Journal of Thrombosis and Thrombolysis (2020) 50:995–1003
https://doi.org/10.1007/s11239-020-02100-z

Low prevalence of JAK2 V617F mutation in patients with thrombosis and normal blood counts: a retrospective impact study

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Published online: 7 April 2020

Abstract

To determine the prevalence of the V617F Janus Kinase 2 (*JAK2*) mutation in patients with thrombosis without other biological signs of underlying myeloproliferative neoplasm (MPN) and identify associated risk factors for thrombosis. Over a 10-year period, data were collected from patients with thrombotic events and who had also been screened for the V617F *JAK2* mutation. Patients with signs of underlying MPN, such as haematocrit levels ≥ 50% and/or platelet counts ≥ 450×10^9/L and/or splanchnic thrombosis were excluded from the study. Of 340 patients fulfilling inclusion criteria, *JAK2* mutation was found in 9 (2.65%), the allele burden being at least 2% in 4 (1.1%). Upon follow-up, MPN was diagnosed in the latter 4. Univariate analysis of the whole cohort showed that age (54 ± 15 vs. 64 ± 13, p = 0.027), platelet count (317 ± 111 vs. 255 ± 75, p = 0.017), C-reactive protein level > 5 mg/L (OR 7.29, p = 0.014), and splenomegaly (OR 54.5, p = 0.0002) were significantly associated with *JAK2* mutation. There was also a trend for an increased risk of cerebral venous thrombosis (OR 6.54, p = 0.064). Logistic regression confirmed a significant association between splenomegaly and *JAK2* mutation (OR 43.15 [95%CI, 3.05–610.95], p = 0.0054). The V617F *JAK2* mutation is rarely found in patients with thrombotic events without overt MPN. Splenomegaly, however, is a statistically and clinically relevant indicator of a potential *JAK2* mutation in patients with non-splanchnic thrombotic events. Such patients should require further assessment and a close follow-up.

Keywords Janus kinase 2 · Myeloproliferative neoplasia · Splenomegaly · Thrombophilia · Thrombosis

Highlights

- Systematic testing for JAK2 mutation in unprovoked thrombosis is controversial
- JAK2 testing in patients without overt biological signs of myeloproliferative disease is addressed
- The prevalence of JAK2 mutations is less than 3% in such cases
- Splenomegaly is associated with JAK2 mutation in non-splanchnic thrombotic events

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Introduction

Thrombosis is a major cause of morbidity in patients with myeloproliferative neoplasms (MPN) harbouring a mutation in the Janus kinase 2 gene (*JAK2*) leading to a self-activated V617F JAK2 mutant protein [1–4]. The latter is the most frequent one in Philadelphia chromosome-negative MPN, present in 95% of *polycythemia vera* (PV) cases and up to 65% cases of essential thrombocythemia (ET) and primary myelofibrosis. It is part of the WHO diagnostic criteria for MPN [5, 6] and has been shown to be an independent predictor of thrombotic events [5, 7]. Its incidence indeed ranges

from 17 to 41% in individuals presenting with splanchnic vein thrombosis [8–10]. This has led to recommending testing for *JAK2* mutation as an indicator of potential latent MPN in patients with splanchnic vein thrombosis [11, 12]. However, the incidence of the *JAK2* mutation in patients presenting with thrombosis at other vascular sites has been reported to be significantly lower (i.e. less than 11%) [4].

Increased risks for hematological and non-hematological cancers have been associated with the *JAK2* mutation [13]. More specifically, it is believed that individuals with a mutational burden below 2% may suffer from a latent form of MPN [14].

All in all, although little is known of the implications of *JAK2* testing and the significance of the mutation in thrombotic patients with normal complete blood counts (CBC), there is a controversial and liberal attitude for *JAK2* mutation testing in patients with unprovoked thrombotic events.

Based on these premises, the study reported here aimed at determining the prevalence of *JAK2* mutations in subjects presenting with thrombotic events and normal CBC. Secondary objectives were to specify the clinical and biological profile of such patients and ultimately offer a better insight into the relevance of *JAK2* testing in this population.

Materials and methods

Study population

This retrospective study spanning ten years (January 2006 to December 2016) analysed data from patients tested at Nice University Hospital (France) for *JAK2* mutation from electronic medical files (**Fig.** 1). Selection criteria were: patients aged 18 years and older with thrombophilia workup for an arterial (AT) or venous (VT) thrombotic event, documented by imaging techniques or histologically proven. Patients with splanchnic thromboses were excluded as well as those with CBC suggestive of MPN (i.e. haematocrit over 49% and/or platelets over $450*10^9$/L) since testing for *JAK2* mutation in such circumstances is anyhow warranted [15].

Clinical data and thrombophilia screening

Clinical and biological features of the subjects were extracted from digital medical files. Iron deficiency as well as plasma inflammatory markers were collected whenever available. Results of bone marrow aspirates (cytology) and histological analyses of bone marrow biopsies were only recorded in patients with *JAK2* mutations. A family history of thrombophilia and cardiovascular risk factors—such as age, hypertension, diabetes mellitus, dyslipidaemia, and smoking—were also recorded. Clinical changes, modifications in CBC at follow-up and outcome were documented over the ten-year period.

The provoked or unprovoked nature of AT or VT and their recurrence—as well as the location of the clot—were specified. Provoked VT with major triggering circumstances were defined as plaster cast immobilisation and/or fracture of a lower limb and/or surgery under general anaesthesia lasting more than 30 min and/or bedrest for more than 3 days, occurring in the 3 previous months and/or active cancer in the 2 preceding years [16, 17]. Moderate or minor triggering circumstances were pregnancy or puerperium, estrogen treatment or hormonal replacement therapy in the year preceding the VT or travelling for a journey of more than 6 h [16]. AT or VT occurring under antithrombotic treatment were also recorded.

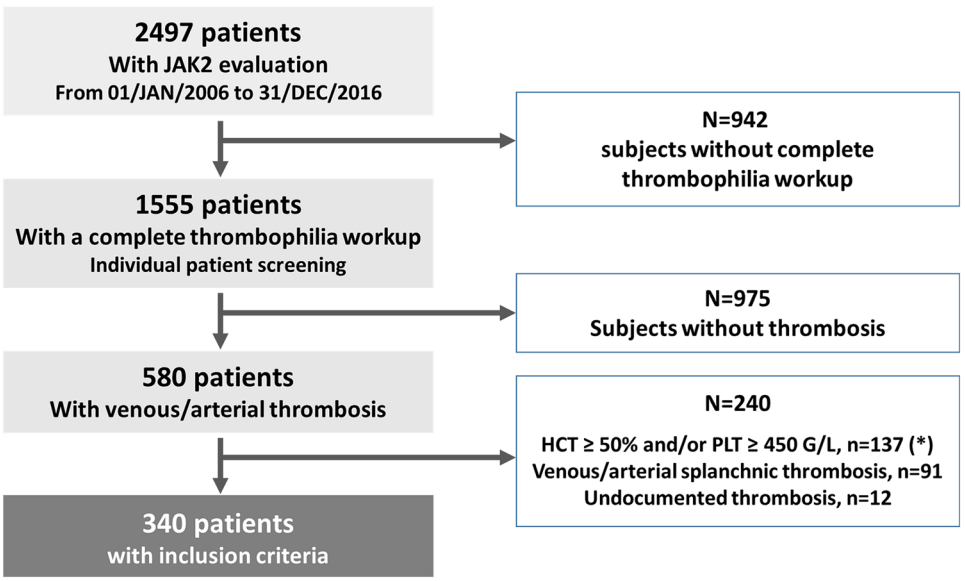

Fig. 1 Flow-chart illustrating the inclusion process. *N* number of subjects excluded from the analysis, *HCT* haematocrit, *PLT* platelets (*) 3 patients with HCT ≥ 50% and 17 patients with PLT ≥ 450 G/L had a JAK2 mutation

Testing for *JAK2* mutation is systematic as part of our institutional screening practices for patients with cerebral vein thrombosis (CVT) in search of thrombophilia. This includes investigation for antiphospholipid antibodies (anti-β2-glycoprotein-I and anti-cardiolipin), lupus anticoagulant, protein C and S activity, antithrombin level and factor V Leiden or prothrombin G20210A mutations. All patients provided written informed consent for molecular analyses. However, *JAK2* testing was only performed in case of unprovoked thrombotic events if the thrombophilic workup (specified above) was negative, and as a "second-line" approach.

JAK2 V617F mutation

After DNA extraction from blood samples (Qiagen, France), *JAK2* V617F mutation was investigated. Quantification was only performed upon *JAK2* mutation detection. A 2% cut-off was used to classify patients with a significant allelic burden. Real-time fluorescent quantitative PCR using allele-specific primers and a TaqMan-MGB probe for dual-inhibiting amplification of wild-type alleles were performed for the detection of as low as 0.05% mutant *JAK2*V617F cells [18] ("Appendix A" section).

Statistical analyses

Data are reported as mean ± standard deviation (SD). For small samples (i.e. $n < 20$), continuous variables are expressed as median values with their interquartile range (IQR), extreme values being specified when appropriate. The chi-square test was used to study statistical relationships between qualitative variables and the risk of thrombosis in *JAK2* mutated patients. Significant variables were then tested in a multivariate model (logistic regression using backward analysis) with odd ratios and confidence intervals (CI). Differences between groups were analysed using the Mann–Whitney test. McNemar's test with Yates' continuity correction was used to compare sensitivity and specificity between groups. P values of less than 0.05 were considered statistically significant using two-sided tests. Statistical analyses were performed with GraphPad® Prism 6.2 and MS Excel 2010. Logistic regression was performed using MedCalc Statistical Software (Ostend, Belgium).

Ethics

Informed patient consent was collected as part of routine clinical practice and data were anonymized when recorded for the purpose of this study. Data are stored in the University Hospital's electronic file repository under the identification number #277, according to *Commission nationale de l'informatique et des libertés* (CNIL) guidelines. The retrospective nature and format of the study did not require approval from an institutional review board.

Results

Patient characteristics

Over the 10-year period, 2497 patients with *JAK2* evaluation were recovered, of whom 1555 also had been investigated for thrombophilia (Fig. 1). Ultimately, of the 580 cases of thrombotic events with thrombophilia screening, 340 were finally included in the study. Demographics and clinical data are shown in Table 1. None of the patients had been splenectomised and none had peripheral (or blood) cytological signs of asplenia. Splenomegaly was identified, either clinically or radiologically, in five patients. Of the latter, only two presented with cytopenia. Thirty-one of the 335 remaining patients were also found to have cytopenia: platelet count $< 150*10^9$/L (n = 8), haemoglobin level < 120 g/L (n = 21), and leukopenia (i.e. $< 4*10^9$/L) (n = 6).

More than a third of patients were at least 60 years old and the male-to-female gender ratio was 0.89. Ninety-one patients presented with AT, 268 with VT, and 19 had mixed AT and VT. One hundred and nine patients (32.1%)—93 VT and 28 AT—presented with thrombophilia, 32 of whom with multiple thrombotic aetiologies. Antiphospholipid syndrome was diagnosed in 50 patients (14.7%).

Of the 64 patients with arterial thrombosis and one or more risk factors for cardio-vascular disease, 50 were smokers, 33 had high blood pressure, 10 had a body mass index ≥ 30 kg/m^2 (Table 1).

Multiple causes of VT were identified in 31 of 109 patients presenting with constitutional and/or acquired thrombophilia. One patient had a homozygous prothrombin G20210A mutation.

At least one thrombotic event occurred in 73 patients who were on antithrombotic drugs prior to the thrombotic event leading to thrombophilia workup. Of note, 4/73 patients simultaneously had anticoagulants and antiplatelet drugs.

JAK2 V617F prevalence and mutational status

A *JAK2* mutation was found in 9 patients with a normal CBC, thus yielding a prevalence of 2.65%. Only four patients (of which two had splenomegaly) had a significant *JAK2* allele burden (≥ 2%). One patient presented with moderate thrombopenia (Table 2) whereas the others had no cytopenia.

The clinical and biological data of these 9 patients with *JAK2* mutations are presented in Table 2. Their median age was 72 years old (IQR, 55.5–75). Central nervous system involvement was reported in 3 of the 5 cases of AT

Table 1 Patient characteristics

Demographics	*N = 340*
Mean age, years (range)	54.2 (18–85)
Subjects aged ≥ 60 years, n (%)	127 (37.4)
Woman/men, n/n	180 / 160
Clinical aspects	*N = 340*
Splenomegaly, n (%)	5 (1.5)
Thrombosis in patients on antiplatelet drugs, n (%)	39 (11.5)
Thrombosis in patients on anticoagulant drugs, n (%)	34 (10)
Complete blood count	*N = 340*
Mean haematocrit percentage, % (± SD)	40 (± 0.5)
Haemoglobin level < 120 g/L, n (%)	21 (6.2)
Mean leukocyte count, $*10^9$ (± SD)	7.6 (± 0.3)
Leukocyte count < 4 $*10^9$/L, n (%)	6 (1.8)
Mean platelet count, $*10^9$/L (± SD)	257 (± 8)
Platelet count < 150 $*10^9$/L, n (%)	8 (2.4)
Identification of JAK2 V617F mutation	*N = 9*
Allele burden < 2%, n	5
Allele burden ≥ 2%, n	4
Patients with associated risk factors for thrombophilia	*N = 109*
Lupus anticoagulant, n (%)	17 (15.6)
Beta-2 glycoprotein 1 antibodies n (%)	17 (15.6)
Cardiolipin antibodies, n (%)	27 (24.8)
Heterozygosity for the factor V Leiden, n (%)	21 (19.3)
Prothrombin G20210A mutation, n (%)	5 (4.6)
Protein S deficiency, n (%)	31 (28.4)
Protein C deficiency, n (%)	29 (26.6)
Antithrombin deficiency, n (%)	9 (8.3)
Arterial thrombosis	*N = 91*
≥ 1 risk factor for cardio-vascular disease, n	64
Cardioembolic source of thrombosis, n	12
Recurrent arterial thrombosis, n	67
Stroke, n	30
Lower limb arterial occlusion, n	25
Upper limb arterial occlusion, n	19
Myocardial infarction, n	11
Central retinal artery occlusion, n	10
Renal artery occlusion, n	6
Venous thrombosis	*N = 268*
Criteria for provoked DVT*, n (%)	117 (43.7)
Recurrent DVT, n (%)	94 (35.1)
Lower limb DVT, n (%)	163 (60.8)
Pulmonary embolism (PE), n (%)	84 (31.3)
Proximal PE, n	36
Distal PE, n	59
Cerebral vein thrombosis, n (%)	37 (13.8)
Upper DVT, n (%)	26 (9.7)
Central retinal vein occlusion, n (%)	20 (7.5)

*Subjects presenting with 1 or more factors amongst which: plaster cast immobilisation and/or fracture of a lower limb, or surgery under general anaesthesia lasting more than 30 min, or bedrest for more than 3 days occurring in the 3 previous months, or active cancer in the 2 preceding years, or pregnancy, or puerperium, or estrogen treatment, and/or a journey lasting more than 6 h.

(i.e. stroke) and three of the four cases of VT (i.e. CVT). Two of the 3 patients with stroke were not known to have cardiovascular risk factors and none of them had a cardioembolic thrombosis. The four cases of *JAK2*-positive VT were unprovoked and without evidence of thrombophilia.

The prevalence of *JAK2* mutation was, respectively, 7.7%, 5.9% and 1.8%, in patients receiving antiplatelet treatment (n = 39), anticoagulants (n = 34) or without antithrombotic treatment (n = 271) and respectively 10% (3/30) and 8.1% (3/37) for patients with ischemic stroke or CVT. Bone marrow biopsy pathology was performed in seven of the 9 patients with *JAK2* mutation. Cytological features of bone marrow analysis were characteristic of ET in two cases, despite normal CBC, normal ferritin levels and no evidence for haemodilution. None of the patients with splenomegaly showed further signs of MPN within the timeframe of the study, although one patient was lost to follow-up after refusing bone marrow biopsy.

Factors associated with *JAK2* mutant-induced thrombotic events

Cardiovascular risk factors as enumerated previously were not related to AT associated with *JAK2* mutation (Table 3). Similarly, the most common causes of thrombophilia were not predictive of VT in *JAK2* mutant patients. Neither the nature nor site of thrombosis were predictive of *JAK2*-associated thrombosis. In the univariate analysis, elevated C-reactive protein levels and splenomegaly remained associated with the *JAK2* mutation. However, in multivariate analysis, only the association with splenomegaly was statistically significant.

Patients with the *JAK2* mutation were younger (54 ± 15 years vs. 64 ± 13 years; p = 0.027), and had higher platelet levels (317 ± 111$*10^9$/L vs. 255 ± 75$*10^9$/L; p = 0.017). In multivariate analysis, age was not a significant predictor contrarily to platelet levels (p = 0.006), with an odds ratio of 1.012 (95%IC, 1.004–1.021).

Patient follow-up

Follow-up data were available for only 6 of the 9 patients with *JAK2* mutation. The median time of follow-up was 12 months (IQR, 1–36). None of these patients presented with recurrent thrombosis during the follow-up period. Anomalies suggestive of MPN appeared on the CBC of the four of the 6 patients with a mutant allele burden greater than 2%: thrombocytosis (n = 3) and polycythemia (n = 1). These patients were subsequently treated with hydroxycarbamide.

Table 2 Patients with JAK2 V617F mutation and thrombotic manifestations

Patient	1	2	3	4	5	6	7	8	9
Age, years	61	78	72	39	50	73	66	73	76
Sex, M/F	F	F	M	F	M	M	F	F	M
Thrombosis, V/A	V	V	V	V	A	A	A	A	A
Recurrent thrombosis	Yes	Yes	No	No	No	No	Yes	No	No
Nature of thrombosis	Unprovoked: DVT lower limb CVT	Unprovoked: DVT lower limb PE	Unprovoked: CVT	Unprovoked: CVT	Stroke	CRAO	Lower and upper limbs	Stroke	Stroke
Splenomegaly	No	No	Yes	No	Yes	No	No	No	No
Cardioembolic source of arterial thrombosis	n/a	n/a	n/a	n/a	No	Yes	No	No	No
No. of CV risk factors	1	1	4	0	0	2	0	0	2
Associated risk factor for thrombophilia	No	No	No	No	No	No	Anti-PL	No	No
Antiplatelet agent prior to thrombosis	No	Yes	Yes	No	No	No	No	No	Yes
Anticoagulant agent prior to thrombosis	No	Yes	No	No	No	Yes	No	No	No
Haematocrit, %	45	40	44	42	44	39	29	36	48
Leukocyte count, $*10^9$	12.6	7.7	9.5	6.7	11.7	8.6	6.1	5.8	4.6
Platelet count, $*10^9$	248	393	408	389	425	304	383	185	115
Allele burden for JAK2 mutation, %	31–50	12.5–31	12.5–31	0.1–2	5–12.5	0.1–2	0.1–2	0.1–2	0.1–2
Bone marrow, diagnosis	–	ET	–	N	N	N	N	ET	N
CBC anomalies during follow-up	High haematocrit	High platelet levels	High platelet levels	–	High platelet levels	None	None	–	–

M male, *F* female, *A* arterial (thrombosis), *V* venous (thrombosis), *CVT* cerebral vein thrombosis, *PE* pulmonary embolism, *DVT* deep vein thrombosis, *CRAO* central retinal artery occlusion, n/a not applicable, CV cardiovascular, *Anti-PL* antiphospholipid marker, *ET* essential thrombocythemia, *CBC* complete blood counts, *N* normal

Discussion

This study is one of the few to analyse the implications of screening for *JAK2* V617F mutations in patients presenting with non-splanchnic thrombosis and without any biological signs of MPN. It also analysed potential thrombosis-associated risk factors.

Evidence was found that thrombotic events specifically related to *JAK2* mutations, other than splanchnic thrombosis, are only exceptionally found in patients without CBC anomalies. The prevalence of *JAK2* mutations is 2.65% in this series with, in most cases (5/9), allele burdens lower than 2%. Age-related clonal haematopoiesis of indeterminate potential or CHIP cannot be excluded but seems unlikely owing to the relatively young age of the patient-population (i.e. mean age of 54 years).

As for pathophysiological mechanisms, the *JAK2* mutation has been shown to promote higher blood-cell counts. Thrombotic events thus could occur mainly through i) an increase of direct activation or endothelium adhesion of blood cells [19–23] or ii) an over-expression of the *JAK2* mutation in endothelial cells [24–26]. It has also been shown that an increase in P-selectin levels through the activation of the *JAK2/STAT3* pathway could lead to a prothrombotic state [27], mostly in response to an inflammatory process mediated by interleukin-9 [28]. It is also believed that

Table 3 Predictors of JAK2 V617F mutant in patients presenting with thrombosis (univariate and multivariate analyses)

Univariate analysis	OR	95% IC	p value
Thrombosis occurring despite antiplatelet therapy	0.61	0.06–5.73	0.67
Thrombosis occurring despite anticoagulant therapy	2.93	0.29–29.12	0.36
Recurrent thrombosis	0.60	0.06–5.88	0.66
Cerebral vein thrombosis	6.54	0.89–47.97	0.065
Stroke	3.28	0.52–20.77	0.21
Unprovoked deep vein thrombosis	1.87	0.19–18.40	0.59
Pulmonary embolism	0.72	0.04–13.63	0.83
Provoked deep vein thrombosis	0.43	0.04–4.14	0.46
High blood pressure	1.18	0.19–7.47	0.86
Diabetes	2.44	0.24–24.53	0.45
Smoking	0.18	0.02–1.71	0.14
Protein S deficiency	0.67	0.04–12.79	0.79
Protein C deficiency	0.73	0.04–13.82	0.83
Antithrombin deficiency	2.47	0.12–49.35	0.55
CRP > 5 mg/L	**7.29**	**1.49–35.71**	**0.014**
Splenomegaly	**54.5**	**6.54–453.96**	**0.0002**
Multivariate analysis	**OR**	**95% IC**	**p value**
Splenomegaly	**43.15**	**3.05–610.95**	**0.0054**
Cerebral vein thrombosis			**NS**
CRP > 5 mg/L			**NS**

Known independent risk factors for thrombosis are presented in italics. Variables used in the multivariate model are shown in bold. *OR* odds ratio, *95%IC* 95% confidence interval, *CRP* C-reactive protein

hydroxycarbamide could reverse this prothrombotic state by stimulating the NO-cGMP pathway [27, 29, 30].

A previous study of 664 consecutive patients addressed a very similar issue and reported a *JAK2* mutation in 6 (< 1.0%) patients [31]. The mutant allele burden was however extremely low and none of these patients developed either overt MPN nor recurrent thrombosis. Another study from Ianotto et al., exploring 372 patients with deep VT for *JAK2* and calreticulin mutations, identified 10 *JAK2*-mutated patients, including 3 with MPN yielding a prevalence of 1.9% [32].

In the present series, latent ET was found in 2 of the 6 *JAK2* mutated patients who had had bone marrow biopsies. It was also found that the ≥ 2% cut-off for allele burden was of clinical interest since such patients were more likely to present with splenomegaly and evolve towards MPN within the first year of follow-up. This, of course, does not exclude *JAK2* allele burdens of < 2% from being implicated in thrombotic processes. In fact, presence of a *JAK2* mutation has been described in up to 10% of healthy subjects [33]. The risk of thrombosis, however, has been shown to increase with the percentage of mutant allele burden and the type of MPN [31, 34].

The large series reported here confirms the low incidence of *JAK2* mutation in thrombotic patients. However, it also indicates that a systemic inflammatory profile and splenomegaly should prompt *JAK2* mutation analysis.

No prognostic value was found for other factors such as thrombosis in an atypical site, multiple thromboses, CVT, anticoagulation, anti-platelet treatment or cardiovascular risk factors. However, in the multivariate analysis, platelet levels were found to be predictive of *JAK2* mutation [odds ratio 1.012 (95%IC, 1.004–1.021)]. This might be difficult to interpret in a daily clinical setting, especially in proinflammatory states such as thrombotic events. Of note, 6 of the 9 *JAK2*-mutated patients presented with cerebral involvement: respectively three strokes (10% of all strokes) and three CVT (8% of CVT). This was not statistically significant, and mirrors findings from previous studies [2, 8]. The prevalence rate of CVT of 8.1% observed in this cohort anyway seems higher than that from recent studies, where it ranges from 5.6 to 6.6% [35–37]. Despite limited data, it is suggested that testing for latent MPN should be performed in cases of CVT [38]. We believe, however, that this requires assessing and eliminating several confounding factors: (i) prior absence of constitutional/acquired thrombophilia, (ii) absence of a systemic inflammatory disease (iii) a CBC not suggestive of MPN and, of course, (iv) no palpable spleen or radiologic splenomegaly. Although it enrolled a large cohort of patients, this retrospective single-center initiative is insufficiently powered to detect MPN development because of the low number of patients with *JAK2* mutations that were ultimately identified. Furthermore, the average follow-up

for patients detected as carrying a *JAK2* mutation is relatively short considering that subsequent diagnoses of MPN may occur as late as after 10 years, independently from the mutational burden. This implies that the association of *JAK2* mutation and splenomegaly could be also be fortuitous (i.e. type I error), despite the latter being a well-known feature of MPN. As pointed out previously, it could also appear that a center-effect may have inflated the incidence of CVT when compared to data from other series. As expected from the retrospective design of this study, incidence rates could have further been increased by an unavoidable liberal attitude to *JAK2* testing.

Our findings, however, besides being consistent with those of previous studies may impact day-to-day clinical (i.e. "real-life") practice, notably by pointing out the importance of *JAK2* testing in patients with splenomegaly as just discussed above. Based on data gathered from guidelines [39, 40], ours and previous studies, *JAK2* testing needs to be considered in patients presenting with unprovoked VT and CVT especially when splenomegaly is also present.

Conclusion

The *JAK2* mutation is only rarely associated with AT or VT events in patients without overt MPN. Splenomegaly is a clinically relevant marker for possible *JAK2* mutation in patients with non-splanchnic thrombotic events. Other than that, screening for the *JAK2* mutation should be subject to the prior exclusion of more common thrombophilic disorders.

Acknowledgements All authors had full access to the study data and had final responsibility for the decision to submit for publication. We would like to thank Jacques Levraut for his help with the statistical analysis.

Author contributions ML, LL and NM designed the study. ML obtained the data. All authors analysed and interpreted the data. ML and NM wrote the report. All authors revised the report and approved the manuscript.

Compliance with ethical standards

Conflicts of interests The authors report no personal conflicts of interest.

Ethical approval All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. According to current regulations, informed consent was systematically required prior to performing genetic tests. Due to the retrospective design of the study, informed consent from patients was not required for their inclusion.

Appendix A—DNA extraction and analysis

The EZ1 Advanced XL instrument (Qiagen) was used to isolate genomic DNA from 0,2 mL of patient's blood samples using the Advanced XL instrument (Qiagen) according to the manufacturer's recommendations. Quality and quantification were assessed using the NanoDrop 8000 spectrophotometer (Thermo Scientific) and samples were diluted to achieve a concentration of 5 ng/µL.

Quantification of the JAK2 V617F mutation was done through real-time fluorescent quantitative PCR with specific allele primers and TaqMan-MGB probe—the minimal detection rate for the mutation being 0.05%.

The Mx 3000P real-time PCR system (Agilent Technologies) testing duplicate samples was used. Genetic material was amplified with 25 ng of genomic DNA in 1 × TaqMan Universal PCR Master Mix (Life Technologies). Thermal cycling parameters were: 95 °C for 10 min, and 45 cycles at 95 °C for 30 s and 60 °C for 1 min.

The primers/probe sequences (with their concentrations) are presented in the following table:

Primer	Sequence	Concentration	Comments
JAK2 V617F *Forward primer*	5′-AGCTTT CTCACA AGCATT TGGTT-3′	600 nmol/L	
JAK2 V617F *Reverse primer*	5′-GTTTTA CTTACTCTC GTCTCC ACAAAA-3′	300 nmol/L	
JAK2 V617F *Probe*	5′-6FAM-AAT TATGGAGTA TGTTTCTG-MGBFNQ-3′	200 nmol/L	
Albumin *Forward primer*	5′-ATGCTG CACAGA ATCCTT GGT-3′	300 nmol/L	Internal control
Albumin *Reverse primer*	5′-TCATCG ACTTCC AGAGCT GAAA-3′	300 nmol/L	Internal control
Albumin *Probe*	5′-6VIC-AACAGG CGACCA TGC-MGBB-FNQ-3′	200 nmol/L	Internal control

Plasmid calibrator standard curves were used to obtain JAK2 V617F and ALB copy numbers.

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