Somatic calreticulin mutations in patients with Budd-Chiari syndrome and portal vein thrombosis

We studied the role of the recently identified *CALR* mutations in 141 patients with Budd-Chiari Syndrome (BCS) or portal vein thrombosis (PVT) in a large multinational cohort. A *CALR* mutation was present in one of the 141 patients (0.7%). This patient was previously diagnosed with primary myelofibrosis. This results in *CALR* positivity in one out of 44 (2.3%) patients with myeloproliferative neoplasm (MPN), and in one of 11 (9.1%) *JAK2V617F* negative patients diagnosed with MPN. We suggest that analysis of *CALR* mutations should be performed in *JAK2V617F* negative BCS and PVT patients.

BCS and non-malignant, non-cirrhotic PVT are rare vascular liver diseases. The etiology of these diseases encompasses both inherited and acquired risk factors, of which MPN are the most common with a prevalence ranging between 20%-50%.[1-3] Detecting presence of MPN in patients with BCS and PVT is important, given the prognostic and potential therapeutic implications regarding anticoagulant therapy.[4,5] However, diagnosing MPN in patients with BCS and PVT is often difficult as portal hypertension caused by the obstruction of the hepatic veins and/or portal vein can explain splenomegaly, and decreases peripheral blood cell counts through hypersplenism.[6] The discovery of the *JAK2V617F* mutation has greatly improved the ability to non-invasively detect MPNs in patients with BCS and PVT.[7-9] However, in the absence of the *JAK2V617F* mutation, diagnosing MPN can still be challenging and a bone marrow biopsy remains required in most patients and may still be inconclusive.[4,10] Recently, exome sequencing resulted in the detection of mutations in *CALR* in patients with essential thrombocythemia (ET) or primary myelofibrosis (PMF) lacking *JAK2V617F* and *MPL* mutations. *CALR* mutations were present in 67%-88% of these patients.[11,12] Identification of *CALR* mutations in patients with BCS and PVT without the characteristic blood counts could improve the ability to diagnose ET and PMF. The aim of our study was to determine the prevalence and role of *CALR* mutations in patients with BCS and non-malignant, non-cirrhotic PVT.

In this case-control study, patients and controls were recruited from the European Network for Vascular Disease of the Liver (EN-Vie) study cohort. This study cohort has been described in detail elsewhere.[2,3] For this study, consecutive, incident cases of BCS and non-malignant, non-cirrhotic PVT were enrolled between 2003 and 2005 and prospectively followed in nine European countries. In addition, healthy, unrelated, population-based controls were included. These controls did not have a history of thrombosis and fulfilled the same age criteria as the included patients.[13] Blood samples were obtained at diagnosis. DNA was extracted from whole blood according to local standard methods. DNA samples were stored in the Erasmus MC University Medical Center in Rotterdam at -80°C until analysis. Presence of *CALR* mutations was determined with a PCR fragment analysis examining exon 9. Results of this PCR were analyzed using Genemapper 4.0, as previously described.[12] MPNs were diagnosed by performing a bone marrow biopsy (in 49% of patients), *JAK2V617F* mutation detection (in 98% of patients), EPO measurements (in 20% of patients), red cell mass measurement (in 11% of patients) and/or spontaneous erythroid colony formation (SECF) testing (in 18% of patients).

DNA samples were available from 77 patients with BCS, 75 patients with PVT and 76 controls. Determination of *CALR* mutation was successful in 92% of all samples, resulting in an inclusion of 70 BCS patients, 71 patients with PVT and 68 controls in the current analysis. Base-line characteristics and underlying etiological factors of this cohort are shown in Table 1. Median age of patients with BCS and PVT was 43.1 (31.0-53.4) years and 80 (57%) were female. One or more underlying prothrombotic factor(s) could be identified in 87% of all patients with BCS and PVT. MPN was present in 44 patients (31.2%) of whom 26 (37%) patients with BCS and 18 (26%) patients with PVT. The median age of patients with MPN was 44.6 (31.1-51.6) years compared to 43.1 (30.2-55.0) years in patients without MPN (P=0.95). There was also no difference in male/female distribution between the patients with and those without MPN (61% and 54% female, respectively; P=0.4). Additional prothrombotic risk factors were diagnosed in 32 (73%) of all patients with MPN. Thirty-three of the 44 patients with MPN had a *JAK2V617F* mutation (75%). Of the 11 remaining patients with MPN without *JAK2V617F*, 3 (27%) had polycythemia vera (PV) (diagnosis based on bone marrow biopsy findings and SECF), 2

Table 1. Base-line characteristics and etiological factors of the study cohort.

	BCS (n=70)	PVT (n=71)
Age, years	36.4 (26.4-50.7)	49.8 (41.6-57.2)
Female sex	41 (58.6)	39 (54.9)
ALT (U/L)	65 (32-169)	40 (24-61)
Platelets (*10^9/L)	224 (126-375)	263 (164-403)
White blood cell count (*10^9/L)	9.7 (6.7-13.6)	9.5 (7.6-12.8)
Hemoglobin (mmol/L)	8.6 (7.5-9.7)	8.2 (7.2-9.3)
Inherited thrombophilia*	15 (21.4)	16 (22.5)
Factor V Leiden mutation	10 (14.3)	4 (5.7)
Prothrombin gene G20210A	2 (2.9)	8 (11.4)
Protein C deficiency	1 (1.5)	1 (1.6)
Protein S deficiency	0	3 (4.9)
Antithrombin deficiency	2 (3.0)	2 (3.3)
Acquired thrombophilia*	54 (77.1)	52 (73.2)
Myeloproliferative neoplasms	26 (37.1)	18 (25.7)
Polycythemia vera	9 (34.6)	3 (16.7)
Essential thrombocytosis	5 (19.2)	7 (38.9)
Primary myelofibrosis	2 (7.7)	3 (16.7)
Unclassifiable	6 (23.1)	4 (22.2)
Occult	4 (15.4)	1 (5.6)
JAK2V617F present	19 (27.1)	14 (19.7)
Antiphospholipid antibody syndrome	21 (30.0)	19 (27.5)
Paroxysmal nocturnal hemoglobinuria	9 (12.9)	0
Hormonal risk factors†	16 (39.0)	18 (46.2)
Non-hematologic systemic disorder	8 (11.4)	2 (2.8)
History of thrombosis	14 (20.0)	16 (22.5)
Local risk factor¥	11 (15.7)	19 (26.8)
Single risk factor	24 (34.3)	23 (32.4)
Multiple risk factors	39 (55.7)	37 (52.1)
No risk factor	7 (10.0)	11 (15.5)

*Results are expressed as median (interquartile range) for continuous variables and as count (proportion) for categorical variables. *Patients could have more than one etiological factor simultaneously. †Presence of hormonal risk factor was missing in 29 patients with BCS and 32 patients with PVT. ¥Local risk factors were defined as presence of an abdominal trauma, abdominal intervention, and/or intra-abdominal infection, i.e. pancreatitis, liver abcess, cholangitis, cholecystitis, intra-abdominal abcess, diverticulitis, appendicitis, gastroenteritis, and/or spontaneous bacterial peritonitis with or without sepsis; BCS: Budd-Chiari syndrome; PVT: portal vein thrombosis; ALT: alanine aminotransferase.*

patients (18%) had ET, 2 (18%) had PMF, 2 (18%) were unclassifiable and 2 (18%) had occult MPN, at that time tested by SECF. The diagnosis of MPN was based on the above-mentioned criteria and reviewed by a hematologic expert (FWGL). A *CALR* mutation was present in only one MPN patient (2.3% of all patients with MPN). In the 97 BCS and PVT patients without MPN no *CALR* mutation was detected. PMF or ET was present in 17 patients with BCS and PVT in our cohort (39% of all patients with MPN). Of these 17 patients, 13 (77%) carried a *JAK2V617F*, one (6%) had a *CALR* mutation, and 3 (17%) did not have a known MPN-associated mutation.

The patient with a *CALR* mutation was a 46-year old female Dutch patient with a cardiomyopathy and PMF since 1981, resulting in severe splenomegaly requiring splenectomy in 2003. Pathological examination of the spleen showed extensive extramedullary hematopoiesis with signs of multiple non-recent splenic infarctions. Post operatively, she was diagnosed with PVT including thrombosis of the splenic vein and superior mesenteric vein. At that time, a bone marrow biopsy revealed end-stage PMF. She did not carry a *JAK2V617F* mutation. The *CALR* mutation detected in this patient was a type 1 mutation, the most common encountered *CALR* mutation, characterized by a 52-bp deletion in exon 9.[11,12] At the moment of diagnosis of PVT, platelet count in this patient was increased ($396x10^9$/L), white blood cell count was $6.3x10^9$/L, and hemoglobin was 5.8 mmol/L. During follow up, she presented with several episodes of gastrointestinal bleeding twelve months after diagnosis of PVT, caused by peptic ulcers under treatment of oral anticoagulants and non-steroidal anti-inflammatory drugs and angiodysplasia of the cecum. Fourteen months after diagnosis of PVT, this patient died of progressive multi-organ failure after presenting with gastrointestinal bleeding resulting in circulatory collapse.

In summary, in this large European cohort of 141 newly diagnosed patients with BCS and non-malignant, non-cirrhotic PVT, a somatic *CALR* mutation was present in only one patient with PVT and in none of the patients with BCS. This resulted in a prevalence of *CALR* mutations of 0.7% (1 of 141) in the total cohort, 2.3% (1 of 44) in all patients with MPN, and 9.1% (1 of 11) in all patients with MPN without *JAK2V617F*. Importantly, no *CALR* mutations were detected in patients without MPN or controls.

Recently, the association between MPN and somatic mutations in *CALR* was described for the first time.[11,12] *CALR* mutations were present only in PMF and ET lacking *JAK2V617F* and MPL mutations. In patients with PMF or ET, the prevalence of *CALR* mutations was reported to be 17%-24%.[11,12] In our cohort with only hepatic and/or portal vein thrombosis, the observed prevalence of *CALR* mutations in patients with PMF or ET was considerably lower (6%). This might be attributable to the fact that patients with a *CALR* mutation have a lower risk of thrombosis compared to patients with a *JAK2V617F* mutation. This decreased thrombosis risk may in turn result from the lower hemoglobin and white blood cell counts observed in patients with a *CALR* mutation compared to MPN patients without a *CALR* mutation.[11,12] This finding is in line with previous reports that state that *CALR*-mutant MPN patients do not develop thrombosis as often as patients carrying *JAK2V617F*, resulting in the lower prevalence of *CALR* mutation frequency in our cohort of patients with BCS and PVT as compared to this prevalence in cohorts of patients with MPN.[14,15] No *CALR* mutations were observed in the control group in our study, as was expected based on the results of the study by Nangalia *et al.*[11] Interestingly, *CALR* mutations were also absent in patients with lymphoid cancers or solid tumors in that study, suggesting that *CALR* mutations might be used as a marker to detect presence of MPN in BCS and PVT. Two other studies recently investigated presence of *CALR* mutations in patients with BCS and PVT, with comparable results. Turon *et al.* found a prevalence of 1.9% *CALR* mutations in a Spanish cohort of patients with BCS and PVT and no *CALR* mutation was present in a cohort of 144 patients with abdominal vein thrombosis, also including renal vein, splenic vein and mesenteric vein thrombosis.[16,17] In the current study, we included 141 consecutive patients with BCS and PVT who were extensively screened for etiological factors and prospectively followed in nine European countries. None of the patients included in the current study was previously described in the other studies assessing presence of *CALR* mutations in BCS and PVT.

In conclusion, *CALR* mutations are rare in patients with BCS and PVT with a prevalence of 0.7% in the total cohort and 2.3% in patients with MPN. This low prevalence is probably due to the relatively lower risk of thrombosis in *CALR*-mutant patients compared to patients with a *JAK2V617F* mutation. Despite this low prevalence, we believe testing for *CALR* mutations should be considered in patients with BCS and PVT who are *JAK2V617F* negative, as diagnosing MPN is often difficult in these patients due to masked blood cell counts because of portal hypertension, occult gastrointestinal bleeds and/or hypersplenism. Screening for somatic *CALR* mutations is an easy to perform diagnostic method that could aid in diagnosing MPN with limited burden for the patient.

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References

1. Smalberg JH, Arends LR, Valla DC, Kiladjian JJ, Janssen HL, Leebeek FW. Myeloproliferative neoplasms in Budd-Chiari syndrome and portal vein thrombosis: a meta-analysis. Blood. 2012;120(25):4921-4928.
2. Plessier A, Darwish-Murad S, Hernandez-Guerra M, et al. Acute portal vein thrombosis unrelated to cirrhosis: a prospective multicenter follow-up study. Hepatology. 2010;51(1):210-218.

3. Darwish Murad S, Plessier A, Hernandez-Guerra M, et al. Etiology, management, and outcome of the Budd-Chiari syndrome. Ann Intern Med. 2009;151(3):167-175.
4. Kiladjian JJ, Cervantes F, Leebeek FW, et al. The impact of JAK2 and MPL mutations on diagnosis and prognosis of splanchnic vein thrombosis: a report on 241 cases. Blood. 2008;111(10):4922-4929.
5. Spaander MC, Hoekstra J, Hansen BE, Van Buuren HR, Leebeek FW, Janssen HL. Anticoagulant therapy in patients with non-cirrhotic portal vein thrombosis: effect on new thrombotic events and gastrointestinal bleeding. J Thromb Haemost. 2013;11(3):452-459.
6. Valla DC. Primary Budd-Chiari syndrome. J Hepatol. 2009;50(1):195-203.
7. Smalberg JH, Darwish Murad S, Braakman E, Valk PJ, Janssen HL, Leebeek FW. Myeloproliferative disease in the pathogenesis and survival of Budd-Chiari syndrome. Haematologica. 2006;91(12):1712-1713.
8. Patel RK, Lea NC, Heneghan MA, et al. Prevalence of the activating JAK2 tyrosine kinase mutation V617F in the Budd-Chiari syndrome. Gastroenterology. 2006;130(7):2031-2038.
9. Baxter EJ, Scott LM, Campbell PJ, et al. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. Lancet. 2005;365(9464):1054-1061.
10. Vardiman JW, Thiele J, Arber DA, et al. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood. 2009;114(5):937-951.
11. Nangalia J, Massie CE, Baxter EJ, et al. Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. N Engl J Med. 2013;369(25):2391-2405.
12. Klampfl T, Gisslinger H, Harutyunyan AS, et al. Somatic mutations of calreticulin in myeloproliferative neoplasms. N Engl J Med. 2013;369(25):2379-2390.
13. Hoekstra J, Guimaraes AH, Leebeek FW, et al. Impaired fibrinolysis as a risk factor for Budd-Chiari syndrome. Blood. 2010;115(2):388-395.
14. Rotunno G, Mannarelli C, Guglielmelli P, et al. Impact of calreticulin mutations on clinical and hematological phenotype and outcome in essential thrombocythemia. Blood. 2014;123(10):1552-1555.
15. Rumi E, Pietra D, Ferretti V, et al. JAK2 or CALR mutation status defines subtypes of essential thrombocythemia with substantially different clinical course and outcomes. Blood. 2014;123(10):1544-1551.
16. Haslam K, Langabeer SE. Incidence of CALR mutations in patients with splanchnic vein thrombosis. Br J Haematol. 2015;168(3):459-460
17. Turon F, Cervantes F, Colomer D, Baiges A, Hernandez-Gea V, Garcia-Pagan JC. Role of calreticulin mutations in the etiological diagnosis of splanchnic vein thrombosis. J Hepatol. 2015;62(1):72-74.