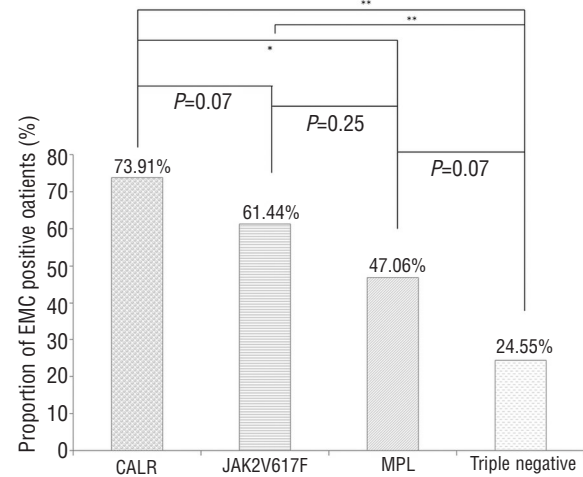


### Endogenous megakaryocytic colonies underline association between megakaryocytes and calreticulin mutations in essential thrombocythemia

Calreticulin (*CALR*) mutations occur in 20%-25% of myeloproliferative neoplasms (MPN).<sup>1,2</sup> At least 40 *CALR* mutations have been reported to date, all located in exon 9. The most frequent *CALR* mutations are a 52-bp deletion (type 1) and a 5-bp insertion (type 2). Expression of type 1 *CALR* mutation was shown to induce constitutive activation of JAK-STAT signaling pathway in a Ba/F3-cell line with STAT5 phosphorylation leading to spontaneous growth in the absence of interleukin-3.<sup>1</sup> Furthermore, megakaryocyte lineage has been reported to play a major role in MPN pathophysiology.<sup>3,4</sup> In particular, hematopoietic colony formation independent of exogenous cytokines, including endogenous megakaryocytic (EMC) and endogenous erythroid colonies (EEC) was shown to be a functional sign of clonal hematopoiesis due to deregulated signaling pathways in MPN. Altogether, these data suggest a particular link between megakaryocytic (MK) proliferation and deregulation of signaling due to driver mutations. However, to our knowledge, spontaneous growth of hematopoietic progenitors (EMC and EEC) has not been characterized in type 1 or type 2 *CALR*-mutated patients.

In the current study, we analyzed patterns of EMC and EEC according to molecular status (*JAK2V617F*, *CALR*, *MPL*, triple negative) in a cohort of 302 essential thrombocythemia (ET) patients from 3 French University Hospitals [Grenoble (n=121), Dijon (n=121), and Nantes (n=60)]. The inclusion criteria were ET patients who had benefited from *in vitro* cultures from bone marrow at diagnosis and for whom DNA was available. The *JAK2V617F* mutation was assessed using purified granulocytes by tetra-primer ARMS-PCR<sup>5</sup> or by allele-specific quantitative PCR.<sup>6</sup> *MPL* mutations were screened by high resolution melt assay<sup>7</sup> and confirmed by sequencing. The mutational status of *CALR* was determined using previously described high-resolution sizing of fluorescent dye-labeled PCR amplicons of exon 9, with Sanger sequencing controls.<sup>1</sup> To standardize EMC and EEC, cultures were performed from bone marrow samples using the same standardized collagen medium in all three centers.<sup>8,9</sup> Megakaryocytic colonies composed of at least 4 MKs were counted by microscopy after MGG staining of dry collagen dishes.<sup>9,10</sup> We analyzed EMC and EEC results both qualitatively [presence or not of EMC and/or EEC (so-called "positive or negative EMC/EEC")] and quantitatively (number of EMC and/or EEC per 10<sup>5</sup> cells plated). For statistical analysis, non-parametric tests were applied: Mann-Whitney test (for comparison of two groups) and McNemar test (paired data).  $\chi^2$  or Fisher tests were used to compare nominal variables, and Spearman rank correlation to compare two continuous variables.  $P < 0.05$  was considered statistically significant.

The mutational distribution was 50.8% (153 of 302) for *JAK2V617F*, 23% (69 of 302) for *CALR*, 5.3% (17 of 302) for *MPL* and 19% (57 of 302) for "triple negative". Six patients lacked *MPL* and/or *CALR* data (1.9%). Among the 69 patients with *CALR* mutations, 35 (50.7%) patients harbored type 1 and 24 (34.8%) patients had type 2. Irrespective of mutational status, overall endogenous hematopoietic growth (defined by presence of EMC and/or EEC) was 58.9% (178 of 302). We observed significantly more EMC (56.3%; 170 of 302) than EEC (13.6%; 41 of 302) whatever the mutational status ( $P < 0.001$ ).



**Figure 1.** Proportion of patients presenting respectively EMC growth according to mutational status in ET. Statistical differences between mutations were analyzed in pairs ( $\chi^2$  test). \*means  $P$  value  $< 0.05$ ; \*\* means  $P < 0.01$ .

In order to determine the relationship between growth profile and genotype, we compared global spontaneous growth defined by EMC and/or EEC positivity according to mutational status. Patients harboring mutations such as *JAK2V617F* or *CALR* showed a significantly higher proportion of overall spontaneous growth (respectively 66% and 73.9%) compared to "triple negative" ones (25%;  $P < 0.001$ ). No difference in frequency of EMC and/or EEC between *JAK2V617F* and *CALR* was noted ( $P = 0.24$ ). On the contrary, *MPL* mutations were less frequently associated with EMC and/or EEC compared to *CALR* (47% and 73.9%, respectively;  $P = 0.03$ ).

As higher platelets counts were reported in patients with *CALR* mutations in comparison with *JAK2V617F* patients,<sup>11</sup> we attempted to compare the proportion of EMC in these two subgroups (Figure 1). EMC were more frequently observed in *CALR* than in *JAK2V617F* (73.9% and 61.4%, respectively) without reaching statistical significance ( $P = 0.07$ ). To strengthen the link between genotypes and megakaryocytic proliferation, we also compared the mean number of EMC according to the molecular status. *CALR* mutations were associated with a significantly higher mean number of EMC compared to "triple negative" and *JAK2V617F* patients: 9.36 (0-81.5), 1.75 (0-23.3), and 5.5 (0-53.3), respectively;  $P < 0.01$  and  $P = 0.02$ . A tendency towards a higher number of EMC was observed between *CALR* and *MPL* but without statistical significance ( $P = 0.07$ ). Interestingly, concerning EEC, *CALR*-mutated patients displayed a significantly weaker proportion of EEC positivity compared to *JAK2V617F*-mutated patients (1.4% vs. 22.8%;  $P < 0.001$ ). This percentage of EEC in *JAK2V617F* ET is consistent with previous data obtained with a serum-free assay.<sup>6,9,12</sup> EEC were rare in the *CALR*-mutated population; only one type 2-mutated patient among 69 showed 2 EEC per 10<sup>5</sup> cells. A significantly higher proportion of EEC were associated with *JAK2V617F* compared to triple negative ( $P < 0.001$ ) and *MPL* ( $P < 0.05$ ).

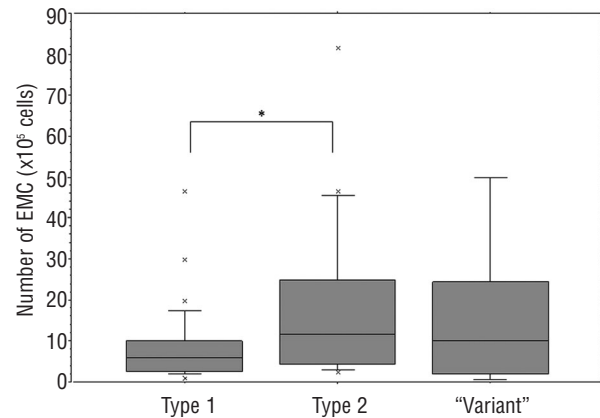
Next, the endogenous megakaryocytic profile between types 1 and 2 *CALR* mutants and "variants" was compared. "Variants" have neither type 1 nor type 2 *CALR* mutations (n=10 patients). EMC frequencies were similar in type 1 and type 2 patients (82.8% and 70.8%, respectively;  $P = 0.34$ ). The proportion of EMC in "variants" was

significantly lower than in type 1 mutants (50%;  $P=0.04$ ), although a higher allelic burden was observed compared to type 1 ( $P<0.01$ ). Among the population presenting EMC (Figure 2), type 2 patients induced more EMC than type 1 [mean<sub>2</sub>=19.2 (2.5-81.5) vs. mean<sub>1</sub>=8.6 (1-46.6), respectively;  $P=0.03$ ]. Interestingly, among these patients, we observed a significant difference in allelic ratio between type 1 and type 2 [mean<sub>1</sub>=41.8% (5-65%) and mean<sub>2</sub>=48.1% (30-73.9%), respectively;  $P=0.03$ ].

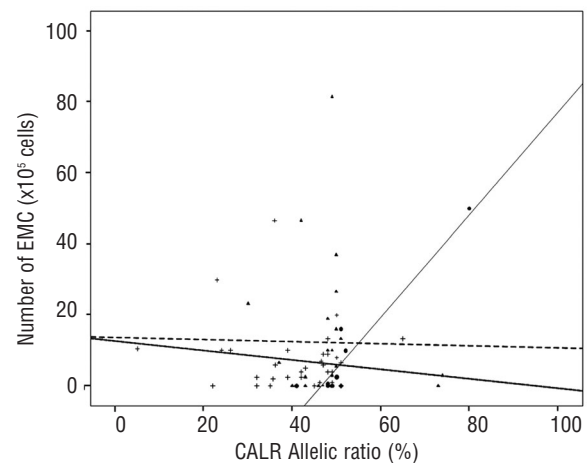
Among the population presenting EMC, no difference in the number of EMC was observed between “variants” and either type 1 or type 2, even if “variants” patients displayed a higher allelic burden than type 1 [mean<sub>variant</sub>=56.2% (48-80%);  $P<0.01$ ] and type 2 ( $P<0.05$ ). We wondered if there was a correlation between the number of EMC and allelic burden in the *CALR*-mutated population and according to type of mutation (Figure 3). Overall, no linear correlation was observed either in the whole *CALR* population ( $n=68$ ), or in type 1 and type 2 ( $\rho=0.07$ ,  $P=0.56$ ;  $\rho_1=-0.069$ ,  $P_1=0.686$  and  $\rho_2=0.084$ ,  $P_2=0.69$ , respectively). However, in the “variants”, we observed a linear correlation between allelic burden and spontaneous megakaryocytic growth ( $\rho=0.705$ ,  $P<0.05$ ).

Klampfl *et al.* showed that type 1 *CALR* mutation induced a cytokine independent growth of the murine Ba/F3 cell line.<sup>1</sup> Our study confirmed this finding with the difference that our patient cohort was made up of a ‘real-life’ group of MPN patients and our patient selection did not discriminate between type 1 or type 2 mutations. Moreover, our results highlight a special link between *CALR* and megakaryocyte proliferation. Firstly, we demonstrated that a significantly higher proportion of *CALR* patients displayed EMC in comparison to “triple negative” and to *MPL*-mutated patients. *CALR*-mutated patients also expressed a higher number of EMC compared to *JAK2V617F*-mutated patients. The more frequent EMC and the higher number of EMC in *CALR*-mutated patients argue for an impact of *CALR* on autonomous MK proliferation. In the same way, it is consistent with the almost complete absence of *CALR* mutations in polycythemia vera (PV)<sup>15</sup> (despite the fact that rare PV cases with *CALR* mutations have recently been reported<sup>14</sup>), the higher platelet counts in *CALR* patients,<sup>11</sup> and the preferential expression of *CALR*-mutated protein in megakaryocytes.<sup>15</sup> Our results also confirm that EEC is a characteristic feature of *JAK2V617F* mutation. No EEC was observed in *MPL*-mutated patients and only one patient carrying *CALR* mutation was EEC positive. These functional differences could be related to lower hemoglobin counts observed in *CALR* patients compared to those with *JAK2V617F* mutations. These differences in the balance between erythroid and MK proliferation strengthen the evidence for a megakaryocytic role for *CALR* mutants in ET pathogenesis, and define different growth profiles of ET: *JAK2V617F* patients harbor a “mixed profile” (erythroid and MK) compared to the “MK profile” of *CALR* patients.

Among populations presenting EMC, we observed significantly more EMC and a higher allelic burden in type 2 than in type 1. This supports the hypothesis that, in *CALR*-mutated patients, the greater allele burden induces increased cytokine-independent MK proliferation and subsequent clone enlargement. This suggests not only a contribution of mutation type but also of *CALR* allele burden on ET phenotypes. Nevertheless, the *CALR* allelic burden did not show any linear correlation with the number of EMC either in type 1 or in type 2 mutants. Hence, the *CALR* allelic burden may not be the only factor determining the intensity of EMC growth: others



**Figure 2.** Distribution of number of endogenous megakaryocytic colonies (EMC) among *CALR* population showing EMC growth. “Variant” corresponds to neither type 1 nor type 2 mutations. Whisker plots represent numbers of EMC expressed per  $10^5$  cells plated according to type of *CALR* mutation. \* means  $P < 0.05$ .



**Figure 3.** Linear regression of *CALR* allelic burden according to number of endogenous megakaryocytic colonies (EMC). Each symbol represents a patient (respectively + for type 1,  $\blacktriangle$  for type 2,  $\bullet$  for “variants”). Linear regression was done according to type of *CALR* mutations (--- for type 1, ... for type 2 and — for “variants”).

mutations or genetic modifiers (i.e. epigenetic, RNA splicing) could impact MK activation, and thereby disease evolution.

Finally, our study showed that a “variant” subpopulation displayed EMC in 50% of cases. Even though MK “variants” appear to be less proliferative (less frequent EMC) than type 1 *CALR*, a linear and positive correlation between the number of EMC and allelic burden was observed. This suggests that, in this heterogeneous subgroup, EMC could identify patients in whom *CALR* plays a pivotal role in autonomous activation of MK. In our opinion, it may be interesting to study “not type 1 nor type 2” variants in a larger cohort and according to their clinical features. In conclusion, our study underlines the functional link between *CALR* mutants and megakaryocyte growth stimulation. These findings improve our understanding of phenotypic differences observed among patients with different driver mutations and different *CALR* genotypes.

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