



Pathological and genomic features of myeloproliferative neoplasms associated with splanchnic vein thrombosis in a single-center cohort

Daniele Cattaneo^{1,2} · Cristina Bucelli¹ · Alfredo Marchetti^{1,2} · Marta Lionetti^{1,2} · Elisa Fermo¹ · Valentina Bellani^{1,2} · Claudio De Magistris^{1,2} · Akihiro Maeda^{1,2} · Alessio Marella^{1,2} · Massimo Primignani³ · Dario Consonni⁴ · Umberto Gianelli^{5,6} · Antonino Neri⁷ · Luca Baldini⁸ · Niccolò Bolli^{1,2} · Alessandra Iurlo¹

Received: 24 January 2023 / Accepted: 5 April 2023 / Published online: 20 April 2023
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Abstract

Here, we reviewed clinical-morphological data and investigated mutational profiles by NGS in a single-center series of 58 consecutive MPN-SVT patients admitted to our hospital between January 1979 and November 2021. We identified 15.5% of PV, 13.8% of ET, 34.5% of PMF, 8.6% of SMF and 27.6% of MPN-U. Most cases (84.5%) carried *JAK2V617F* mutation, while seven patients were characterized by other molecular markers, namely *MPL* in four and *CALR* mutations in three cases. NGS was performed in 54 (93.1%) cases: the most frequent additional mutations were found in *TET2* (27.8%) and *DNMT3A* (16.7%) genes, whereas 25 (46.3%) patients had no additional mutation. Cases with *JAK2V617F* homozygosity had a higher median number of additional mutations than those with low allele burden. More importantly, all cases of leukemic evolution were characterized by a higher median number of co-mutations, and a co-mutational pattern of high-risk lesions, such as truncating mutations of *ASXL1*, bi-allelic *TP53* loss, and *CSMD1* mutations. Nevertheless, no difference was found between cases with and without additional somatic mutations regarding fibrotic progression, SVT recurrence, other thrombohemorrhagic complications, or death. After a median follow-up of 7.1 years, ten deaths were recorded; fibrotic progression/leukemic evolution was ascertained in one (1.7%) and six (10.3%) patients, respectively, while 22 (37.9%) patients suffered from recurrent thrombosis. In conclusion, our data underline the importance of using NGS analysis in the management of MPN-related SVT as it can support the MPN diagnosis, particularly in “triple-negative” cases, and provide additional information with potential consequences on prognosis and therapeutic strategies.

Keywords Splanchnic vein thrombosis · Myeloproliferative neoplasms · Next-generation sequencing · Outcome · Therapy

Introduction

BCR::ABL1-negative myeloproliferative neoplasms (MPN) are characterized by a defined risk of disease progression to secondary myelofibrosis (SMF) or acute myeloid leukemia

(also known as blast-phase MPN—MPN-BP) and show an increased thrombotic risk [1, 2]. Arterial and venous thrombotic events at initial presentation are reported in approximately 11%–25% of patients with essential thrombocythemia (ET) [3–5], 12%–39% of those with polycythemia vera (PV) [6], and in approximately 9.5% of those with primary myelofibrosis (PMF) [1].

Niccolò Bolli and Alessandra Iurlo co-contributing senior authors.

✉ Daniele Cattaneo
daniele.cattaneo@unimi.it

¹ Hematology Division, Foundation IRCCS Ca' Granda Ospedale Maggiore Policlinico, Via Francesco Sforza 35, 20122 Milan, Italy

² Department of Oncology and Hemato-Oncology, University of Milan, Milan, Italy

³ Gastroenterology and Hepatology Unit, Foundation IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy

⁴ Epidemiology Unit, Foundation IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy

⁵ Division of Pathology, ASST Santi Paolo e Carlo, San Paolo Hospital, Milan, Italy

⁶ Department of Health Sciences, University of Milan, Milan, Italy

⁷ Scientific Directorate, Azienda USL-IRCCS di Reggio Emilia, Reggio Emilia, Italy

⁸ University of Milan, Milan, Italy

Venous thrombosis can also involve unusual sites, including cerebral veins [7, 8] and splanchnic vessels (SVT). The latter includes Budd-Chiari syndrome (BCS), portal vein thrombosis (PVT) and other thrombotic events that can occur in isolated or multiple vessels of the portal venous axis, most commonly in the splenic (ST) and mesenteric ones (MT), with a prevalence between 1 and 23% [9–11].

Accordingly, BCS and PVT in the absence of underlying liver disease share many risk factors, with MPNs being the most common [12]: more specifically, as confirmed in a recent meta-analysis [13], *BCR::ABLI*-negative MPNs are the most frequent underlying prothrombotic factor in BCS and PVT, with a reported prevalence of 30%–50% and 15%–30%, respectively [14–17]. Therefore, given the frequency of MPNs in SVT patients, referral to a hematologist to discuss molecular screening and bone marrow (BM) biopsy should be systematically considered since targeting the underlying malignant condition, primarily with generally long-term anticoagulation, positively influences patients' outcomes [18].

However, recognition of an underlying MPN can be difficult [19]: indeed, hypersplenism, hemodilution, iron deficiency, hepatic ischemia and/or occult bleeding can affect hematological parameters or inappropriately increase serum erythropoietin levels [20]. Furthermore, MPNs associated with SVT appear to have atypical intrinsic clinical-laboratory features compared to “classic” MPNs, including onset at a younger age, a female predominance, and the presence of normal peripheral blood (PB) cell count [21].

All these patients should be evaluated with a careful investigation, including a *JAK2V617F* mutation test. In this context, in a meta-analysis of 831 patients with unprovoked SVT, the aforementioned mutation was detected in 33% of overall and in 49% of idiopathic cases [21]. Similarly, a previous study involving 93 consecutive SVT cases diagnosed from 1992 to 2005 identified the *JAK2V617F* mutation in 35.6% of 73 patients with PVT and in 40% of 20 with BCS [19]. Furthermore, even though *JAK2* exon 12 mutations have previously been described only anecdotally in SVT patients [13, 22–24], Magaz et al. [25] have recently reported that in non-cirrhotic, non-malignant SVT next-generation sequencing (NGS) can identify these mutations, including those in the hotspot exon 12 region of *JAK2*, not previously detected by conventional techniques, for example by direct Sanger sequencing.

As for the *MPL* gene, its mutations have also been documented, although in significantly fewer SVT patients [13, 26]. Similarly, after the description of *CALR* deletions and/or insertions [27, 28], Turon et al. [29] and Haslam et al. [30] first assessed the incidence of these mutations in two large cohorts of patients with SVT of different etiologies but found them only in a minority of cases. Consequently, although *CALR*

and *MPL* mutations should be investigated in all *JAK2V617F*-negative SVT patients, this approach has lower sensitivity [26]. Conversely, BM biopsy could represent a key component of the diagnostic algorithm of SVT-related MPNs [19, 23]. However, a diagnosis of MPN on histological grounds alone and the distinction between PV, ET and PMF is quite complex and difficult [31]. Recently, NGS has contributed to a thorough description of the mutational spectrum of MPNs and a classification on the basis of the causal biological mechanisms has been proposed [32]. Also, the order of acquisition of somatic mutations influences the thrombotic risk [33], but how this is relevant in the context of MPNs associated with SVT is still unclear.

Clinical features and outcome of MPN-SVT patients have already been reported in several studies [34–39]. However, due to the rarity of the disease and the several unmet clinical needs related to its management [40], a more in-depth analysis is required to accurately estimate the risk of SVT recurrence and evolution (fibrotic or leukemic) of the underlying MPN, and to test the effectiveness of current therapeutic strategies in reducing the associated vascular risk.

Here, we conducted a retrospective, cohort study with the aim of reviewing clinical-morphological features and correlating them with mutational profiles by NGS in MPN patients who suffered from SVT.

Methods

Patients

A single-center consecutive series of 58 patients admitted to our hospital between January 1979 and November 2021 with a diagnosis of SVT associated with *BCR::ABLI*-negative MPN was evaluated. Inclusion criteria were as follows: available demographic, clinical, hematological, and histological data at diagnosis; stored BM biopsy; and at least one granulocyte DNA sample (collected at the time of *JAK2V617F* mutational screening, after a median time from MPN diagnosis of 6.1 years) to perform NGS analysis. The protocol was approved by the local IRB (439_2022 BIS). Baseline clinical characteristics and outcome measures [thrombosis, hemorrhages, fibrotic progression, leukemic evolution, death, and overall survival (OS)] were assessed. Major thromboses and bleeding events were recorded when objectively documented and according to standard definitions as detailed by the CYTO-PV Collaborative Group [41]. Diagnosis of SMF was made according to the International Working Group for Myelofibrosis Research and Treatment (IWG-MRT) criteria [42]. Diagnosis of MPN-BP was made with a 20% threshold of BM or PB blasts [43]. Patients were treated with antiplatelet/anticoagulant and/or cytoreductive

agents according to institutional guidelines which were based on current recommendations.

Follow-up information was updated in April 2022.

Methods

Molecular analyses

The *JAK2V617F* mutation was detected by allele-specific PCR according to the protocol of Baxter et al. [44] and confirmed by direct Sanger sequencing. Quantitative analysis of the allele burden of the *JAK2V617F* mutation was performed by RQ-PCR using JAK2 MutaQuant (Ipsogen Inc, New Haven, CT, USA). The cut-off used for defining a case as negative for *JAK2V617F* mutation was 0.5%.

MPL mutations were tested by direct sequencing of exon 10. Primer used were: MPL10F 5' TAGCCTGGATCTCCT TGGTG 3'; MPL10R 5' CCTGTTTACAGGCCTTCGGC 3'.

Mutations in exon 9 of *CALR* gene were also assessed by bidirectional sequencing approach as previously described [28]. All sequencing analyses were performed on ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Warrington, UK) using the Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK).

Sequencing, mutation calling and filtering

NGS analysis was performed using DNA extracted from PB granulocytes. 100 ng of tumor DNA was used to prepare libraries using the HyperPlus kit (KAPA Biosystems). The targeted gene panel included coding exons and splice sites of 73 genes involved in myeloid malignancies based on literature data. Libraries were sequenced using 150 bp paired end reads, to a mean total depth of 1006x.

Mutect 2 GATK 4.1.5.0, LoFreq (v. 2.1.5) and VarDict (2019.06.04) were used for variant calling, and only variants called by at least 2/3 of these variant callers were further considered. To filter out artifacts and germline polymorphisms [45, 46], we first excluded variants with at least one of the following characteristics:—variant allele frequency (VAF) lower than 0.02 and less than 15 supporting reads;—coverage < 300x;—occurrence in > 5% of a panel of normal controls (PoN, analyzed for the same gene panel and in the same sequencing conditions) at a VAF < 5%;—expected minor germline allele frequency > 0.001 based on the information retrieved from the public database gnomAD (<http://gnomad.broadinstitute.org>; gnomAD r3.1.2);—or occurrence in at least one sample of our PoN at a VAF > 45% (both features being suggestive of polymorphic variants).

The shortlist of variants was therefore considered as containing bona fide somatic mutations, whose significance

was evaluated at the amino acid level in order to differentiate known/putative pathogenic mutations from variants of unknown significance (VUS) on the basis of data available in literature and reported in the publicly accessible Catalogue Of Somatic Mutations In Cancer (COSMIC, version 69) (<http://cancer.sanger.ac.uk/cancergenome/projects/cosmic>).

Variants deemed as VUS were excluded from downstream analyses. VAF was used to estimate the mutation clonality and mutation precedence by analyzing the confidence intervals (CI) of VAF values, inferred by assuming a binomial distribution of VAF and calculated against the hypothesis of a random VAF of 0.5. Variants with non-overlapping CIs were estimated to be acquired in different time windows, under the assumption that mutations with higher VAF are present in more cancer cells and thus were acquired earlier [47–49].

Cytogenetic analyses

G-banding with trypsin performed on fresh BM aspirates was the standard technique for chromosome analysis with at least 20 metaphases described [50]. Normal karyotype was defined as the absence of any chromosomal abnormality in a minimum of 20 metaphases examined. Chromosomal abnormalities were considered clonal if the same structural or extra-chromosome abnormality appears in at least two and monosomy in at least three metaphases.

Bone marrow biopsy

Histological confirmation of the clinical diagnosis of MPN as defined by the 2016 WHO classification was performed by an experienced pathologist (U.G.). Formalin-fixed, paraffin-embedded BM biopsy specimens were available for all patients. Sections were stained with hematoxylin–eosin, Giemsa, and Gomori's silver impregnation for the evaluation of morphology and BM fibrosis grade.

Statistical approach

Descriptive statistics were generated for all clinical characteristics and outcome measures as appropriate: for continuous variables, median and range; for categorical variables, frequency and percentage.

We used Fisher's exact test and non-parametric methods (Wilcoxon and Kruskal–Wallis) tests to analyze categorical and quantitative variables, respectively. We evaluated death risk (with follow-up time truncated at 25 years) according to selected variables by fitting univariate Cox models to calculate hazard ratios (HR) and 95% CIs. Statistical analysis was performed with Stata 17 (StataCorp. 2021).

Results

Overall, 58 patients with MPN-SVT were analyzed (Table 1). In 24 (41.3%) cases the diagnosis of MPN and SVT were coincident, in 19 (32.8%) an SVT index event preceded the diagnosis of MPN by a median of 2.8 years (range, 0.3–27.9), while in the remaining 15 (25.9%) patients it occurred after a median of 13.3 years from MPN diagnosis (range, 3.0–25.4).

Female patients (32/58, 55.2%) were prevalent, with a median age at MPN diagnosis of 47.1 years (range, 18.5–78.7). Notably, only a minority of patients (20.7%) were over 60 at the time of the index event, while 32.8% were under 40.

Portal vein thrombosis occurred in 19 (32.8%) cases, ST in four (6.9%), MT in one (1.7%) and BCS in eight (13.8%). An association of PVT and/or ST and/or MT was found in 26 (44.8%) patients.

Hematological parameters at MPN and SVT diagnosis are summarized in Supplementary Table S1: at MPN diagnosis significantly higher median hemoglobin (Hb) levels were detected (13.7 vs. 13.2 g/dL; $p=0.01$) as compared to SVT diagnosis, with a trend towards a statistically significant difference in median platelet (PLT) counts (405 vs. $392 \times 10^9/L$; $p=0.06$).

Pre-PMF along with myeloproliferative neoplasm, unclassifiable (MPN-U) were the most frequent MPN subtypes (27.6%, $n=16$ each), followed by PV (15.5%, $n=9$), ET (13.8%, $n=8$), SMF (8.6%, $n=5$), and overt PMF (6.9%, $n=4$).

As reported in Supplementary Table S2, hematological parameters were consistent with those already reported for each MPN subtype: in detail, Hb levels were significantly higher in PV ($p=0.01$), while PLT counts in ET patients ($p=0.0006$). PMF cases showed significantly higher lactate dehydrogenase (LDH) levels ($p=0.0008$), with a trend towards a statistically significant difference in white blood cells (WBC) count ($p=0.07$). As expected, PMF patients also exhibited significantly larger spleen size ($p=0.003$), albeit with no robust differences in the number of cases with splenomegaly.

No difference was found also for gender, type of SVT, constitutional symptoms, abnormal karyotype, and therapies (either anticoagulant or cytoreductive agents).

Regarding driver mutations, *JAK2V617F* was found in most patients (49/58, 84.5%). Three (5.2%) had a *CALR* mutation (including two patients with *CALR* type 1 mutation and the remaining case with a *CALR* type 1-like mutation) and four (6.9%) an *MPLW515L/K* mutation. The remaining two (3.4%) patients were “triple-negative”. As reported in Supplementary Table S2, a higher median *JAK2V617F* allele burden was detected

Table 1 Characteristics of the patients

	Patients n. 58
Male/female	26/32
Age at MPN diagnosis (years), median (range)	47.1 (18.5–78.7)
MPN subtype, n (%)	
- PV	9 (15.5)
- ET	8 (13.8)
- pre-PMF	16 (27.6)
- overt PMF	4 (6.9)
- MPN-U	16 (27.6)
- SMF	5 (8.6)
Molecular status, n (%)	
- <i>JAK2V617F</i> mutated	49 (84.5)
- <i>JAK2V617F</i> allele burden (%), median (range)	28.4 (3.8–99.0)
- <i>JAK2V617F</i> allele burden $\geq 50\%$	13 (22.4)
- <i>CALR</i> mutated	3 (5.2)
- <i>MPL</i> mutated	4 (6.9)
- triple-negative	2 (3.4)
NGS, n (%)	
Wild-type	25 (46.3)
HMR	9 (16.7)
<i>ASXL1</i>	6 (11.1)
<i>EZH2</i>	1 (1.9)
<i>U2AF1</i>	2 (3.7)
<i>DNMT3A</i>	9 (16.7)
<i>TET2</i>	15 (27.8)
<i>TP53</i>	2 (3.7)
Others	4 (7.4)
Cytogenetic abnormalities, n (%)	5 (8.6)
Thrombophilia abnormalities, n (%)	20 (34.5)
Follow-up from MPN diagnosis (years), median (range)	7.1 (0.3–29.3)
Death, n (%)	10 (17.2)
- Leukemic evolution	5 (8.6)
- Hemorrhagic complications	3 (5.2)
- Infections	1 (1.7)
- Secondary malignancies	1 (1.7)
Type of SVT, n (%)	
- PVT	19 (32.8)
- PVT and ST	11 (19.0)
- PVT and MT	5 (8.6)
- PVT, ST, and MT	10 (17.2)
- ST	4 (6.9)
- MT	1 (1.7)
- BCS	8 (13.8)
Age at SVT diagnosis (years), median (range)	46.8 (20.7–78.9)
Follow-up from SVT diagnosis (years), median (range)	8.8 (0.6–35.8)
Recurrence of SVT, n (%)	10 (17.2)
Esophageal varices, n (%)	28 (48.3)
Bleeding from varices, n (%)	10 (17.2)
Other thrombotic complications before SVT, n (%)	11 (19.0)
- arterial thrombosis	6 (10.3)
- AMI	5 (8.6)
- others	1 (1.7)
- venous thrombosis	5 (8.6)
- DVT	2 (3.4)

Table 1 (continued)

	Patients n. 58
- PE	2 (3.4)
- others	1 (1.7)
Other thrombotic complications after SVT, n (%)	12 (20.7)
- arterial thrombosis	8 (13.8)
- AMI	5 (8.6)
- ischemic stroke	2 (3.4)
- others	1 (1.7)
- venous thrombosis	4 (6.9)
- CVT	2 (3.4)
- PE	1 (1.7)
- others	1 (1.7)
Bleeding events (excluding variceal ones), n (%)	6 (10.3)
Anticoagulant therapy, n (%)	45 (77.6)
- before SVT (low-molecular weight heparin)	2 (3.4)
- after SVT	45 (77.6)
- VKA	31 (53.5)
- DOAC	14 (24.1)
- apixaban	3 (5.2)
- rivaroxaban	11 (18.9)
Antiplatelets, n (%)	15 (25.9)
- before SVT	8 (13.8)
- after SVT	10 (17.2)
Cytoreductive therapy, n (%)	45 (77.6)
- before SVT	9 (15.5)
- hydroxyurea	8 (13.8)
- interferon-alpha	1 (1.7)
- after SVT	45 (77.6)
- hydroxyurea	44 (75.9)
- interferon-alpha	2 (3.4)
- ruxolitinib	3 (5.2)
- other drugs	6 (10.3)

MPN myeloproliferative neoplasm, *PV* polycythemia vera, *ET* essential thrombocythemia, *PMF* primary myelofibrosis, *MPN-U* myeloproliferative neoplasm, unclassifiable, *SMF* secondary myelofibrosis, *HMR* high molecular risk, *SVT* splanchnic vein thrombosis, *PVT* portal vein thrombosis, *ST* splenic vein thrombosis, *MT* mesenteric vein thrombosis, *BCS* Budd-Chiari syndrome, *AMI* acute myocardial infarction, *DVT* deep vein thrombosis, *PE* pulmonary embolism, *CVT* cerebral vein thrombosis, *VKA* vitamin K antagonist, *DOAC* direct oral anticoagulant

among patients with SMF, followed by PMF cases ($p = 0.003$).

As detailed in Fig. 1, NGS analysis—performed on 54/58 patients—showed no additional mutations in 25 (46.3%) cases, including the two “triple-negative” subjects, with the remaining 29 (53.7%) patients harboring at least one additional pathogenic variant. In detail, most of the subjects (28/29, 96.5%) had one or two mutations, whereas only one (3.5%) patient showed three concomitant variants. The most frequent sequence variants involved *TET2* gene (15/29, 51.7%), followed by *DNMT3A* (9/29, 31.0%) and *ASXL1* (6/29, 20.7%). Other variants detected in at least one

case also include *U2AF1*, *TP53*, *EZH2*, *SF3B1*, *SETBP1*, *RUNX1*, and *CUX1* (Fig. 1).

As reported in Supplementary Table S3, when considering the number of additional mutations, no specific association was found with each MPN subtype; similarly, there was no difference in their distribution according to hematological parameters, hepato-splenomegaly, constitutional symptoms, gender, or age at MPN diagnosis. Interestingly, when driver and additional mutations are considered, a different co-mutational spectrum emerged (Fig. 2A): specifically, *JAK2V617F* mutation occurred concurrently with *DNMT3A* mutations, while *MPL* mutations were associated with *ASXL1* mutations (2/4 cases). Our cohort was also enriched in cases with high VAF ($\geq 50\%$) of *JAK2V617F* (13/49 cases), suggesting that acquired uniparental disomy (aUPD) was more frequent than observed in non-SVT MPN cases. Furthermore, high VAF *JAK2V617F* mutations had a higher median number of additional mutations than cases with low allele burden (2 vs. 1, $p = 0.0007$, two-tailed). More importantly, as shown in Fig. 2B-C, the co-mutational spectrum was also different between patients with low and high VAF of *JAK2V617F*, with *DNMT3A* being the most frequent partner of *JAK2V617F* low-burden cases, while *TET2* and *ASXL1* mutations were more frequently associated with *JAK2V617F* high-burden cases. Overall, high molecular risk (HMR) mutations were more frequently detected among patients with at least two non-driver mutations. Indeed, this correlated with clinical outcomes, as the six cases of leukemic evolution were all characterized by a higher *JAK2V617F* allele burden, a higher median number of co-mutations, and a co-mutational pattern of high-risk lesions showing themselves a high VAF, such as truncating mutations of *ASXL1* (3/5), bi-allelic loss of *TP53* (1/6), and *CSMD1* mutations (1/6), confirming that genomics can help predict clinical outcomes (Fig. 3). Nevertheless, no difference was found between cases with and without additional somatic mutations regarding fibrotic progression, SVT recurrence, other thrombo-hemorrhagic complications, or death.

The study of mutations VAF also allowed to estimate the order of acquisition of the mutations by analyzing the overlap between VAF CIs, under the assumption that mutations with a higher VAF would be present in a larger number of cells and therefore be acquired earlier in the clonal phylogeny of the tumor. This was particularly useful for *JAK2V617F* and *TET2*, the two most frequently mutated genes: indeed, we could estimate that *JAK2V617F* mutation preceded those of *TET2* in 13/14 cases (Fig. 4 and Supplementary Fig. S4). This has relevant implications, since *JAK2*-first cases are associated with a more pro-thrombotic phenotype and more aUPD, features that are enriched in our MPN-SVT cohort.

Cytogenetic data were available at diagnosis in all patients (Table 1), with a normal karyotype reported in the

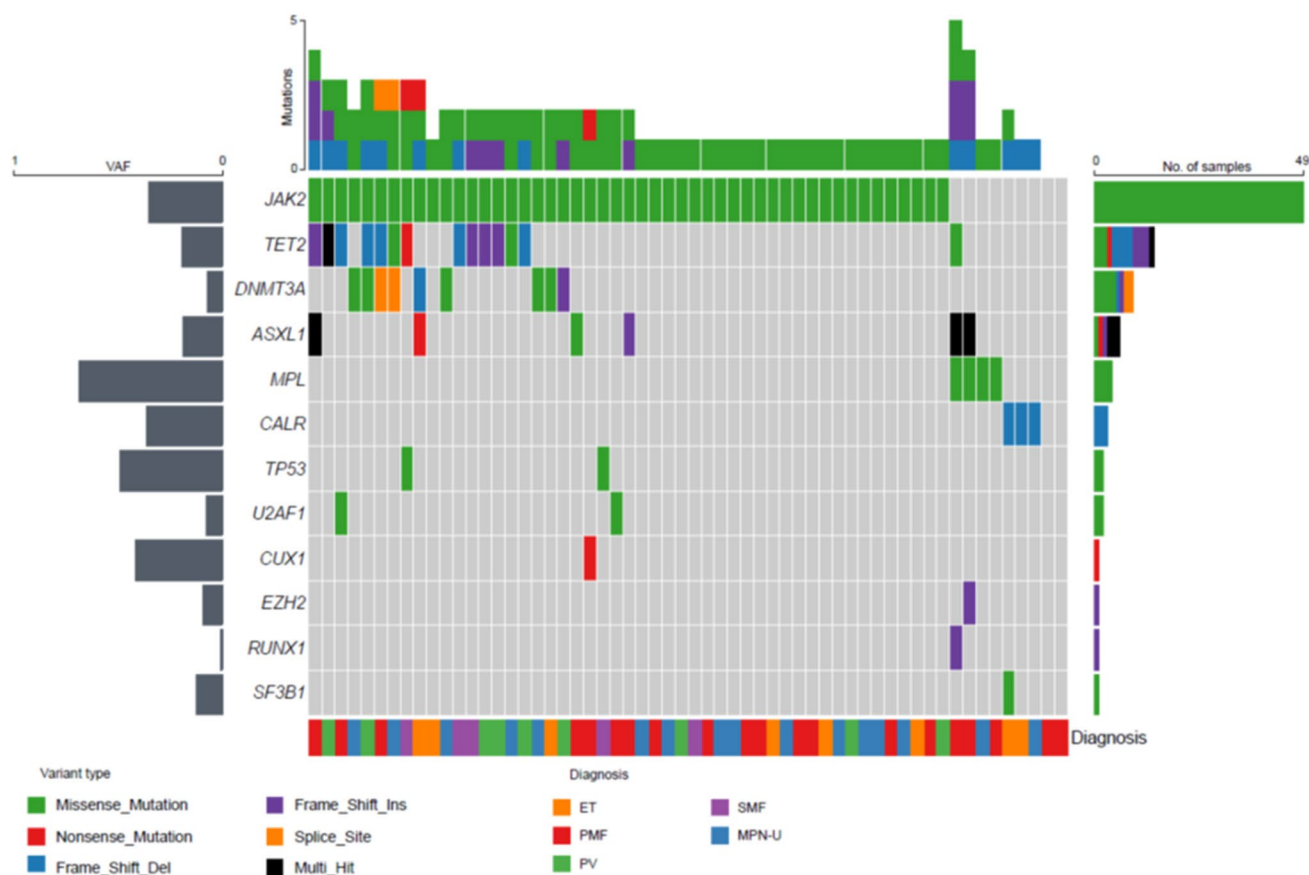


Fig. 1 Oncoplot depicting the mutational profile in the study cohort. Each column corresponds to a unique sample. The left-side bar plots indicate *VAF* gene-wise, the side bar plots the number of samples

affected by mutations gene-wise, and the top side bar plots the mutation number per sample. Diagnosis annotation is included at the bottom of the oncoplot

majority of cases (53/58, 91.4%). In particular, as regards chromosomal abnormalities, they were represented by 20q- in two cases and 5q- or +1 in one case each.

Information on concomitant hypercoagulable disorders was available in 49 (84.5%) patients: thrombophilia abnormalities were detected in 20 (40.8%) cases, the most frequent including heterozygous prothrombin G20210A mutation and hyperhomocysteinemia in five (10.2%) cases each, heterozygous factor V Leiden mutation in two (4.1%) and protein S deficiency in nine (18.4%).

Most SVT events (82.8%) developed in the absence of any antithrombotic prophylaxis. In contrast, most patients (53/58, 91.4%) received anticoagulant and/or antiplatelet agents for secondary prophylaxis. In detail, anticoagulants alone were administered to 43 (81.1%) patients [vitamin K antagonists (VKAs) in 69.8% and direct oral anticoagulants (DOACs) in 30.2% of cases], antiplatelets alone (aspirin in 75% and clopidogrel in 25% of cases) in eight (15.1%) and a combination of anticoagulants and antiplatelets in two (3.8%).

Globally, cytoreductive agents were used in 45 (77.6%) cases (including nine patients already on treatment at the time of SVT), mostly hydroxyurea (75.9% of cases), with only three (5.2%) patients treated with ruxolitinib.

Median follow-up for the whole cohort was 7.1 years (range, 0.3–29.3) after MPN diagnosis and 8.8 years (range, 0.6–35.8) after the diagnosis of SVT.

A total of ten (17.2%) patients experienced SVT recurrence, involving more than one site in three cases, while isolated recurrences were PVT in one case, ST, MT and BCS in two cases each; at the time of SVT recurrence, six patients were on anticoagulation with VKAs, while one patient was on antiplatelet agents alone. Excluding SVT recurrences, 12 new episodes of thrombosis occurred in 11 patients, more frequently involving the arterial district (8/12, 66.7%), in course of anticoagulant therapy with VKAs in most cases (9/11, 81.8%) (Table 1). Focusing specifically on patients with recurrent arterial thrombosis, they were on antiplatelet therapy only in a minority of cases (3/8, 37.5%) while additional cardiovascular risk factors (i.e., diabetes,

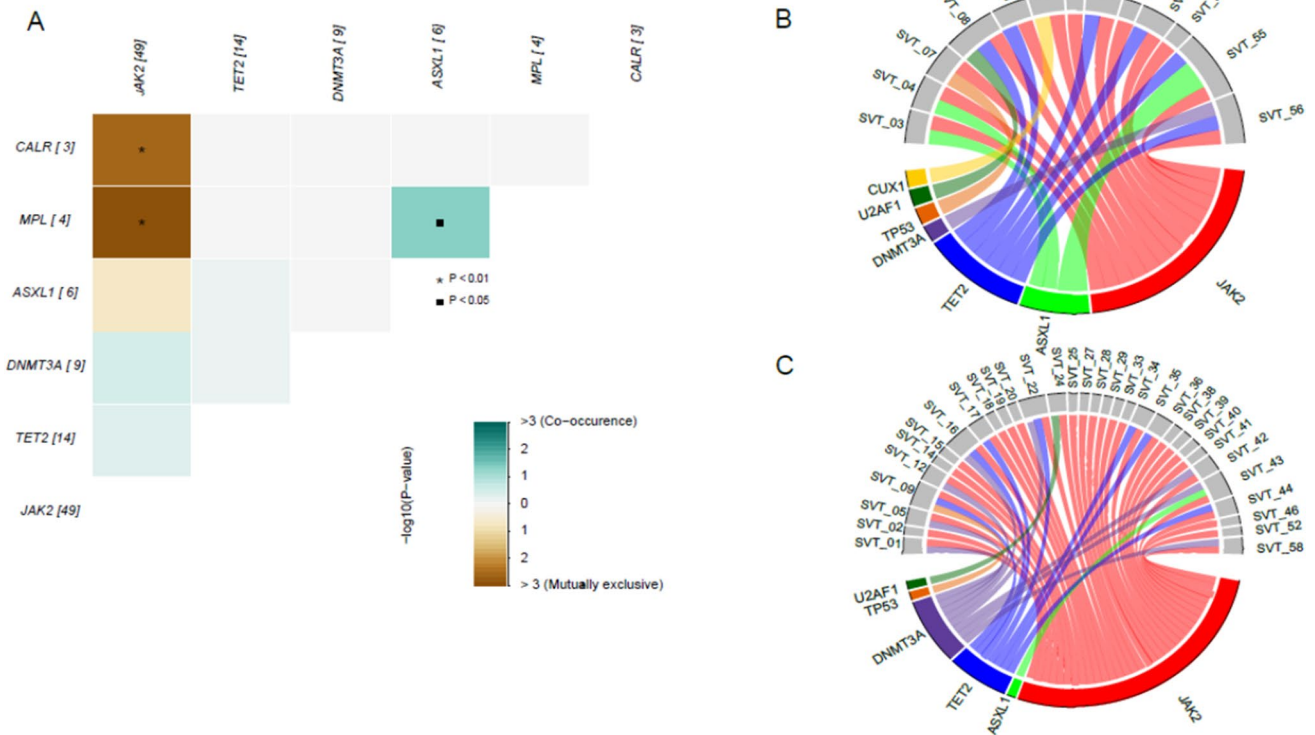


Fig. 2 (A) Interaction of somatic variants from a selected pool of genes. Patterns of *co-occurrence* are highlighted in blue while *mutual exclusion* in brown. Statistically significant pairs are highlighted with

either dots ($p < 0.05$) or stars ($p < 0.01$). (B) and (C) depict chord diagrams gauging the different mutational patterns seen in samples with respectively high and low *JAK2V617F* allelic ratio

hypertension, dyslipidemia or smoking habit) were reported in most patients (6/8, 75%).

Overall, 48.3% (28/58) of patients developed esophageal varices, with 35.7% of them experiencing one or more

variceal bleeding episodes (range, 1–5) during anticoagulant therapy with VKAs in most cases (8/10, 80%).

At the last follow up, 10/58 (17.2%) patients with MPN-SVT had died; causes of death are summarized in Table 1.

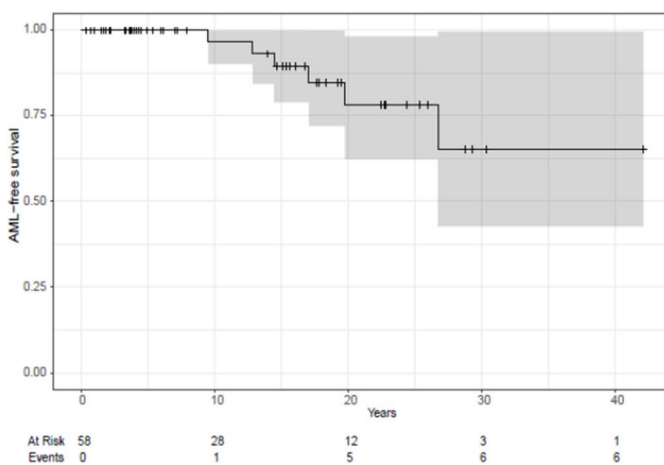
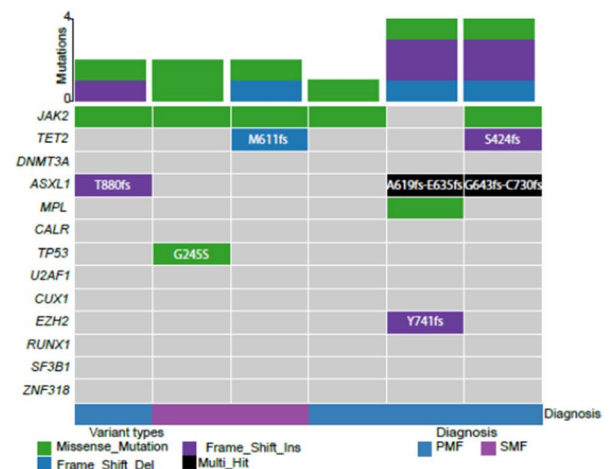


Fig. 3 Kaplan Meier curve with leukemic progression cumulative risk over a 40 years follow-up (95%CI, gray ribbons). On the right, an oncoplot showing the mutational distribution among samples from



patients who evolved into acute myeloid leukemia (AML). Mutations in non-driver MPN genes are annotated for the amino acid change

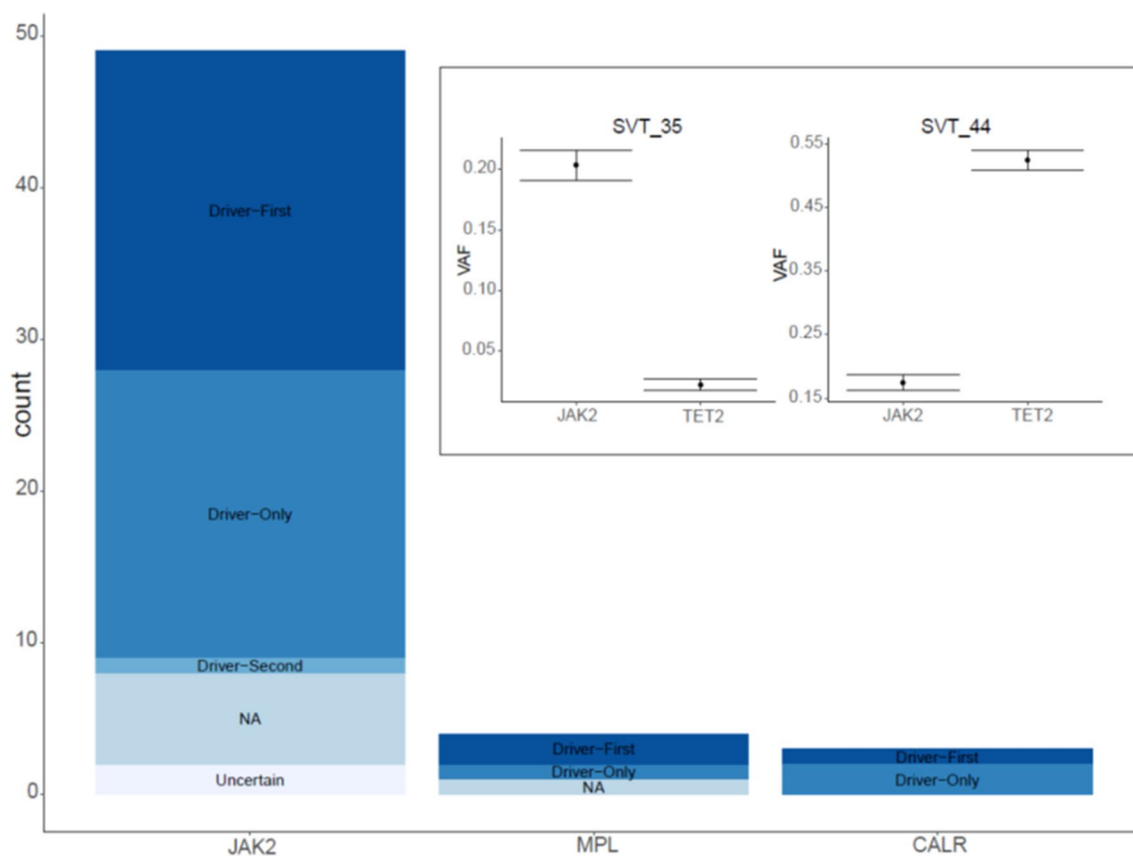


Fig. 4 Stacked barplot showing driver gene clonal composition. Per-sample clones were defined as Driver-First in the setting of at least one additional mutation where driver genes were mutated at higher VAF with no CI overlap; Driver-Only, where no additional mutations were found; Driver-Second, where additional mutations were deemed

to be antecedent to the driver ones; Uncertain, where precedence could not be determined with confidence; NA, where VAF could not be reliably assessed. Figure 4 detail provides two examples of our cohort samples clonal architecture

Progression to SMF and MPN-BP was ascertained in one (1.7%) and six (10.3%) patients, respectively.

As reported in Supplementary Table S4, clinical features and the type and number of driver or non-driver mutations, as well as cytoreductive or anticoagulant therapies did not have a significant impact on OS of these patients.

Discussion

In this real-life, single-center cohort study, we investigated mutational profiles using NGS and reviewed clinical-morphological data of 58 consecutive MPN-SVT patients with a median follow-up of 7.1 years after MPN diagnosis.

First, we confirmed a consistent association of SVT with a distinct phenotype characterized by female predominance and younger age, across all MPN subtypes, and a striking association with the *JAK2*V617F mutation (84.5% in our cohort).

Second, pre-PMF and MPN-U were the most frequent underlying diagnoses. In this regard, we recognize that an accurate differentiation among MPN subtypes in SVT patients can represent a diagnostic challenge: as already reported in a previous study by our group [31], peculiar clinical-laboratory features were found in the three morphology-defined MPN subtypes when associated with SVT. More precisely, there was an almost complete correspondence between the clinical and morphological features of PMF patients, whereas discrepancies were frequent in patients with an ET- or, above all, a PV-like morphology, since only a minority of cases showed a clinical phenotype typical of PV. This finding is also in line with previous studies indicating the lack of specificity of current diagnostic criteria for PV in SVT patients [40, 51–53]. Consistent with this notion, up to 27.6% of MPN-SVT cases included in our study received a primary diagnosis of MPN-U based on BM findings. Overall, past and current evidence suggests that a histology-based classification has limitations and does not reflect a genomic

classification that would better fit the actual biology of these diseases.

It should also be emphasized that the distribution by gender and age differs from that observed in the general population and in MPN patients without SVT, suggesting that traditional risk factors for thrombosis are of limited value in the context of MPN-SVT [39, 54], with consequent growing interest in other possible biomarkers of vascular injury and thrombotic risk, with circulating endothelial cells among the most promising [55]. Indeed, we found that MPN-SVT cases had a not negligible risk of subsequent vascular events, even though most of our cases were treated with both anticoagulant/antiplatelet and/or cytoreductive drugs, mainly hydroxyurea. Although used only in a minority of our cases (5.2%), ruxolitinib, the first JAK1/2 inhibitor to become commercially available, could represent an effective therapeutic option also in this subgroup of patients, reducing disease-related symptoms and spleen size, as well as the resistivity indices of the hepatic and splenic intraparenchymal arteries, with a substantial stabilization of esophageal varices grade [56].

According to our data, the onset of SVT should not be considered a feature that suggests an underlying aggressive MPN. In the past, MPN-SVT have in fact been reported as atypical myeloproliferative disorders with high thrombotic risk and slow disease progression [57]. In particular, our cohort of 16 MPN-U patients appears to represent a distinct clinical group, since SVT was the initial manifestation in all of these cases: moreover, they showed a very indolent clinical course, without evidence of fibrotic progression or leukemic evolution, and requiring the introduction of cytoreductive agents in fewer cases. Importantly, none of these patients developed clinical features during the follow-up that allowed physicians to reclassify them as one of the classical *BCR::ABL1*-negative MPNs. Similar results have also been recently reported in previous studies [34, 35, 39] supporting the notion that MPN-U with SVT may represent a very early stage of hematological neoplasm without overt histopathological and hematological features indicative of a specific disease subtype according to WHO criteria.

Consequently, survival of MPN-SVT patients is primarily influenced by the recurrent thrombotic event and/or the consequences on splanchnic circulation and liver function, rather than the natural history of the underlying malignancy. In support of this statement, our cohort was also enriched in cases with *JAK2V617F* homozygosity; furthermore, as we could estimate that *JAK2V617F* mutation preceded those of *TET2* in most cases (Fig. 4 and Supplementary Fig. S4), we confirm that mutations order has relevant implications also in MPN-SVT patients, since *JAK2*-first cases have already been demonstrated to be typically associated with an increased risk of thrombosis [33]. Consistently, the frequency of *JAK2*-first cases was much lower in unselected,

non-SVT MPN cases [32]. In addition, no obvious genetic thrombophilia conditions were found despite thorough investigation in our patients. Indeed, prothrombin G20210A and factor V Leiden mutations were present with a frequency not different from the general population, and no high-risk mutations were found, once more stressing the role of genomics of the underlying MPN as the main driver of the clinical outcome.

Furthermore, in agreement with previous studies [25, 32, 58] the most frequently mutated gene was *TET2* (15/29 patients, 51.7%), followed by *DNMT3A* (9/29, 31.0%). Some of these genes have essential functions independent of their enzymatic activity, with *TET2* appearing to be an important regulator of hematopoietic stem cell biology [59]. *TET2* mutations have been identified in a high proportion of patients with *JAK2V617F*-positive and *JAK2V617F*-negative MPNs, suggesting that the occurrence of these mutations may represent an early event in disease pathogenesis [60]. Among others, Colaizzo et al. identified previously unreported *TET2* mutations in 3/23 non-cirrhotic *JAK2V617F*-positive MPN-SVT patients [61]. Interestingly, only patient no. 3 showed overt MPN, while the others developed an MPN-specific phenotype only years after SVT diagnosis, thus further supporting the notion of *TET2* mutations as an early event along with *JAK2V617F* in the natural history of MPNs. Classically, MPNs diagnosed at the time of an SVT are considered to be early in their disease course, and our clinical and histological data support this hypothesis. However, our genomic findings show a rather complex genetic architecture, also supporting in our cohort the recent view that the preclinical evolution of these malignancies dates back well before the time of diagnosis [62].

Furthermore, *TET2* deficiency results in an increased pro-inflammatory phenotype in murine macrophages which could favor the development of atherosclerosis and thrombosis [63]. More specifically, even in the context of clonal hematopoiesis of indeterminate potential (CHIP) [64–67], *DNMT3A/TET2/ASXL1* gene mutations have already been shown to cause a 1.9-fold higher risk of developing coronary disease and a 4-fold higher risk of myocardial infarction [68].

Interestingly, it has recently been reported that *TET2* and *DNMT3A* genes are both associated with an increased thrombotic risk in PV [58]. Indeed, our cases show a high prevalence of *TET2* mutations in our patients' population (28.5%) compared to other previous studies (roughly 14%) [61, 69, 70]. This evidence together with the “*JAK2*-first” genotype may confer a genetically-determined prothrombotic phenotype in MPN patients affected by SVT.

However, when considering HMR mutations, which are already well-known to be associated with poor prognosis in *BCR::ABL1*-negative MPNs [71], particularly PMF, they were more frequently detected among patients with at least

two non-driver mutations, and correlate with clinical outcomes, as all the cases of leukemic evolution were characterized by a higher *JAK2V617F* allele burden, a higher median number of co-mutations, and a co-mutational pattern of high-risk lesions (Fig. 3). A previous study has already demonstrated that a *JAK2V617F* allele burden $\geq 50\%$ and presence of chromatin/spliceosome/*TP53* mutations are associated with a higher incidence of fibrotic progression/leukemic evolution or death in MPN-SVT patients [72]. Nevertheless, while identifying at least one additional mutation in 53.7% of our patients, the type and number of non-driver mutations were not shown to have any significant impact on fibrotic progression, SVT recurrence, other thrombo-hemorrhagic complications, or death. A possible explanation for the observed differences between our cohorts could be due to a higher frequency of HMR mutations in the Debureaux series (14 cases vs. 8 in our cohort) [72]. Another point to consider is the different representation of specific MPN subtypes: in particular, while Debureaux et al. [72] did not include any patient with MPN-U among their case series, this diagnosis was made in nearly 30% of our subjects, thus supporting the notion of the potential impact of morphological features on the prognostic role of molecular profile also in the MPN-SVT setting.

All this considered, while acknowledging the limited availability of the method and its high cost, our data underline the importance of using NGS analysis in the management of MPN-related SVT as it can support the MPN diagnosis, particularly in “triple-negative” cases, i.e., without *JAK2V617F* or exon 12, *CALR*, or *MPL* mutations. Although no additional mutations were found in our two “triple-negative” cases, targeted NGS myeloid analysis has proven useful in highlighting alternative drivers in these subgroups of patients in the past [73, 74] that may be helpful for diagnosis. Furthermore, in the future whole genome approaches are likely to highlight different genomic lesions, possibly in the form of copy-number changes or rearrangements even in the absence of recurrent gene mutations.

We are aware that our considerations are partially limited by the relatively small number of patients with MPN and SVT. Additionally, the presence of the *JAK2V617F* mutation in most of our cases may represent a selection bias; however, since the discovery of *JAK2* mutation in 2005, all patients who were admitted to our hospital due to idiopathic SVT have so far routinely undergone BM biopsy, along with molecular screening for *CALR* and *MPL* mutations as well. Finally, NGS analysis was performed using DNA extracted from PB granulocytes collected at the time of *JAK2V617F* mutational screening, i.e., after a median time from MPN diagnosis of 6.1 years. This was primarily due to the inclusion in our series of 21 (36.2%) patients diagnosed with MPN prior to the discovery of *JAK2* mutation in 2005 (mainly between 1990 and 2004), a period in

which collection of onset samples for molecular testing was not included in routine clinical practice. While admitting that this latter aspect may represent a limitation of our study, however, as reported for the first time in the pivotal study by Grinfeld et al. [32], the acquisition of additional molecular abnormalities is typically associated with disease progression (fibrotic progression or leukemic evolution), which is a rare event in patients with MPN and SVT and was also rare in our cohort.

In conclusion, our results suggest that a comprehensive approach is always required to make a diagnosis of MPN in patients presenting or suffering from SVT during follow-up, considering BM morphology, clinical and molecular data (either on driver or non-driver mutations), with potential consequences also on prognosis and therapeutic management.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00277-023-05217-2>.

Acknowledgements This study was partially funded by Italian Ministry of Health—Current research IRCCS to NB. NB is recipient of an Investigator grant from the Associazione Italiana Ricerca sul Cancro (AIRC IG n. 25739).

Authors contributions D. Cattaneo, N. Bolli, and A. Iurlo were responsible for study concept and design and wrote the paper. D. Consonni was responsible for statistical analyses. A. Marchetti, M. Lionetti, E. Fermo, A. Maeda, A. Marella, A. Neri, and N. Bolli made and interpreted mutational analyses. U. Gianelli reviewed bone marrow biopsies. D. Cattaneo, C. Bucelli, V. Bellani, C. De Magistris, M. Primignani, L. Baldini, and A. Iurlo followed the patients and collected data. All authors critically reviewed and approved the final version of the manuscript.

Data Availability The data that support the findings of this study are available from the senior authors upon reasonable request.

Declarations

Conflict of interest disclosures All the authors declare they have no potential conflicts of interest.

Ethical statement All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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