
<tr>
<td>WBC &gt; 10x10⁹/L</td>
</tr>
<tr>
<td>Exposure to leukemogenic agents</td>
</tr>
<tr>
<td>Cytogenetic abnormalities</td>
</tr>
</tbody>
</table>

SHR: subdistribution hazard ratio.

**Title for table S2:** somatic mutations identified by NGS analysis in paired samples obtained in the chronic phase and at time of leukemic transformation in 29 patients with polycythaemia vera or essential thrombocythaemia.

**Footnote for table S2:** Chr: chromosome. Ref Seq: reference sequence. AA: aminoacid. VAF: variant allele frequency. db: database. SNV: single nucleotide variation. Indel: insertion and deletion. ITD: internal tandem duplication. NA: paired sample not available. Cases 9 and 20 developed MDS before AML. Cases 14 and 25 progressed to MDS without posterior AML. The remaining cases progressed directly from the chronic phase to AML.
Table S3: Somatic mutations at time of transformation at time of AML/MDS transformation according to JAK2V617F mutational status of the leukemic clone in 29 patients with PV or ET

<table>
<thead>
<tr>
<th>Gene</th>
<th>JAK2V617F-positive N=12</th>
<th>JAK2V617F-negative N=17</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TET2, n (%)</td>
<td>3/12 (25)</td>
<td>5/17 (29)</td>
<td>0.8</td>
</tr>
<tr>
<td>DNMT3A, n (%)</td>
<td>1/12 (8)</td>
<td>5/17 (29)</td>
<td>0.2</td>
</tr>
<tr>
<td>ASXL, n (%)</td>
<td>2/12 (17)</td>
<td>3/17 (18)</td>
<td>0.9</td>
</tr>
<tr>
<td>EZH mutation, n (%)</td>
<td>0/12</td>
<td>4/17 (23)</td>
<td>0.1</td>
</tr>
<tr>
<td>IDH1/2, n (%)</td>
<td>2/12 (17)</td>
<td>3/17 (18)</td>
<td>0.9</td>
</tr>
<tr>
<td>RUNX1 mutation, n (%)</td>
<td>1/12 (8)</td>
<td>4/17 (23)</td>
<td>0.2</td>
</tr>
</tbody>
</table>

4. Supplemental figures

Legend for figure S1: overall survival in ET according to mutational status (p<0.0001).
Legend for figure S2: cumulative incidence of progression to acute myeloid leukaemia or myelodysplastic syndrome (AML/MDS) taking death without AML/MDS as a competing event. **S2A**: progression to AML/MDS in PV. **S2B**: progression to AML/MDS in ET. Solid line corresponds to AML/MDS probability taken death as a competing event. Dotted line corresponded to cumulative incidence of death without previous AML/MDS transformation.
**Legend for figure S3:** cumulative incidence of progression to AML/MDS in ET according to progression to MF, adjusted for the exposure to leukemogenic agents ($p<0.0001$).
**Legend for figure S4:** survival after transformation in 29 patients with acute leukaemia (n=27) or myelodysplastic syndrome (n=2) according to the mutational status of the leukemic clone. Solid line represents JAK2V617F-unmutated, dotted line corresponds to JAK2V617F-mutated (p=0.003 for the comparison).
<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Chr position (GRCh37/hg19)</th>
<th>Gene</th>
<th>Ref Seq</th>
<th>Nucleotide change</th>
<th>AA change</th>
<th>VAF (%) at chronic phase</th>
<th>VAF (%) at acute phase</th>
<th>Mutation type</th>
<th>Consequence</th>
<th>COSMIC db</th>
</tr>
</thead>
<tbody>
<tr>
<td>102305260</td>
<td>JAK2 NM_004973.2</td>
<td>IDH2</td>
<td>NM_002168.2</td>
<td>c.419G&gt;A</td>
<td>p.Arg140Gln</td>
<td>49.54 41.83</td>
<td>SNV missense</td>
<td>COSM41590</td>
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<td>JAK2 NM_004973.2</td>
<td>IDH2</td>
<td>NM_002168.2</td>
<td>c.419G&gt;A</td>
<td>p.Arg140Gln</td>
<td>50.08 41.83</td>
<td>SNV missense</td>
<td>COSM41590</td>
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<tr>
<td>102305260</td>
<td>JAK2 NM_004973.2</td>
<td>IDH2</td>
<td>NM_002168.2</td>
<td>c.419G&gt;A</td>
<td>p.Arg140Gln</td>
<td>50.18 41.83</td>
<td>SNV missense</td>
<td>COSM41590</td>
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<td>COSM41590</td>
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<td></td>
</tr>
</tbody>
</table>

For Peer Review

chronic VAF (%) at

(Steiner et al., 2012)