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S. Lemoine, C. Mornet, I. Quintin-Roue, M. C. Rousselet, L. Cottin, A. Georgeais, L. Dubouis, F. Boyer, C. Orvain, C. Caillon, et al.

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Histological and genetic characterization and follow-up of 130 patients with chronic triple-negative thrombocytosis

Chronic thrombocytosis may be reactive in nature, be driven by inherited genetic characteristics (e.g., *THPO, MPL, JAK2* mutations) or be caused by an acquired myeloid malignancy (mainly myeloproliferative neoplasm [MPN]). The diagnostic workup of isolated thrombocytosis therefore requires testing for inflammation/iron deficiency, consideration of a family history, a bone marrow examination and the search for "classical" MPN driver mutations (*BCR-ABL1*, *JAK2*V617F, *CALR* exon 9, *MPL*W515L/K). According to the World Health Organization (WHO) classification, the diagnosis of essential thrombocytosis (ET) requires typical histological features.[1] In the absence of a driver mutation, histological characterization is the only element allowing classification of thrombocytosis as ET. However, a number of patients with a clinical presentation of "triple-negative" acquired thrombocytosis do not display the characteristic histological features of ET or of MPN, raising the question of appropriate therapeutic management. Indeed, in the presence of "high-risk" features (age >60 years, a history of thrombosis), cytoreductive therapy has been demonstrated to reduce the thrombotic risk in MPN patients; however, when the diagnosis of MPN is uncertain because of the lack of typical histological features, it is not known whether patients would benefit from cytoreductive treatment. Previous studies have shown that next-generation sequencing (NGS) could detect variants in 12 to 73% of cases of triple-negative thrombocytosis[2-5] Variants can be acquired in genes frequently mutated in myeloid malignancies or may be identify as germline variants in genes involved in megakaryocytic proliferation, even in the absence of a familial history of thrombocytosis (mainly *JAK2*, *MPL*).[6,7] Efforts at finding a common genetic alteration in triple-negative ET have, in fact, reinforced the idea that this group of patients is heterogeneous, with clonal or non-clonal hematopoiesis and identification of additional acquired as well as constitutive *JAK2, MPL* or *SH2B3* variants, but no common recurrent anomaly.[6,7]

In a cohort of 130 patients with chronic, non-reactive triple-negative thrombocytosis, we first had bone marrow biopsies reviewed by experts of the French group of hematopathologists (GEBOM), then asked whether targeted NGS could help reach a diagnosis. We also asked whether the outcome of patients was predicted better when the diagnostic classification was based on genetic and/or histological features.

Patients followed at Angers or Brest university hospitals, for ET or chronic thrombocytosis, were included if their platelet count at diagnosis was >400x10^9/L, there was no reactive cause (iron deficiency, splenectomy, inflammation) for the raised platelet count and they did not have *JAK2*V617F, *CALR* exon 9 or *MPL*W515L/K mutations (as determined by allele-specific quantitative polymerase chain reaction [PCR] analysis, PCR fragment length polymorphism and quantitative PCR followed by allelic discrimination using probe competition, as previously described).[8] Bone marrow biopsies had been performed in all patients, 118 of which were available for central review. All patients were >18 years old and provided written informed consent, in accordance with law n. 2004-800 of 2004 and law n. 2012-300 of 2012. The median age at diagnosis was 53 years old (range, 18-84) and 72% were females.

Among the 130 patients with triple-negative thrombocytosis, bone marrow biopsy led to a diagnosis of ET/MPN in 79, while in 45 patients, the biopsy was not in favor of MPN and in six the diagnosis was unclear (insufficient quality). After central review of the bone marrow biopsies by GEBOM experts, the repartition was broadly similar (Figure 1A), but the tissue provided was considered insufficient for a clear diagnosis in eight patients. Overall, 24 (18%) patients finally changed categories after review of the bone marrow biopsies (13 initially in favor of a MPN were reclassified as non-MPN; whereas 6 initially considered not in favor of a diagnosis of MPN were finally considered MPN).

Since a histological diagnosis between MPN and non-MPN may be subject to variability and some patients still had unclear diagnosis, we wondered whether a mutational analysis with targeted NGS of 24 genes commonly mutated in MPN (*Online Supplementary Table S1*) could help with the diagnostic discrimination of patients. Among 130 tested patients, a total of 57 variants were found in 38 patients, who displayed one (n=28), two (n=6) or more (n=4) variants, while 92 (71%) patients had none (*Online Supplementary Table S2*). The most frequently affected genes were *MPL, ASXL1, SH2B3, JAK2, TET2* and *DNMT3A* (Figure 1B). Overall, pathogenic/likely pathogenic variants were found slightly more frequently in patients considered as having MPN after bone marrow biopsy review (33 variants in 17/69 (25%) *vs*. 10 variants in 8/50 (16%) in non-MPN patients, *P*=not significant). Acquired *MPL* mutations were found in ten patients initially considered “triple-negative” because only W515K/L mutations had been screened for. Three of

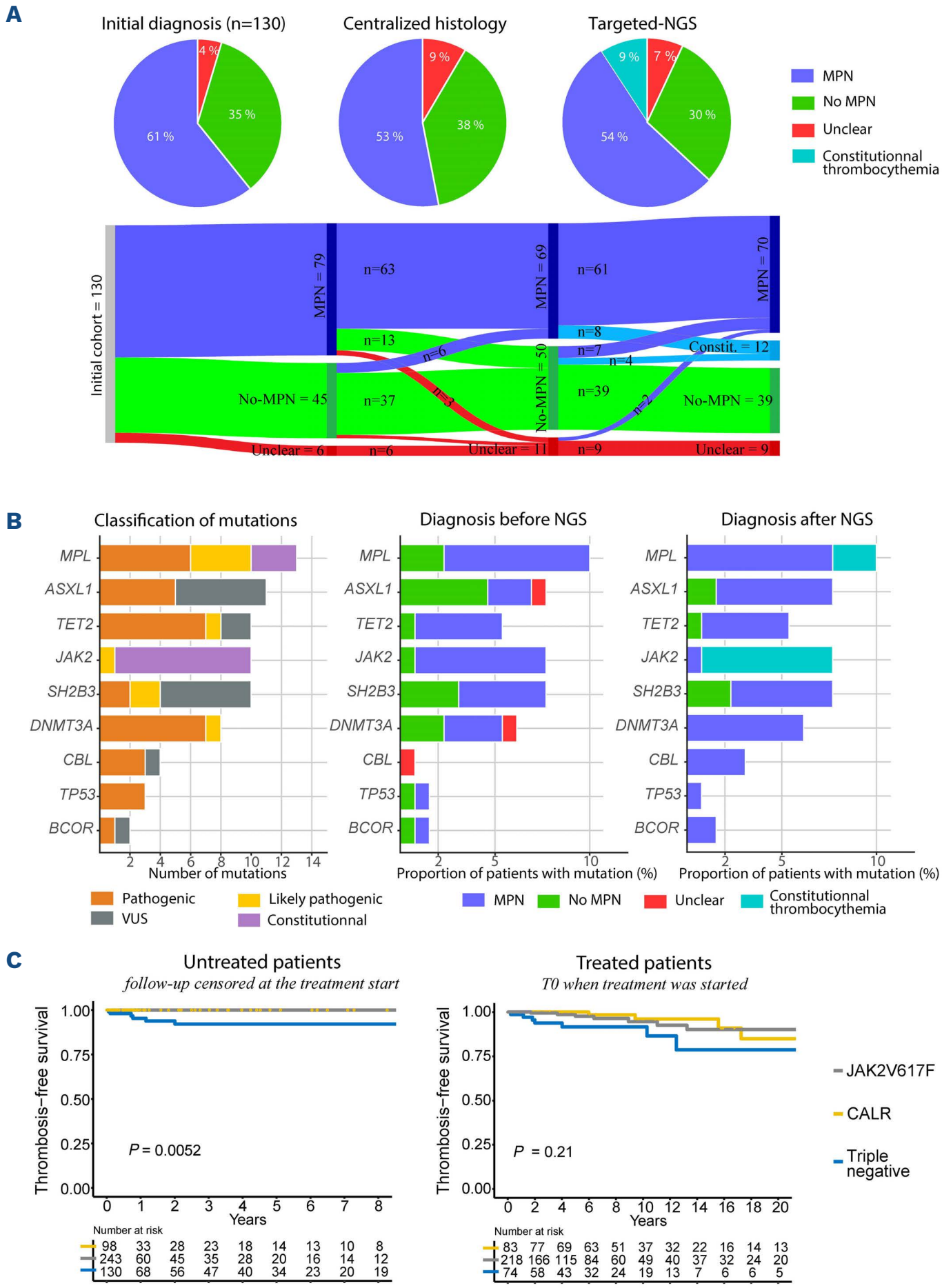

Figure 1. Diagnoses modified, mutations detected by next-generation sequencing in the triple-negative cohort, and incidence of thrombotic events in untreated and treated patients in the triple-negative groups compared to *JAK2*- and *CALR*-mutated controls. (A). Initial and modified diagnoses after bone marrow review and next-generation sequencing (NGS) analysis. Top. Repartition of diagnoses according to successive classifications: (left) at initial diagnosis (before bone marrow biopsy review by GEBOM and NGS); (middle) after bone marrow biopsy review; (right) after targeted NGS. Bottom. Sankey diagram showing the proportion of patients whose diagnosis was modified by bone marrow biopsy review and/or NGS analysis. (B). Molecular landscape of the whole cohort. (Left) number of mutations per gene classified per category (pathogenic, likely pathogenic, germline and of uncertain significance); (left) repartition of genes mutated according to diagnosis before targeted NGS analysis (MPN, no-MPN, uncertain diagnosis); (right) repartition of mutated genes according to diagnosis after targeted NGS analysis (MPN, no-MPN, constitutional thrombocytopenia). (C). Thrombosis-free survival in untreated and treated patients with triple-negative thrombocytosis compared to *JAK2*- and *CALR*-mutated controls. Survival curves are represented by Kaplan-Meier plots with log-rank associated tests and Cox models for multivariate analysis. Statistics were performed with R software (v4.0.3, Vienna, Austria). MPN: myeloproliferative neoplasm (i.e., essential thrombocythemia); no-MPN : histology/NGS not in favor of myeloproliferative neoplasm; VUS: variant of unknown significance.

these mutations affected W515 (W515A in 2 patients, W515S in 1 patient), one affected S505 and the others were scattered in the whole gene, in regions coding extracellular as well as intracellular domains.

Germline variants were found mostly in the *JAK2* and *MPL* genes (in 9 and 3 patients, respectively) (Figure 1B), most of which were confirmed to be germline by Sanger sequencing on nail DNA. Interestingly, germline *MPL* variants were found in the non-MPN group whereas germline *JAK2* variants were mainly found in the MPN group, suggesting that these variants favor the development of a "true" MPN phenotype. Some of these *JAK2* variants have been described in contexts different from that of familial thrombocytosis, such as *JAK2*R1063H which was shown to enhance signaling and lead to a distinct phenotype in *JAK2*V617F-positive MPN.[9] However, it is interesting to note that patient B358, who presented with isolated thrombocytosis and a bone marrow biopsy suggestive of MPN, has an 11-year-old daughter with thrombocytosis, an isolated R1063H variant at NGS screening and a bone marrow biopsy also showing characteristics of MPN. Also, a young patient (A005) with thrombocytosis and a bone marrow biopsy suggestive of ET, displayed a previously described *JAK2*T875N variant,[10-12] which was confirmed as germline on examination of nail DNA. He had thrombosis resulting in cerebral and thoracic spinal cord ischemia and, 2 years after diagnosis, he developed polycythemia. In addition, rare variants (mean allele frequency <0.1) of *JAK2* and *MPL* in our cohort were significantly more frequent than in a local control cohort (from the French Exome Project Database), further suggesting their significance in chronic thrombocytosis.

In order to assess whether NGS data could better discriminate thrombocytosis patients with a higher risk of complications, we reclassified patients with an acquired pathogenic or likely pathogenic variant in the "MPN" group, irrespective of their histology. Similarly, patients with germline variants in *JAK2* or *MPL* were reclassified as having constitutional thrombocythemia. Overall, applying these criteria in the "non-MPN" group, NGS was not informative for 39 patients, allowed confirmation of non-MPN in four (constitutional thrombocythemia) and prompted reclassification into the "MPN" group in nine patients. In the MPN group, NGS was not informative for 44 patients, allowed confirmation (acquired pathogenic/likely pathogenic variant) in 17 and prompted reclassification into constitutional thrombocythemia in eight patients. For the 11 patients for whom bone marrow biopsy was not able to provide a classification, NGS did not give additional information in nine, but detected an acquired mutation in two patients: *DNMT3A*D835M (variant allele frequency, 2%) and *CBL*L380P (variant allele frequency, 3%), allowing a reclassification to MPN. It is worth noting that mutations with such a low allele burden could be considered as clonal hematopoiesis of indeterminate potential and the clinical interpretation of these cases remains challenging.

In order to determine whether the NGS-based or histology-based classifications allow for a better prognostic discrimination, clinical characteristics and evolution were assessed in the groups defined by histological findings or NGS findings. The demographic, biological, and main clinical data of the patients, divided into groups according to the initial diagnosis, histology review or the NGS-based diagnosis, are detailed in Table 1A and *Online Supplementary Table S2*. Age at diagnosis and sex ratio were similar between MPN and non-MPN patients whereas MPN patients had higher platelet counts and lower leukocyte counts. These findings held true regardless of the diagnostic classification applied. In contrast, while the ratio of treated *versus* untreated patients was similar in the two groups (MPN and non-MPN) after initial assessment (67% in MPN *vs.* 51% in non-MPN, P=0.128), it became significantly higher in MPN patients after central review (69% *vs.* 46%, P=0.025), suggesting that therapeutic decisions in current clinical practice were not based only on the WHO classification and/or decisional algorithms. In survival analyses, we used information on event status and follow-up time to estimate a survival function (median follow-up of 5 years).

Overall survival was not significantly different between MPN and non-MPN patients whatever the classification applied (P values of 0.34, 0.081 and 0.27 for initial, bone marrow biopsy-reviewed and NGS classifications, respectively). It is interesting to note, however, that transformation to myelofibrosis or acute leukemia only occurred in MPN patients (n=5), according to all classifications. Similarly, no significant difference was observed for event-free survival between the MPN and non-MPN groups defined with the three classifications (P values of 0.82, 0.66 and 0.68, respectively). However, the patients reassigned to the MPN group after expert pathologist review or NGS had more thrombotic events compared to other patients with an initial diagnosis of MPN or not, suggesting that bone marrow biopsies should be examined by highly trained pathologists, and that identification of clonal hematopoiesis by NGS is of relevance (P=0.0078) (*Online Supplementary Figure S1A*). Since patients in the MPN and non-MPN groups had similar evolutions, we wondered whether the presence of an acquired "additional" mutation could have an impact on event-free survival (thrombosis or transformation) or overall survival in the whole triple-negative population. The presence of pathological variants did not have a statistically significant effect on overall survival, but did seem to be associated with a higher risk of

Table 1. (A) Demographic, biological and clinical characteristics in each group of patients with triple-negative thrombocytosis (with myeloproliferative neoplasm, not with myeloproliferative neoplasm, unclear and constitutional) according to the initial diagnosis and the diagnosis made with next-generation sequencing. (B) Comparison of demographic, clinical and biological characteristics at diagnosis and evolution of triple-negative patients, *JAK2*-mutated patients and *CALR*-mutated patients.

A. Diagnoses of the triple-negative group of patients (N=130)

	Diagnosis	Age at Dx, years, median (range)	Sex	Hemoglobin, g/L, median (range)	Platelets $\times10^9$/L, median (range)	Leukocytes $\times10^9$/L, median (range)	Neutrophils $\times10^9$/L, median (range)	CD34$^+$ cells/x10^{-6}L, median (range)	Spleno-megaly, n/N (%)	History of thrombosis, n/N (%)	Cyto-reductive treatment, n/N (%)
Initial Dx	MPN (N=79)	53 (18-84)	57F/22M	135 (107-170)	685 (442-1800)**	8.1 (4.1-16)*	4.8 (2.3-11.7)*	2.7 (0.8-14)	10/79 (13)	14/79 (18)	53/79 (67)
	No-MPN (N=45)	53 (18-79)	30F/15M	135 (89-169)	580 (454-1990)	9.6 (4.9-16)	6.0 (2.7-14.3)	3 (1-6.8)	1/45 (2)	7/45 (16)	23/45 (51)
	Unclear (N=6)	55 (36-82)	6F/0M	139 (125-155)	543 (444-1544)	8.5 (4.7-23.8)	5.1 (2.7-22.4)	1.8 (0.5-3)	0/6	0/6	3/6 (50)
Dx with NGS	MPN (N=70)	58 (18-84)	51F/19M	135 (107-163)	710 (444-1800)**	8.1 (4.1-16.0)*	5.1 (2.3-11.7)	2.4 (0.5-14)	6/70 (9)	14/70 (20)	49/70 (70)*
	No-MPN (N=39)	51 (18-78)	24F/15M	136 (89-169)	579 (442-1990)	9.5 (4.9-16)	5.8 (2.7-14.3)	2.6 (1-10.7)	3/39 (8)	5/39 (13)	18/39 (46)
	Constitutional (N=12)	40 (24-71)	9F/3M	141 (131-170)	524 (468-788)	9.1 (6.0-13.9)	6.2 (3.5-10.5)	2.8 (1.5-7.4)	2/12 (17)	2/12 (17)	7/12 (58)
	Unclear (N=9)	62 (36-82)	9F/0M	137 (20-155)	550 (472-1544)	8.8 (4.7-23.8)	5.7 (2.7-22.4)	4 (3-6.8)	0/9	0/9	5/9 (56)

B. Comparison of triple-negative patients *versus* patients with mutated essential thrombocythemia

Groups	Age at Dx, years, median (range)	Sex	Hemoglobin, g/L, median (range)	Platelets $\times10^9$/L, median (range)	Leukocytes $\times10^9$/L, median (range)	Neutrophils $\times10^9$/L, median (range)	CD34$^+$ cells x10^{-6}/L, median (range)	Spleno-megaly, n/N (%)	History of thrombosis, n/N (%)	Cyto-reductive treatment, n/N (%)
Triple-negative (N=130)	53 (18-84)^	93F/37M	136 (89-170)§	635 (442-1990)	8.5 (4.1-23.8)§	5.4 (2.3-22.4)§	2.7 (0.5-14.0)	11/130 (8)	21/130 (16)§	79/130 (61)^
*JAK2*V617F (N=246)	65 (17-97)	158F/88M	142 (76-175)	684 (145-2381)	9.3 (3.5-23.8)	6.6 (0.15-19.9)	2.5 (0.5-14)	20/246 (8)	81/246 (33)	226/246 (92)
CALR exon 9 (N=98)	60 (18-88)^^	49F/49M#	140 (105-165)	800 (511-2300)$	7.9 (4.3-19.5)§	5.1 (2.5-13.0)§	2.85 (1.0-17.1)	6/98 (6)	12/98 (12)§	86/98 (88)

Comparisons of quantitative and categorical parameters were performed with Mann-Whitney and Fisher tests, respectively.*P<0.05 *vs.* No-MPN group; **P≤0.001 *vs.* No-MPN group. ^P<0.01 *vs. JAK2* and *CALR*-mutated; ^^P<0.05 *vs. JAK2*-mutated; #P<0.05 *vs. CALR*-mutated; §P<0.01 *vs. JAK2*-mutated; $$P$<0.001 *vs.* triple-negative and *JAK2*-mutated. Constitutional: constitutional thrombocythemia; MPN: myeloproliferative neoplasm; no MPN: histology not in favor of MPN; Dx: diagnosis; M: male; F: female.

events, mainly after a long follow-up (beyond 4 years) (*Online Supplementary Figure S1B*).

We then compared our cohort of triple-negative patients with a control group of 246 patients with *JAK2*V617F-mutated ET and 98 with *CALR*-mutated ET. Age at diagnosis, hemoglobin levels and neutrophil counts were significantly higher in these *JAK2*V617F-mutated ET patients than in our triple-negative patients, and platelet counts were higher in the *CALR*-mutated patients than in the triple-negative patients (Table 1B). More importantly, overall, triple-negative patients had a significantly higher incidence of thrombosis than that in patients with *JAK2*V617F-mutated ET, especially during the first years following the diagnosis (hazard ratio=2.76 [95% confidence interval: 1.2-6-3]; P=0.0167) (*Online Supplementary Figure S1C*). The proportion of patients treated

Table 2. Main characteristics of patients with triple-negative thrombocytosis who suffered a thrombotic event.

Patients	Age at Dx	Sex	History of thrombosis pre-Dx	Dx with BMB review	Mutations (VAF%)	Dx with NGS	Hb* g/L	Platelets* ×10⁹/L	Leukocytes* ×10⁹/L	Neutrophils* ×10⁹/L	Age at thrombosis, years	Cytoreductive therapy*	Thrombosis location
A_004	32	M	N	No MPN	*ASXL1* D1180E (47) / *SH2B3* R265Q (48)	No MPN	NR	NR	NR	NR	33	N	Stroke
A_005	24	M	N	MPN	*JAK2* T875N (49)	Constitutional	158	478	5.4	3.4	25	N	Cerebral + thoracic spinal cord ischemia
A_013	59	F	N	ET	/	ET	115	894	10.2	5.6	60	N	Stroke
A_037	69	F	N	Unclear	*DMNT3A* T835M (2)	MPN	113	828	7.8	6.4	72	Y	Pulmonary embolism
A_055	71	M	N	No MPN	/	No MPN	148	395	6.5	4.1	74	Y	TIA
B_008	25	F	N	ET	*ASXL1* S846N (51)	ET	148	428	4.66	2.19	27	Y	Abdominal thrombosis
B_024	47	M	Y	No MPN	/	No MPN	NR	NR	NR	NR	47/50/52	Y	Stroke and 2 TIA
B_044	59	F	N	ET	/	ET	133	324	7	4.7	70	Y	Stroke
B_053	64	M	Y	ET	/	ET	134	269	9.3	7.7	82	Y	Myocardial infarction
B_054	65	F	N	No MPN	*DNMT3A* N797D (37)	MPN	110	439	8	6	69	Y	Lower limb thrombosis
B_063	74	M	N	ET	/	ET	116	461	11.1	8.8	74/81/82	Y	TIA and 2 lower limb thromboses
B_344	52	M	N	No MPN	/	No MPN	153	441	13.6	9.1	54	N	MI and stroke
B_358	43	F	N	ET	*JAK2* R1063H (51)	Constitutional	146	400	12.6	8.3	43	N	Stroke

*Hematologic values and treatment at the time of thrombosis. Dx: diagnosis; BMB: bone marrow biopsy; VAF: variant allele frequency; NGS: next-generation sequencing; Hb: hemoglobin; M: male; F. female; Y: yes; N. no; ET: essential thrombocythemia; preMF: pre-myelofibrosis; MPN: myeloproliferative neoplasm; no MPN: histology not in favor of a myeloproliferative neoplasm; Constitutional: constitutional thrombocythemia; TIA: transient ischemic attack; MI: myocardial infarction.

with cytoreductive therapy was significantly higher in *CALR*-mutated patients (86/98; 88%) and *JAK2*V617F-mutated patients (226/246; 92%) than in the triple-negative patients (79/130; 61%) (Table 1B). To evaluate the potential benefit of cytoreductive therapy on thrombosis risk, we compared the incidence of thrombosis in untreated *versus* treated patients (the main characteristics of patients with triple-negative thrombocytosis who suffered a thrombotic event are presented in Table 2). For this purpose, we performed a landmark analysis considering first untreated patients and then treated patients with a starting time of follow-up at treatment initiation. This analysis showed an excess risk of thrombosis in triple-negative patients among untreated, but not among treated patients (Figure 1C). In order to make sure that the increased thrombotic risk applied to "true" cases of MPN, the analysis was also carried out including only patients showing bone marrow histology of MPN and/or an acquired clonal mutation, with similar results (*Online Supplementary Figure S1D*). Of note, all five untreated triple-negative patients who had thrombotic events were in the low-risk group. These elements indicate that cytoreductive strategies might be improved in this group of patients.

In conclusion, this study shows that characterization of triple-negative thrombocytosis relies on thorough clinical, biological, histological and genetic characterization. A relatively small panel of commonly mutated genes allows confirmation or challenges the initial assessment in as many as 20% patients each. Besides the demon-

stration of clonal hematopoiesis, it is of particular interest to study the whole coding sequence of *MPL* and *JAK2* to identify a subset of patients with constitutive thrombocytosis and variants in these genes. Finally, our results showed a higher risk of thrombosis in patients with triple-negative thrombocytosis than in ET patients with driver mutations, with the risk being especially high among patients who do not receive cytoreductive drugs.

Authors

Sandrine Lemoine,[1-4] Clelia Mornet,[4,5] Isabelle Quintin-Roue,[6] Marie-Christine Rousselet,[7] Laurane Cottin,[1-4] Aurélie Georgeais,[5] Ludovic Dubouis,[8] Françoise Boyer,[3,4,9] Corentin Orvain,[2-4,9] Clara Caillon,[3,9] Maxime Renard,[1,3] Valoris Le Brun,[3,5] Lenaig Le Clech,[10] Jean-Christophe Ianotto,[3,4,11,12] Emmanuelle Génin,[13] Barbara Burroni,[4,14,15] Valérie Ugo,[1-4] Damien Luque Paz[1-4#] and Eric Lippert[3-5,13#]

[1]Laboratoire d'Hématologie, CHU Angers, Angers; [2]Université d'Angers, Nantes Université, CHU Angers, Inserm, CNRS, CRCI2NA, Angers; [3]Fédération Hospitalo-Universitaire "Grand Ouest Against Leukemia" (FHU GOAL), Angeres; [4]France Intergroupe des Syndromes Myéloprolifératifs (FIM), Paris; [5]Laboratoire d'Hématologie, CHRU Brest, Brest; [6]Département de Pathologie Cellulaire et Tissulaire, CHRU Brest, Brest; [7]Département de Pathologie Cellulaire et Tissulaire, CHU Angers, Angers; [8]Laboratoire de Biopathologie, CHRU Nancy, Nancy; [9]Service des Maladies du Sang, CHU Angers, Angers; [10]Service d'Hématologie Clinique, CHIC de Quimper, Quimper; [11]Service d'Hématologie Clinique, CHRU Brest, Brest; [12]Université Brest, Inserm, GETBO, Brest; [13]Université Brest, Inserm, EFS, UMR 1078, GGB, Brest; [14]Service de Pathologie, AP-HP, Hôpital Cochin, Paris and [15]Centre de Recherche des Cordeliers, Sorbonne Université, Inserm, Université de Paris, Paris, France.

[#]DLP and EL contributed equally as co-senior authors.

Correspondence:
E. LIPPERT - eric.lippert@chu-brest.fr
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Disclosures

No conflicts of interest to disclose.

Contributions

FB, JCI, CO, LLC, JCI and EL included patients. IQR, MCR, LD and BB analyzed the bone marrow biopsies. SL, CM, LC, AG, CC, MR, VLB, EG, VU, DLP and EL performed molecular studies and analyzed the data. SL, CM, DLP and EL drafted the manuscript. All co-authors proof-read and approved the manuscript.

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Data-sharing statement

All data generated and analyzed during this study are included in this published article and its online supplementary file. Further details can be requested from the senior authors (damien.luquepaz@chu-angers.fr or eric.lippert@chu-brest.fr).

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