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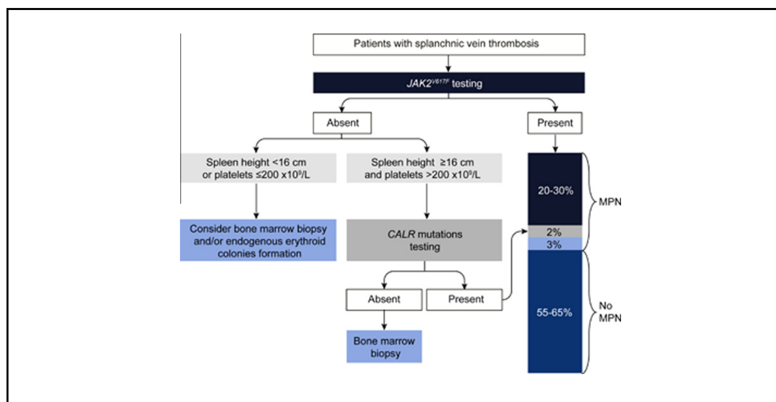
Selective testing for calreticulin gene mutations in patients with splanchnic vein thrombosis: A prospective cohort study

Graphical Abstract



Highlights

- *CALR* mutations are detected in 2% of patients with splanchnic vein thrombosis.
- *CALR* mutations should not be tested in patients with *JAK2*^{V617F}.
- *CALR* mutations should be tested in patients with splenomegaly & platelets $>200\times10^9$/L.
- This strategy avoids 96% of unnecessary *CALR* mutations testing.

Authors

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Lay summary

Mutations of the *CALR* gene are detected in 0 to 2% of patients with SVT, thus the utility of systematic *CALR* mutation testing to diagnose MPN is questionable. This study demonstrates that *CALR* mutations testing can be restricted to patients with SVT, a spleen height $\geq$16 cm, a platelet count $>200\times10^9$/L, and no *JAK2*^{V617F}. This strategy avoids 96% of unnecessary *CALR* mutations testing.

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Research Article

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Cirrhosis

Selective testing for calreticulin gene mutations in patients with splanchnic vein thrombosis: A prospective cohort study

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Background and Aims: Myeloproliferative neoplasms (MPN) are the leading cause of splanchnic vein thrombosis (SVT). Janus kinase 2 gene (*JAK2*)^{V617F} mutations are found in 80 to 90% of patients with SVT and MPN. Mutations of the calreticulin (*CALR*) gene have also been reported. However, as their prevalence ranges from 0 to 2%, the utility of routine testing is questionable. This study aimed to identify a group of patients with SVT at high risk of harboring *CALR* mutations and thus requiring this genetic testing.

Methods: *CALR*, *JAK2*^{V617F} and thrombopoietin receptor gene (*MPL*) mutations were analysed in a test cohort that included 312 patients with SVT. Criteria to identify patients at high risk of *CALR* mutations in this test cohort was used and evaluated in a validation cohort that included 209 patients with SVT.

Results: In the test cohort, 59 patients had *JAK2*^{V617F}, five had *CALR* and none had *MPL* mutations. Patients with *CALR* mutations had higher spleen height and platelet count than patients without these mutations. All patients with *CALR* mutations had a spleen height $\geqslant$16 cm and platelet count $>200\times10^9$/L. These criteria had a positive predictive value of 56% (5/9) and a negative predictive value of 100% (0/233) for the identification of *CALR* mutations. In the validation cohort, these criteria had a positive predictive value of 33% (2/6) and a negative predictive value of 99% (1/96).

Conclusion: *CALR* mutations should be tested in patients with SVT, a spleen height $\geqslant$16 cm, platelet count $>200\times10^9$/L, and no *JAK2*^{V617F}. This strategy avoids 96% of unnecessary *CALR* mutations testing.

Lay summary: Mutations of the *CALR* gene are detected in 0 to 2% of patients with SVT, thus the utility of systematic *CALR* mutation testing to diagnose MPN is questionable. This study demonstrates that *CALR* mutations testing can be restricted to patients with SVT, a spleen height $\geqslant$16 cm, a platelet count $>200\times10^9$/L, and no *JAK2*^{V617F}. This strategy avoids 96% of unnecessary *CALR* mutations testing.

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Introduction

Splanchnic vein thrombosis (SVT) indicates Budd-Chiari syndrome (BCS) and portal venous system thrombosis (PVT). Primary BCS is a rare disorder defined as a blocked hepatic venous outflow tract at various levels from small hepatic veins to the terminal portion of the inferior vena cava.[1] Non-malignant non-cirrhotic extrahepatic PVT is characterized by thrombus development in the main portal

Keywords: Myeloproliferative neoplasms; Budd-Chiari syndrome; Portal vein thrombosis; *JAK2*^{V617F}; *MPL* mutation; *CALR* mutations; Platelets count; Splenomegaly; DNA mutational analysis; Genetic testing.

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vein and/or its right or left branches and/or splenic or mesenteric veins, or by the permanent obliteration that results from a prior thrombus.[1] The pathogenesis of SVT is largely dependent on the presence of systemic prothrombotic conditions that promote thrombus formation in the respective splanchnic veins.[2,3]

Myeloproliferative neoplasms (MPNs) are the leading cause of SVT and are diagnosed in 25 to 50% of patients with SVT.[4] In most patients with SVT and MPN, Janus kinase 2 gene (*JAK2*)^{V617F} mutation is found. In 10% to 20% of patients with SVT this specific mutation is absent, whereas bone marrow biopsy or assessment of endogenous erythroid colonies formation provide evidence for MPN.[5] Mutations across *JAK2* exon 12 or the thrombopoietin receptor gene (*MPL*) are rarely identified in patients with SVT.[5]

Two independent groups described heterozygous calreticulin (*CALR*) mutations as the second most prevalent acquired genetic alteration in essential thrombocythemia and primary myelofibrosis.[6,7] *CALR* mutations are mutually exclusive of *JAK2* and *MPL* mutations. Thereafter, *CALR* mutations have been found in 0 to 2% of patients with SVT.[8–17] Although *CALR* mutations appear to be rare in patients with SVT and their detection not readily accessible to all centers, their identification influences patients' clinical management. This prompted us to take advantage of a large prospective cohort of SVT patients to identify the subgroup at the highest risk of harboring *CALR* mutations and thus requiring this genetic testing.

Patients and methods

Inclusion criteria

This study prospectively included patients with BCS or PVT seen between 2005 and 2013 at the French Reference Center for Vascular Disorders of the Liver (Clichy, France) and for whom peripheral blood DNA was available for mutation screening (Supplementary CTAT Table). The protocol was performed in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institutional review board (CPP Ile de France IV, Paris; France). Informed consent was obtained from all patients included in the study.

We looked for criteria characterizing patients at high risk of having *CALR* mutations in this French cohort, thereafter referred to as "test cohort" and then tested these criteria for validation in a previously reported cohort from Hospital Clinic, Barcelona, thereafter referred to as "validation cohort".[8]

Definitions

BCS was defined as hepatic outflow obstruction regardless of the cause or level of obstruction, from the small hepatic veins to the entrance of the inferior vena cava into the right atrium. BCS was confirmed by ultrasonography and/or multidetector computed tomography and/or magnetic resonance imaging, and/or venography. Sinusoidal obstruction syndrome, as well as outflow obstruction occurring in the setting of heart failure, orthotopic liver transplantation and hepatobiliary cancer were excluded from this definition. Diagnostic criteria for PVT included recent portal, and/or splenic and/or mesenteric venous thrombosis or portal cavernoma. PVT patients with cirrhosis or abdominal malignancies were excluded.

Hematologic studies

JAK2^{V617F}, *MPL* and *CALR* mutations were tested in all patients. *JAK2*^{V617F} and *MPL* mutation analyses were performed as previously described.[5]

For *CALR* mutations, we used DNA extracted by an automated standardized procedure (Qiasymphony, Qiagen) from blood samples collected by venipuncture in tubes containing 0.11 mol/L trisodium citrate and stored at −80 °C until analysis. The mutational status of *CALR* was determined using previously described high-resolution sizing of fluorescent dye-labeled PCR amplification of exon 9, with Sanger sequencing controls.[7]

Bone marrow biopsy and/or endogenous erythroid colonies formation were performed when considered relevant by the physician according to French recommendations.[18]

Investigations for other thrombotic risk factors

Patients were tested according to previously reported methods for the following thrombotic risk factors:[19] factor V R506Q mutation (factor V Leiden); G20210A factor II gene mutation; deficiencies in protein C, protein S, or antithrombin (regarded as primary deficiencies only in conjunction with a prothrombin index ⩾80%); paroxysmal nocturnal hemoglobinuria; and anti-phospholipid antibodies.[1] Oral contraceptive use was considered a thrombotic risk factor when taken within the three months preceding diagnosis of SVT.[20]

Imaging analyses

All abdominal multidetector computed tomography or magnetic resonance imaging performed within six months of SVT diagnosis were reviewed to measure the greatest spleen height in coronal view.

Statistical analysis

Quantitative variables were expressed as median (interquartile range), and categorical variables as absolute and relative frequencies. Comparisons between groups of quantitative and qualitative variables were performed using Mann Whitney and the Fisher exact tests, respectively. All tests were two-sided and used a significance level of 0.05. Data handling and analysis were performed with SPSS 17.0 (SPSS Inc., Chicago, IL).

For further details regarding the materials used, please refer to the CTAT table.

Results

Patient characteristics

Three hundred twelve patients were enrolled, including 99 (32%) with BCS and 213 (68%) with PVT. Patients' characteristics are shown in Table 1.

In patients with BCS, hepatic venous outflow obstruction was due to occlusion of one, two and three hepatic veins in 10, 19 and 67 patients, respectively, and due to obstruction of the suprahepatic segment of the inferior vena cava in two patients. The last patient had small hepatic veins BCS. Thirteen out of the 99 patients with BCS also had a PVT. Out of the 213 patients with PVT without BCS, 39% had a portal cavernoma and 61% an acute PVT. Portal, splenic and mesenteric veins were involved in 199 (93%), 79 (37%) and 118 (55%) of these 213 patients, respectively.

Risk factors for thrombosis are detailed in Table 1. The most common cause was MPN. *JAK2*^{V617F} was detected in 81% of the MPN patients. No *MPL* mutation was found.

CALR mutation

CALR mutations were detected in five patients (1.6%), a proportion in agreement with previous studies. Their individual characteristics are presented in Table 2. None of the patients with *CALR* mutations had *JAK2*^{V617F} *or MPL* mutations. Out of the patients without *CALR* mutations, 59 had *JAK2*^{V617F}, nine had a triple negative MPN (absence of *JAK2*^{V617F}, *CALR* or *MPL* mutations but positive bone marrow biopsy in eight patients, or endogenous erythroid colonies formation in one patient) and 57 did not have MPN after bone marrow biopsy and/or endogenous erythroid colonies formation examination. We compared the clinical and laboratory features of patients with *CALR* mutations with those

Table 1. Characteristics and risk factors for thrombosis in patients with BCS or PVT.

	BCS (n = 99)	PVT (n = 213)
Age, years	35 (25–45)	43 (32–56)
Males	28 (28%)	119 (56%)
Inherited thrombophilia		
Protein C deficiency	4/81 (5%)	14/185 (8%)
Protein S deficiency	4/78 (5%)	19/188 (10%)
Antithrombin deficiency	1/84 (1%)	4/197 (2%)
Factor V gene mutation	11/96 (11%)	9/210 (4%)
Factor II gene mutation	4/99 (4%)	15/213 (7%)
Acquired thrombophilia		
Myeloproliferative neoplasms	30/99 (30%)	44/213 (21%)
Polycythemia vera	13 (13%)	19 (9%)
Essential thrombocythemia	10 (10%)	13 (6%)
Primary myelofibrosis	3 (3%)	3 (1%)
Unclassifiable	4 (4%)	9 (4%)
JAK2^{V617F}	28/99 (28%)	31/213 (15%)
MPL mutation	0/99	0/213
CALR mutation	1/99 (1%)	4/213 (2%)
Antiphospholipid antibody syndrome	9/96 (9%)	23/210 (11%)
Paroxysmal nocturnal haemoglobinuria	4/83 (5%)	1/167 (0.5%)
Hormonal (OC and/or pregnancy)	33/96 (34%)	45/212 (21%)
Systemic disorder[†]	7/99 (7%)	11/213 (5%)
Local risk factor[‡]	3 (3%)	16 (8%)
Single risk factor	44 (44%)	91 (43%)
Multiple risk factors	26 (26%)	49 (23%)
No risk factor	29 (29%)	73 (34%)

Values are n (%) or median (interquartile range).
BCS, Budd-Chiari syndrome; OC, oral contraception; PVT, portal venous system thrombosis.
[†] Behçet disease, sarcoidosis, vasculitis, connective tissue disease or lymphoide hemopathy.
[‡] Intra-abdominal inflammation, infection, or abscess.

of patients with *JAK2*^{V617F}, triple negative MPN and without MPNs (Table 3). Patients with *CALR* mutations had significantly higher platelet counts than patients with triple negative MPNs and patients without MPNs. Patients with *CALR* mutations also had a higher spleen height than patients with *JAK2*^{V617F}, with triple negative MPNs and patients without MPNs ($p = 0.05$, $p = 0.05$, $p = 0.001$, respectively). Patients with *CALR* mutations also had significantly lower haemoglobin and haematocrit levels than patients *with JAK2*^{V617F}. There was no difference between patients with *CALR* mutations and other groups regarding other clinical characteristics, frequency of inherited and other acquired risk factors for thrombosis or laboratory features.

Given the high platelet count and spleen height observed in patients with *CALR* mutations, we tested the hypothesis that criteria derived from those proposed in 2005, before *JAK2* and *CALR* mutations discovery, namely spleen height $\geqslant$16 cm and platelet count $>200\times10^9$/L, could identify a group at high risk of *CALR* mutation among patients with SVT.[18] As shown in Fig. 1, all five patients with *CALR* mutations fulfilled these criteria. These criteria thus had a positive predictive value of 56% (5/9) and a negative predictive value of 100% (0/233) for the identification of *CALR* mutations. Out of the other four patients with a spleen height $\geqslant$16 cm and platelet count $>200\times10^9$/L, two had a histologically proven MPN, one had a histiocytosis and one had an antiphospholipid antibody syndrome.

Validation cohort

We then tested the criteria "spleen height $\geqslant$16 cm and platelet count $>200\times10^9$/L" in an independent cohort of patients with SVT previously reported.[8] As shown in Fig. 2, two out of the three patients with *CALR* mutation fulfilled these criteria. The third patient with *CALR* mutations had a platelet count of 477×10^9/L, but spleen height of 11 cm. A fourth patient with a *CALR* mutation in this cohort had a platelet count $>200\times10^9$/L and an enlarged spleen based on the radiology report. However, images could not be retrieved so that precise spleen size could not be measured. Patients' features are detailed in Table 2. In the validation cohort, the criteria "spleen height $\geqslant$16 cm and platelet count $>200\times10^9$/L" thus had a positive predictive value of 33% (2/6) and negative predictive value of 99% (1/96) for the identification of *CALR* mutations.

Discussion

This study of more than 500 patients with SVT has allowed for the characterization of patients in whom *CALR* mutations should be tested.

Indeed, the main finding of this study was that *CALR* mutations were almost exclusively found in patients with SVT, without *JAK2*^{V617F}, when spleen height was $\geqslant$16 cm and platelet count $>200\times10^9$/L. *CALR* mutations are driving essential thrombocythemia and primary myelofibrosis, but not polycythemia vera.[21] Indeed, these mutations induce activation of the thrombopoietin receptor, *MPL*, resulting in the proliferation of the megakaryocytic lineage. Clinical manifestations of *CALR* mutated MPNs include high platelet count and enlarged spleen.[21] Thus, identification in our study of these two parameters as markers of the presence of *CALR* mutations is not surprising. Because of

Table 2. Characteristics of patients with SVT and *CALR* mutations.

Patient	Cohort	Age (yr)	Gender	$JAK2^{V617F}$ status	Haematologic diseases	Type of SVT	Other risk factor for thrombosis	Spleen height (cm)	Platelet count (10^9/L)
1	Test	24	Female	Negative	PMF	BCS	OC and Gastroenteritis	20.0	417
2	Test	30	Female	Negative	ET	PVT	Sarcoidosis	16.6	436
3	Test	39	Male	Negative	PMF	PVT	None	19.0	453
4	Test	32	Female	Negative	PMF	PVT	None	17.0	476
5	Test	36	Male	Negative	PMF	PVT	None	18.0	477
6	Validation	34	Male	Negative	ET	BCS	None	18.0	300
7	Validation	57	Female	Negative	PMF	PVT	None	18.0	607
8	Validation	57	Female	Negative	ET	BCS	None	11.0	477
9	Validation	73	Male	Negative	ET	PVT	None	n.a.*	337

BCS, Budd-Chiari syndrome; ET, essential thrombocythemia; PMF, primary myelofibrosis; n.a., not available; OC, oral contraception; PVT, Portal venous system thrombosis; yr, years.

* No spleen height available but the patient was known to have an enlarged spleen.

Table 3. Characteristics associated with the *CALR* mutations in patients with SVT from the test cohort.

	With *CALR* mutations (n = 5)	$JAK2^{V617F}$ (n = 59)	Triple negative MPN (n = 9)	No MPN (n = 57)
Age, years	32 (27–37)	42 (31–49)	47 (30–55)	35 (26–47)
Males	2 (40%)	15 (25%)	4 (44%)	20 (35%)
Liver disease				
BCS	1 (20%)	28 (48%)	1 (11%)	25 (44%)
PVT	4 (80%)	31 (52%)	8 (89%)	32 (56%)
Inherited thrombophilia				
Protein C deficiency	0/5	3/43 (7%)	1/9 (11%)	2/50 (4%)
Protein S deficiency	0/5	6/43 (14%)	0/9	7/51 (14%)
Antithrombin deficiency	0/5	1/45 (2%)	0/9	0/57
Factor V gene mutation	0/5	7/58 (12%)	0/9	1/55 (2%)
Factor II gene mutation	0/5	5/59 (8%)	0/9	4/55 (7%)
Acquired thrombophilia				
MPNs	5 (100%)	59 (100%)	9 (100%)	0***
Antiphospholipid antibody syndrome	0/5	5/56 (9%)	1/9 (11%)	5/57 (10%)
Paroxysmal nocturnal haemoglobinuria	0/5	0/46	0/7	1/42 (2%)
Hormonal (OC and/or pregnancy)	1/5 (20%)	15/56(25%)	2/9 (22%)	20/56 35%)
Systemic disorder†	1/5 (20%)	0/59	0/9	2/57 (3%)
Local risk factor‡	1/5 (20%)	1/55 (2%)	2/9 (22%)	1/54 (2%)
Single risk factor	3/5 (60%)	30/59 (51%)	5/9 (56%)	23/57 40%)
Multiple risk factors	2/5 (20%)	29/59 (49%)	4/9 (44%)	9/57 (16%)
No risk factor	0/5	0/59	0/9	25/57 (44%)
Haemoglobin, g/dl	11.9 (10.0–13.0)	14.2 (12.7–15.5)*	13.2 (12.1–15.4)	12.7 (11.6–14.6)
Haematocrit, %	36.5 (31.3–40.4)	42.8 (38.8–48.0)*	40.0 (35.5–45.5)	38.5 (35.5–43.1)
WBC count, $\times 10^9$/L	7 (5.3–12.1)	10 (7.6–13.5)	7.2 (6.3–8.7)	6.8 (4.9–8.1)
ANC, $\times 10^9$/L	5.2 (3.4–10)	7.2 (5.3–10.8)	4.7 (3.9–5.3)	3.9(2.6–5.5)
Platelet count, $\times 10^9$/L	453 (427–477)	377 (286–500)	234 (225–431)*	227 (142–314)**
Spleen height, cm	18.0 (16.8–19.5)	15.0 (13.2–18.0)	14.2 (7–18)	11.0 (9.0–14.0)**
AST, U/L	71 (21–114)	44 (28–91)	37 (21–53)	28 (22–42)
ALT, U/L	73 (33–169)	56 (32–90)	51 (32–100)	35 (21–49)
Serum bilirubin, μmol/L	16 (11–31)	16 (10–33)	11 (8–24)	13 (7–20)
Serum creatinine, μmol/L	54 (51–86)	68 (58–77)	71 (68–84)	71 (64–84)
Serum albumin, g/L	34 (29–43)	38 (33–43)	41 (33–42)	38 (33–45)
Factor V, %	86 (53–111)	62 (52–82)	88 (43–117)	90 (71–106)

Values are n (%) or median (interquartile range). Triple negative correspond to patient without $JAK2^{V617F}$, *CALR* and *MPL* mutation and with confirmed MPN. All patients from the group confirmed MPN had a BM biopsy and/or a EEC.

**p* <0.05, ***p* <0.01, ****p* <0.001 *vs.* patients with *CALR* mutations (only differences with the *CALR* mutated group are indicated). Comparisons between groups of quantitative and qualitative variables were performed using Mann Whitney and the Fisher exact tests, respectively.

ALT, alanine transaminase; ANC, absolute neutrophil count; AST, aspartate transaminase; BCS, Budd-Chiari syndrome; BM, bone marrow; EEC, endogenous erythroid colonies formation; MPN, myeloproliferative neoplasm; OC, oral contraception; PVT, portal venous system thrombosis; *vs.*, versus; WBC, white blood cell.

† Behçet disease, sarcoidosis, vasculitis, connective tissue disease or lymphoide hemopathy.

‡ Intra-abdominal inflammation, infection, or abscess.

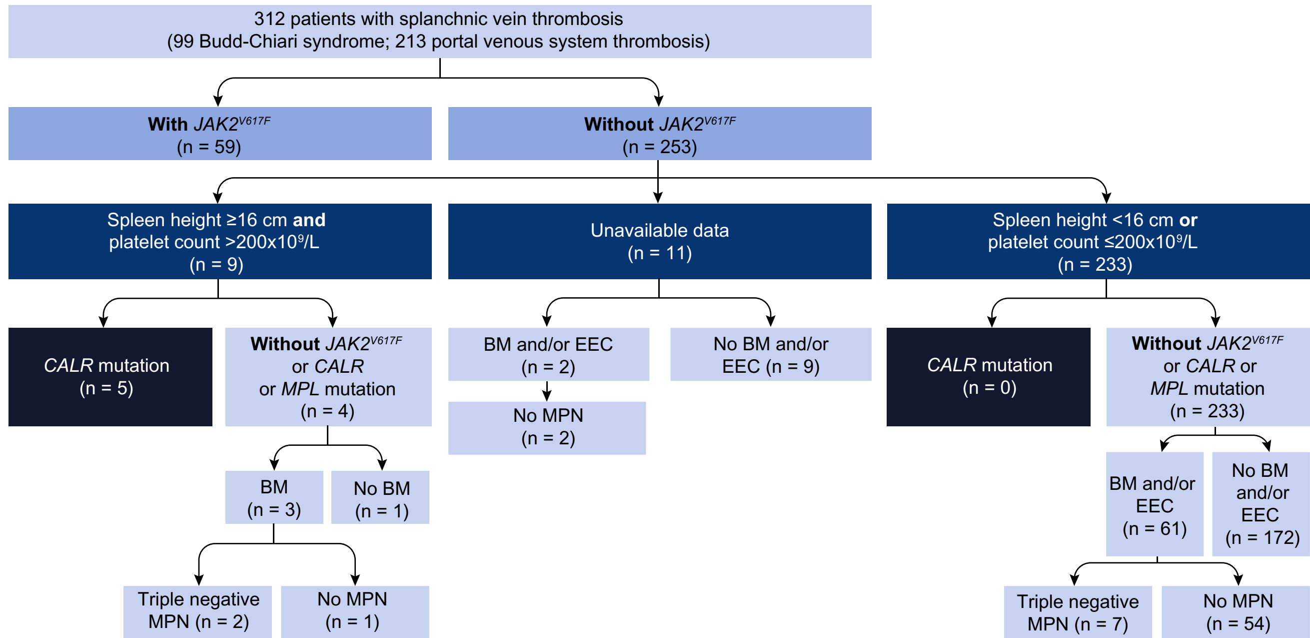

Fig. 1. Flow chart for the test cohort. Unavailable data correspond to patients with platelet count $>200\times10^9$/L but without spleen height available. Triple negative MPN patients are patients without *JAK2*^{V617F}, *CALR* and *MPL* mutation, but with a MPN proven by a BM biopsy and/or an EEC. BM, bone marrow; EEC, endogenous erythroid colonies formation; MPN, myeloproliferative neoplasm.

hypersplenism and haemodilution related to portal hypertension, we chose a lower threshold for platelet count (200×10^9/L platelets) than in patients without SVT (450×10^9/L).[18,21]

When combining the test and validation cohorts together, the criteria "spleen height $\geqslant$16 cm and platelet count $>200\times10^9$/L" had a negative predictive value of 99.7%. Out of the 344 patients without *JAK2*^{V617F}, these criteria would have avoided 329 unnecessary *CALR* mutations tests with only one false negative result. Combining the two cohorts, four out of the eight patients with spleen height $\geqslant$16 cm and a platelet count $>200\times10^9$/L and no *CALR* mutation had a triple negative MPN. These results suggest that a bone marrow biopsy should be performed in patients with both a spleen height $\geqslant$16 cm and a platelet count $>200\times10^9$/L when *CALR* mutations are absent. Six patients with SVT and *CALR* mutations have been reported so far in the literature, in addition to those included in the present study.[9,10,12,15] Spleen size and platelet count were mentioned for three of them: all had enlarged spleens and a platelet count $>200\times10^9$/L, which provide further evidence supporting the relevance of our findings.[9,12]

Due to the rarity of *CALR* mutations in patients with SVT, we were not able to determine whether these mutations are associated with a specific pattern of splanchnic vessels involvement or a particular outcome.

Our results have several implications. Firstly, identification of *CALR* mutations allows the diagnosis of an underlying MPN and, in some cases, can remove the need for bone marrow biopsy,

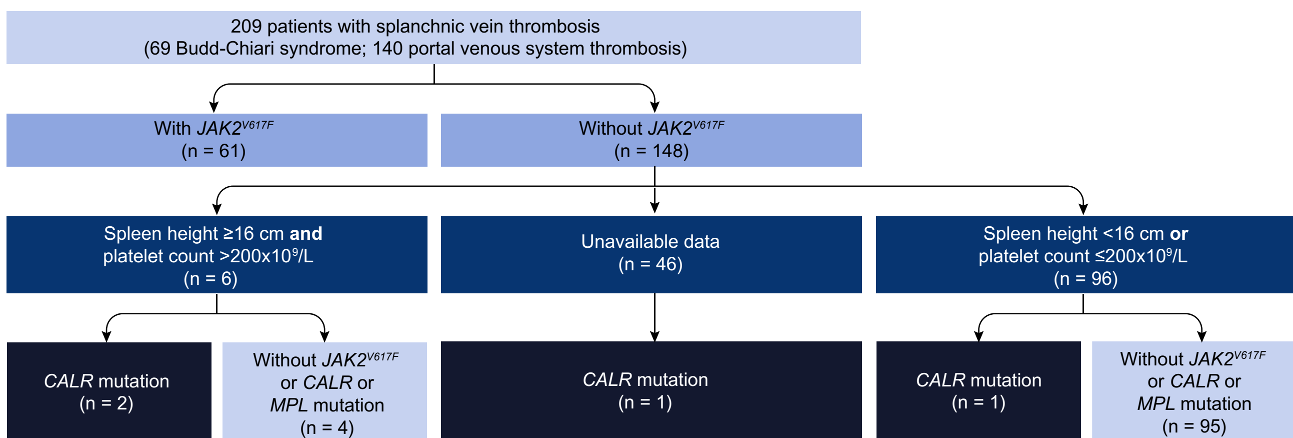

Fig. 2. Flow chart for the validation cohort. Unavailable data correspond to patients with platelet count $>200\times10^9$/L but without spleen height available. MPN, Myeloproliferative neoplasm.

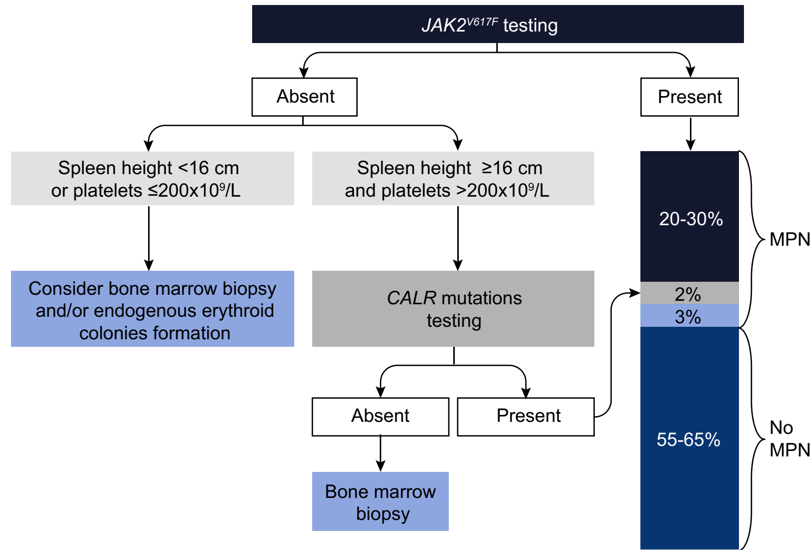

Fig. 3. Proposed algorithm for the identification of myeloproliferative neoplasms (MPNs) in patients with primary splanchnic vein thrombosis (SVT). The first step consists of *JAK2*^{V617F} testing. Patients without *JAK2*^{V617F} but with spleen height $\geqslant$16 cm and platelet count >200×10^9/L should be tested for *CALR* mutations; if *CALR* mutations are absent, a bone marrow biopsy should be performed. In the remaining patients, MPNs are extremely uncommon and a bone marrow biopsy and/or endogenous erythroid colonies formation can be considered on a case by case basis.

an invasive procedure. Secondly, the criteria we proposed here (spleen height was $\geqslant$16 cm and platelet count >200×10^9/L) are readily accessible and identify a patient population at high risk of having a MPN (with or without *CALR* mutations). These patients should undergo rapid haematological investigations, to consider cytoreductive therapy. Indeed, data from the French network on vascular liver diseases suggest that early introduction of cytoreductive therapy in patients with SVT and MPN reduces severe liver-related complications and improves event free survival.[22] Thirdly, by avoiding 96% of unnecessary *CALR* mutations testing, this strategy will have economic consequences. For instance, in France, based on an incidence of primary SVT of 22 per million inhabitants (around 2 per million for BCS and 2 per 100, 000 for PVT[23]), on a population of 67 million inhabitants and on a cost of *CALR* of 124 euros/test, this strategy would save approximately 200,000 euros per year.

In conclusion, this study provides the rationale for a new algorithm to diagnose MPNs in patients with SVT (Fig. 3). Given its high frequency in this setting, *JAK2*^{V617F} must be tested first. Thereafter, patients without *JAK2*^{V617F} but with spleen height $\geqslant$16 cm and platelet count >200×10^9/L should be tested for *CALR* mutations. A bone marrow biopsy should be proposed in patients without *CALR* mutations when the spleen is enlarged and platelets counts are normal or increased. In the remaining patients, namely those without *JAK2*^{V617F}, when spleen height is <16 cm and platelet count $\leqslant$200×10^9/L, MPNs are extremely uncommon and further studies are needed to identify those requiring a bone marrow biopsy.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Authors' contributions

J.P., and P-E.R. wrote the paper. C.M., and N.C. performed the mutational screening. A.P., O.G., C.B. and K.Z. collected the clinical data. F.T., F.C., D.C., and J.C.G.P., collected and analysed the data from the Spanish cohort. All authors discussed and critically revised the manuscript.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2017.04.021.

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