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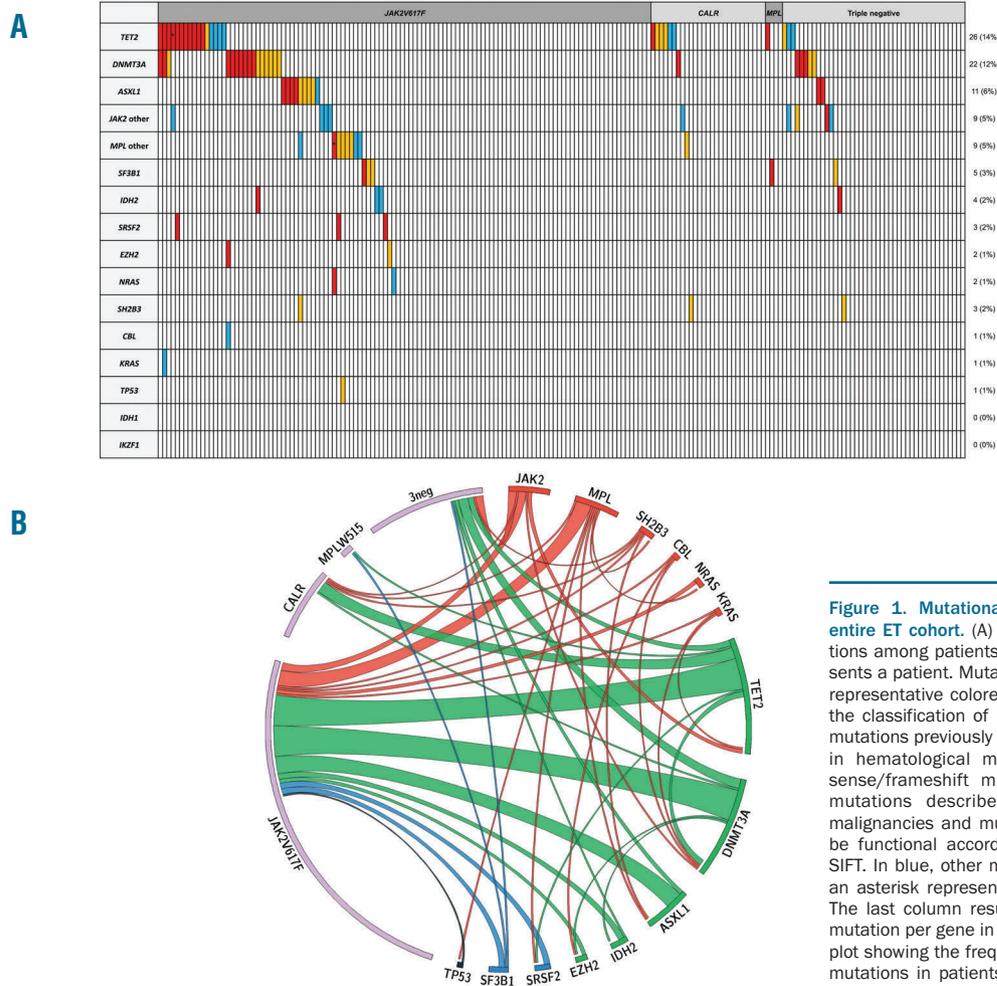
### Positive impact of molecular analysis on prognostic scores in essential thrombocythemia: a single center prospective cohort experience

Classical Philadelphia-negative myeloproliferative syndromes (MPN) are characterized by the presence of driver mutations (*JAK2*, *CALR* or *MPL*) and comprise polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF). The prognosis of ET is highly variable from one patient to another and is related to two major risks of complications: an increased risk for thrombosis (the most frequent) and a risk of hematological transformation (the most severe) into secondary myelofibrosis or acute leukemia. Different prognostic scoring systems based on clinical (age, history of thrombosis) and biological factors (leukocyte count, driver mutation) have been developed in ET.<sup>1,2</sup> Since the milestone study from Vannucchi *et al.*,<sup>3</sup> additional mutations have been clearly demonstrated as important prognostic factors in PMF<sup>4</sup> but, to date, few data are available in ET. In this study, we analyzed prognostic factors and validated scoring systems in a single center cohort of ET patients, and we evaluated the additive impact of NGS

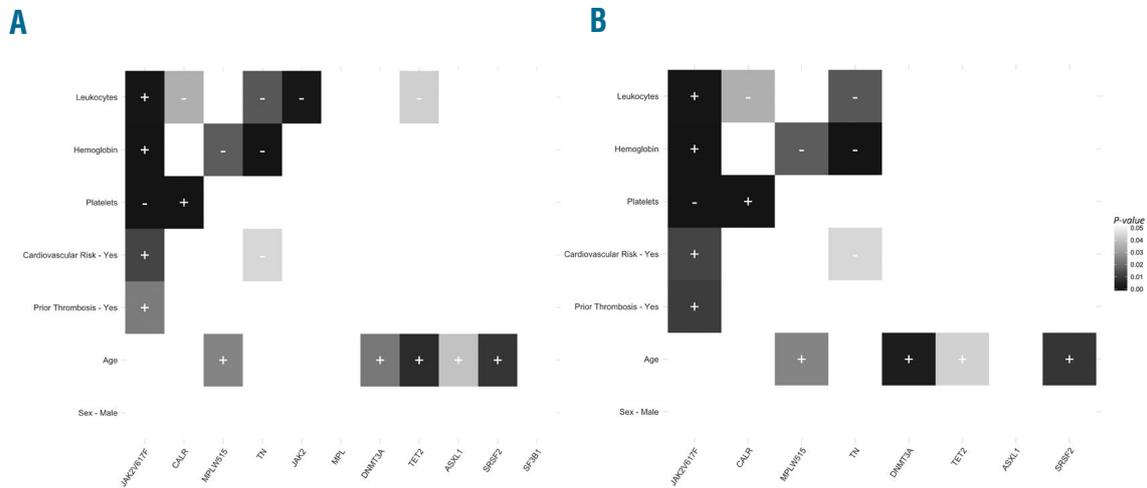
analysis.

The cohort consisted of 190 consecutive ET patients diagnosed at the Henri Mondor hospital between January 2000 and December 2016 (patients' clinical and biologic characteristics at diagnosis are summarized in *Online Supplementary Table S1*). All patients meet the ET WHO 2008 criteria for diagnosis. With a median follow up of 6.4 years, thrombosis occurred in 18 cases (9.4%) and transformation into myelofibrosis, myelodysplastic syndrome or acute myeloid leukemia was observed in 11 cases (5.7%). A total of 16 deaths (8.4%) were observed during the follow up.

Driver molecular mutations were as follow: *JAK2V617F* (allelic burden over 0.5%) in 116 patients (61.1%), *CALR* mutations in 27 patients (14.2%, of which 15 are del52 (type 1), 8 ins5 (type 2) and 4 other mutations inducing a similar frameshift (details in *Online Supplementary Table S2*)) and *MPLW515K/L* mutation in 4 patients (2.1%). Triple negative patients represented 22.6% of the cohort (n= 43). *CALR* mutations were previously associated with a favorable prognosis in ET. In this study we failed to draw a conclusion about the prognostic impact of these mutations, probably due to the small number of *CALR* mutated cases.<sup>5</sup>



**Figure 1. Mutational landscape of the entire ET cohort.** (A) Distribution of mutations among patients. Each column represents a patient. Mutations are depicted by representative colored boxes according to the classification of pathogenicity. In red: mutations previously described as somatic in hematological malignancies or nonsense/frameshift mutations. In orange, mutations described in hematological malignancies and mutations supposed to be functional according to Polyphen2 or SIFT. In blue, other mutations. Boxes with an asterisk represent a double mutation. The last column resumes the number of mutation per gene in the cohort. (B) Circos plot showing the frequency of co-occurring mutations in patients where the width of the ribbon corresponds to the relative frequency of the association of two mutations.



**Figure 2. Association between clinico-biological parameters and mutations.** Additional mutations were tested when all of them were considered (panel A) and when only pathogenic mutations were considered (panel B). Colored boxes represent a significant link, based on appropriate statistical test (Exact Fisher, t-test, Mann-Whitney). A plus sign represents a positive link whereas a minus sign represents a negative link.

Searching for additional mutations, a 16-genes NGS panel (*ASXL1*, *EZH2*, *DNMT3A*, *TET2*, *IDH1*, *IDH2*, *TP53*, *SF3B1*, *SRSF2*, *CBL*, *NRAS*, *KRAS*, *JAK2*, *MPL*, *SH2B3*, *IKZF1*) was used at the time of diagnosis (or during the first years of follow up). After removal of known polymorphisms, mutations were classified according to their putative impact in three groups: A (pathogenic mutations), B (likely pathogenic mutations) and C (mutations of unknown significance) (Details in *Online Supplementary Methods*). We then evaluated the impact of these additional mutations by taking into account all mutations ("ABC") or by considering only the most pathogenic ("A").

At least one additional mutation was found in 83 patients (44%). The mutation frequencies and details are reported in Figure 1A, *Online Supplementary Figure S1* and *Table S3*. Notably, an additional mutation was detected in 15/43 triple negative patients (35%). The most mutated genes were *TET2* (ABC: 13.7%, A: 6.8%), *DNMT3A* (ABC: 11.5%, A: 6.8%) and *ASXL1* (ABC: 5.8%, A: 3.2%). Association of mutations, considering all additional mutations (ABC), are represented in the Figure 1B. The most frequent associations of mutated genes concerned *JAK2V617F* and both *TET2* and *DNMT3A* (16 cases each). Of note, mutations of *CALR* and *ASXL1* or splicing genes (*SRSF2* and *SF3B1*) were mutually exclusive. As already described, the NGS analysis showed good performance in measuring the allelic charge of the *JAK2V617F* mutation and high correlation with standard procedures (*Online Supplementary Figure S2*).<sup>6</sup> Only 2 mutations of low allele burden (0.14 and 0.24%) were not detected due to insufficient sequencing depth (4218 and 918X).

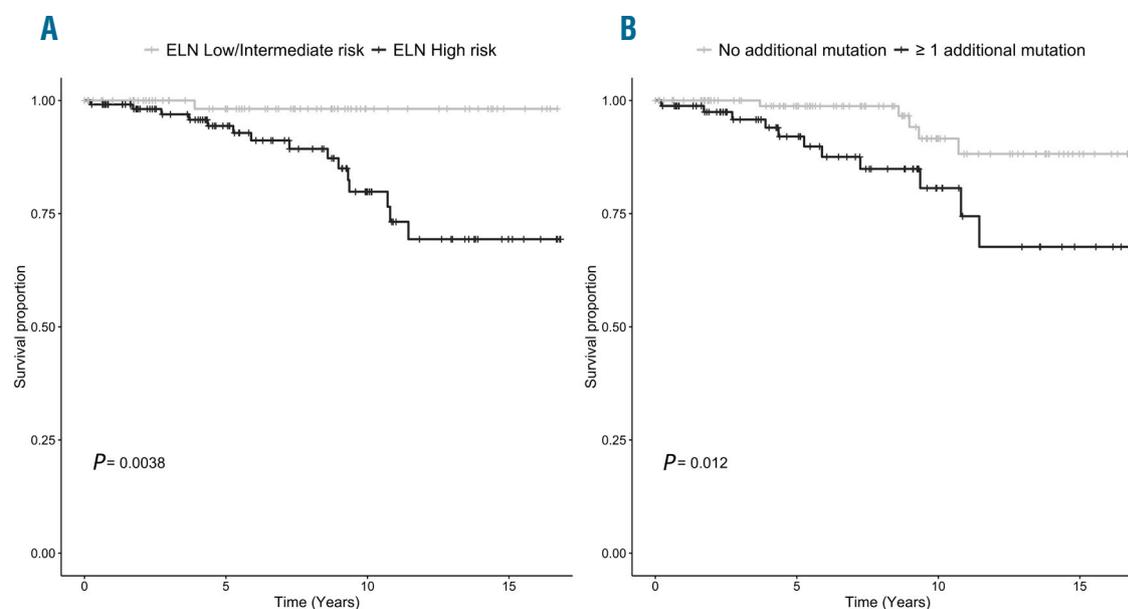
Association of clinico-biological parameters and molecular landscape were tested with driver mutations and additional mutations for which mutations of "ABC" and "A" classifications were analyzed separately (Figure 2). With the "ABC" classification, *DNMT3A*, *TET2*, *ASXL1* and *SRSF2* mutations were associated with older age ( $P=0.022$ ,  $P=0.0066$ ,  $P=0.04$ ,  $P=0.0087$  respectively), *JAK2* mutations other than *V617F* and *TET2* mutations were also associated with lower leukocytes count ( $P=0.0014$  and  $P=0.042$  respectively, Figure 2A). When

we applied the "A" classification, *TET2*, *DNMT3A* and *SRSF2* were still associated with older age ( $P=0.043$ ,  $P=0.003$  and  $P=0.0087$  respectively) (Figure 2B).

We studied the performance of ELN<sup>7</sup> (high risk defined as age > 60 years or a history of thrombosis), IPSET-survival<sup>8</sup> and IPSET-thrombosis<sup>1</sup> scoring systems (details in *Online Supplementary Table S11*) using the concordance index for overall survival and event-free survival (death, hematologic progression and thrombosis: all events, then separate analyses) (details of prognostic groups in *Online Supplementary Table S4*). In our cohort, the ELN score seemed to be the better prognostic scoring system. It was able to categorize patients according to overall and event-free survival ( $P=0.0038$  and  $P=0.00063$ , respectively, Figure 3A and *Online Supplementary Figure S3*), but not thrombosis or transformation separately. The IPSET-survival score was able to discriminate for overall survival ( $P=0.033$ , *Online Supplementary Figure S4*). Lastly, in our cohort IPSET-Thrombosis did not allow us to discriminate survival, all events, transformation nor thrombosis (*Online Supplementary Figure S5*).

Thereafter, we tested the prognostic impact of additional mutations. Results of all univariate analysis are summarized in *Online Supplementary Table S5*. We assessed with multivariate models whether prediction of prognosis was improved by incorporating the NGS findings into the ELN classification (*Online Supplementary Methods*). For "ABC" classification, the presence of at least one additional mutation and the number of additional mutations were associated with adverse prognosis in term of survival ( $P=0.012$  and  $P=0.019$  respectively, Figure 3B and *Online Supplementary Figure S6*). The same result was observed with the "A" classification with lower  $P$ -values ( $P=0.0024$  and  $P=0.00016$ ; *Online Supplementary Figures S7* and *S8*). Interestingly, the HMR signature was associated with survival only when we considered pathogenic mutations ( $P=0.011$ ).

In multivariate analysis, whatever the additional mutation classification (ABC or A), both ELN status and the presence of an additional mutation were associated (i) to a higher risk of event (ELN status (HR: 3.75 (High risk/Low+Int risk),  $P=0.0185$ ) and additional mutation (HR: 2 (Yes/No),  $P=0.035$ ); *Online Supplementary Tables S6*



**Figure 3. Overall survival of the entire cohort according to the ELN classification and additional mutations.** Kaplan-Meier representation of survival data according to ELN prognostic score (panel A) and to the presence of one or more additional mutations (driver mutations were excluded and all additional mutations detected were considered: "ABC" classification) (panel B).

and *S7*), and (ii) to a lower overall survival (ELN status (HR: 9.6 (High risk/Low+Int risk),  $P=0.029$ ) and additional mutation (HR: 3.5 (Yes/No),  $P=0.022$ ; *Online Supplementary Tables S8 and S9*).

Hematological transformation was associated with male sex ( $P=0.0047$ ), *MPLW515* mutations ( $P<0.0001$ ) and splice mutations ( $P<0.0001$ ). In multivariate analysis, only male sex (HR: 5.9,  $P=0.029$ ) remained associated to hematological transformation (*Online Supplementary Table S10*). The presence of cardiovascular risk factor was associated with thrombosis events in univariate ( $P=0.013$ ) and multivariate (HR: 3.2 (Yes/No); 95% CI [1.2-8.1];  $P=0.016$ ) analysis.

In our study, targeted-NGS sequencing increased the proportion of ET patients with a clonal marker from 77.4% with driver mutations to 85.3%. Then, detection of a mutation by NGS screen can improve the diagnosis of clonal hematopoiesis in case of triple negative patients.<sup>8,9</sup> However, the interpretation of an additional mutation has to consider the age of the patient and the allele burden due to clonal hematopoiesis of indeterminate potential (CHIP) that can be found in healthy subjects. The result of the NGS must obviously be compared with all WHO diagnostic criteria to avoid a false diagnosis of MPN, particularly when detecting mutations in *TET2*, *DNMT3A* and *ASXL1* that have been demonstrated to be the most frequent in both CHIPs<sup>12</sup> and MPN (after the driver mutations) in previous studies<sup>10,11</sup> as in ours. In the present cohort, these mutations were associated with older age at diagnosis, raising the question of the presence of CHIPs associated to MPN either with 2 separates clones or with the acquisition of a driver mutation in a CHIP clone. This last hypothesis is supported by studies at clonal level that have shown that patients with both *TET2* and *JAK2V617F* mutations were older when the *TET2* mutation was first acquired.<sup>13</sup> However, we detected few additional mutations with allele burden <20%, and the distribution of the allele burdens between

patients over or under 65 years was similar (*Online Supplementary Figure S9*). This suggests that there were few CHIPs among the mutations detected in this study. Therefore, these additional mutations in our cohort can be considered as true clonal markers of MPN.

The prognostic impact of additional mutations in PMF was mostly studied in PMF where a signature of 5 genes (*ASXL1*, *EZH2*, *IDH1/2* and *SRSF2: HMR*) was associated to poor prognosis.<sup>3</sup> This HMR signature also showed an impact in pre-fibrotic PMF<sup>14</sup> but not in our ET cohort.

In ET, few studies are available to date. Tefferi *et al.* described an adverse molecular signature in a cohort of 183 ET patients with a validation in another cohort of 174 patients.<sup>15</sup> Notably, *ASXL1* and *SRSF2* were not found to be associated with adverse prognosis in this ET cohort, despite conveying a worse outcome in PMF or in another cohort of ET.<sup>8</sup>

In our study, the presence of at least one additional mutation at the time of diagnosis improved the prognostic evaluation of ELN classification in terms of overall survival and event-free survival. No impact of additional mutations on hematological transformation was found in multivariate analysis with a limited number of events. The prognostic impact of additional mutations was similar when we considered all mutations or only the most pathogenic. This result is of interest in the context of the rise of NGS in clinical practice because the classification of mutations encountered can be difficult particularly when a germline sample is not analyzed in parallel. Our study focused on diagnosis, but additional mutations could appear during the follow up even if this seems to be a rare event during chronic stage of the disease.<sup>16,11</sup> Nevertheless, some of these mutations could have a strong prognostic impact, such as *TP53* mutations.<sup>17,18</sup>

To conclude, our results suggest that NGS provides additional information on prognosis in ET. Larger studies are still needed for recommending a systematic molecular screening at ET diagnosis or during follow up.

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