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Advances in natural history and diagnostic techniques in rare vascular liver diseases

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ADVANCES IN NATURAL HISTORY AND DIAGNOSTIC TECHNIQUES IN RARE VASCULAR LIVER DISEASES

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La tesis **"Advances in natural history and diagnostic techniques in rare vascular liver diseases**" presentada por Marta Magaz Martínez ha estado realizada bajo su dirección y reúnen las condiciones necesarias para su lectura y defensa pública para optar al título de Doctor por la Universidad de Barcelona en el marco del Programa de Doctorado en Medicina e Investigación Traslacional.

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Abbreviations and acronyms

ERN, European Reference Networks

VLD, vascular liver disorders

SVT, non-cirrhotic splanchnic vein thrombosis

BCS, Budd Chiari syndrome

PVT, non-cirrhotic non-tumoral portal vein thrombosis

PSVD, porto-sinusoidal vascular liver disorder

IPH, idiopathic non-cirrhotic portal hypertension

PH, portal hypertension

SMV, superior mesenteric vein

SV, splenic vein

HE, hepatic encephalopathy

TIPS, transjugular intrahepatic portosystemic shunt

MPNs, Myeloproliferative neoplasms

JAK2, Janus kinase 2

CALR, Calreticulin

MPL, Thrombopoietin Gene

NGS, Next generation sequencing

VAF, Variant allele frequency

HMR, High molecular risk

VUS, Variant of unknown significance

Auto-Ab, serum auto-antibodies

ANA, Antinuclear antibodies

anti-SMA, anti-smooth muscle antibodies

HLA-DR, Human Leukocyte Antigen – DR molecule

EC, endothelial cells

AECA, anti-endothelial cell antibodies

SEC, sinusoidal endothelial cells

TESIS EN FORMATO DE COMPENDIO DE ARTÍCULOS

La presente tesis consta de tres objetivos y tres artículos artículos:

1) **Magaz M**, Alvarez-Larrán A, Colomer D, López-Guerra M, García-Criado MÁ, Mezzano G, Belmonte E, Olivas P, Soy G, Cervantes F, Darnell A, Ferrusquía-Acosta J, Baiges A, Turon F, Hernández-Gea V, García-Pagán JC. Next-generation sequencing in the diagnosis of non-cirrhotic splanchnic vein thrombosis. J Hepatol. 2021 Jan;74(1):89-95. doi: 10.1016/j.jhep.2020.06.045. Epub 2020 Jul 15. PMID: 32679300. **IF: 20.582** (Hepatología). D1.
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Resumen en castellano

Las enfermedades conocidas como minoritarias o raras son aquellas que afectan a una proporción muy baja de la población, en concreto a 5 personas de cada 10.000 habitantes. Aunque el número de personas afectadas por estas enfermedades es bajo, globalmente afectan a una proporción no despreciable de la población. Además, estos pacientes se encuentran en una situación de desventaja ya que, habitualmente, en sus centros de referencia es poco probable que se dispongan de los medios y conocimientos óptimos para diagnosticar y tratar enfermedades tan específicas y poco frecuentes como éstas. Ello ligado a la dificultad añadida que representa progresar en las técnicas diagnósticas y terapéuticas de estas enfermedades debido a que en el número de pacientes que pueden concentrar los distintos centros hospitalarios a nivel individual siempre será muy bajo. En este contexto, establecer centros de referencia para cada enfermedad minoritaria en los que se acumule su manejo es esencial para progresar en estas áreas. La colaboración entre los distintos centros hospitalarios para la realización de estudios multicéntricos que permitan reunir un número relevante de pacientes con características similares es fundamental para poder lograr una mejora en el conocimiento en la historia natural, las técnicas diagnósticas y diferentes estrategias terapéuticas de estas enfermedades minoritarias.

Esta tesis se centra por tanto en las enfermedades vasculares hepáticas y, particularmente, en las trombosis esplácnicas y la enfermedad vascular porto-sinusoidal:

Las neoplasias mieloproliferativas (MPN) son la causa más frecuente de las trombosis esplácnicas no cirróticas y no tumorales (SVT). El diagnóstico de las MPN se basa en las alteraciones del recuento celular sanguíneo, la histología de las biopsias de médula ósea y en la detección de mutaciones genéticas específicas (habitualmente las mutaciones clásicas JAK2 (gen de la quinasa 2 de Janus), tanto la más común *JAK2V617F* o *JAK2* en exón 12, el gen de la calreticulina (*CALR*) y las mutaciones del gen de la trombopoyetina (*MPL*).

La secuenciación mediante NGS, de sus siglas en inglés “Next generation sequencing”, permite la evaluación simultánea de múltiples genes implicados en la patología clonal mieloide. El objetivo de nuestro primer estudio consistió en evaluar el papel potencial de NGS en elucidar la etiología en pacientes con trombosis venosas esplácnicas no cirróticas.

Se incluyeron un total de muestras de ADN de 80 pacientes (75 con trombosis esplácnicas no cirróticas idiopáticas o con un factor exclusivamente local [Idiop / loc-SVT] y 5 pacientes con una MPN ya conocidas y SVT (SVT-MPN) pero que habían resultado negativos para las tres

mutaciones clásicas (tanto para *JAK2* [V617F y exón 12], (*CALR*) y (MPL) mediante las técnicas convencionales.

Las mutaciones implicadas en trastornos mieloides diferentes de los genes *JAK2, CALR y MPL* se clasificaron como variantes de alto riesgo molecular (HMR) o variantes de significado incierto.

En 2 de los 5 pacientes triple negativos (40%), que tenían una trombosis esplácnica asociada a una neoplasia mieloproliferativa (que había sido diagnosticada mediante criterios clínicos e histológicos mediante biopsia de médula ósea) se detectó una mutación en el *exón 12 de JAK2*. La mutación JAK2-exón 12 fue también identificada en un paciente entre los 75 pacientes con Idiop /loc-SVT.

Es más, 28/74 (37,8%) de los restantes pacientes con Idiop / loc-SVT tenían al menos 1 Variante HMR. Sesenta y dos de los pacientes con Idiop / loc-SVT no recibían anticoagulación a largo plazo y 5 de ellos (8,1%) habían presentado retrombosis esplácnica. Esta incidencia acumulada fue significativamente mayor en pacientes en los que se detectaron variantes HMR que en aquellos que no las tenían.

Por tanto, NGS fue capaz de identificar mutaciones de JAK2-exón12 que no se habían detectado previamente mediante técnicas convencionales. Además, NGS detectó variantes de HMR en aproximadamente un tercio de los pacientes con Idiop / loc-SVT. Asimismo, estudios recientes han demostrado que la presencia de HMR puede tener capacidad pronóstica. En nuestra corte se observó que aquellos pacientes con HMR parecen tener un mayor riesgo de retrombosis esplácnica. Por todo ello, en un futuro NGS podría convertirse en una herramienta diagnóstica útil en las trombosis esplácnicas no cirróticas.

Por otra parte, los pacientes con neoplasias mieloproliferativas, concretamente, los pacientes con policitemia vera (PV) o trombocitemia esencial (ET) que presentan una trombosis esplácnica en el debut podría tener un perfil clínico-biológico diferente de aquellos pacientes que debutan sin trombosis esplácnica. Para investigar esta hipótesis, se revisaron 3705 pacientes con PV/ET procedentes de tres registros nacionales, de los que solamente 118 presentaban una trombosis esplácnica. Después de la corrección por edad y sexo, los pacientes con PV/ET con trombosis esplácnica al debut mostraron un aumento del riesgo de muerte (HR 2,47; IC del 95%: 1,5 a 4,01, p <0,001), trombosis venosa (TIR 3,4; IC del 95%: 2,1 a 5,5, p <0,001), mayor riesgo de hemorragia (TIR 3,6; IC del 95%: 2,3–5,5, p <0,001) y de una segunda neoplasia (TIR 2,37; IC del 95%: 1,4–4,1, p=0,002), respecto a aquellos pacientes sin trombosis esplácnica al debut.

No se documentó ningún caso de leucemia en estos pacientes con PV / ET que presentaban trombosis esplácnica y siete de ellos (6%) progresaron a mielofibrosis. La trombosis esplácnica no se asoció con un menor riesgo de mielofibrosis después de la corrección por edad y sexo. Los pacientes con trombosis esplácnica murieron con mayor frecuencia por complicaciones relacionadas con enfermedad hepática, hemorragia mayor o un segundo cáncer, lo que resulta en una reducción de 5 años de la mediana de supervivencia ajustada por edad y sexo.

Se podría extraer como conclusión que aquellos pacientes con PV y ET que presentan una trombosis esplácnica al debut presentan una supervivencia más corta que los pacientes con PV y ET de la misma edad y sexo sin trombosis asociada. Este exceso de mortalidad parece estar relacionado con la enfermedad hepática, riesgo de hemorragias grave y la mayor aparición de una segunda neoplasia, más que con la evolución natural de la MPN.

Por otra parte, la enfermedad vascular porto-sinusoidal (PSVD) es una enfermedad rara que requiere excluir la cirrosis y otras causas de hipertensión portal para poder realizar su diagnóstico, ya que carece de una prueba diagnóstica específica. Y aunque si bien ocasionalmente se ha asociado con enfermedades autoinmunes, se desconoce la fisiopatología real de la PSVD.

El objetivo del tercero de nuestros estudios consistió en evaluar el potencial papel de la autoinmunidad en la fisiopatología y el diagnóstico de la PSVD. Se incluyeron un total de treinta y siete pacientes consecutivos con PSVD y 39 con cirrosis emparejados por sexo, signos de hipertensión portal y función hepática (training set). Mediante el uso de inmunofluorescencia indirecta, ELISA y slot-blot, se identificaron 22 anticuerpos en pacientes con PSVD y cirrosis. La presencia de anticuerpos anti-células endoteliales (AECA) fue analizada por un *cell-based* ELISA. Se incluyeron 31 pacientes con PSVD, 40 pacientes con cirrosis, 15 pacientes con esplenomegalia asociada con enfermedad hematológica y 14 donantes sanos se incluyeron en la cohorte de validación.

La proporción de pacientes con al menos un anticuerpo positivo fue significativamente mayor en pacientes con PSVD en comparación con los pacientes con cirrosis (92% vs 56%; P <0,01). Los AECA fueron significativamente más frecuentes en la PSVD que en la cirrosis (38% frente a 15%; p = 0,013). Los resultados se confirmaron en el set de validación. La presencia de AECA tuvo un valor predictivo positivo del 63% para el diagnóstico PSVD y un valor predictivo negativo del 71%, con una especificidad del 94% cuando los títulos de 1/16 fueron utilizados como valor de corte. Nuestros hallazgos sugieren que la determinación de AECA puede ser una herramienta adicional para facilitar el diagnóstico de PSVD. Además, nuestro estudio proporciona evidencia

que apoyaría la contribución de la autoinmunidad humoral en un subgrupo de pacientes con PSVD.

I. Introduction

I.1 Rare liver diseases

Rare diseases are diseases that affect less than 1 in 2000 inhabitants, declared as less than 5/10000 inhabitants. It is estimated that there are about 6000-8000 different rare diseases that globally affect around 10% of population. The estimated number of people affected by such conditions is 30 million in Europe and around 3 million in Spain (1), then although the number of affected people by each disease may be low, they globally involve a non-despicable proportion of the population. It is remarkable that initial misdiagnosis and/or delay in diagnosis frequently difficult the management of these diseases. Some rare diseases are due to genetic disorders and they usually manifest early in life and are commonly seen in paediatric and young population(2), others have a multifactorial origin, however in most of the rare diseases the etiological mechanisms are still unknown.

Additionally, rare diseases face a disadvantage due to the lack of experience in qualified centres and limited understanding of the disease. Given the low frequency and diversity of these entities, it is very difficult to study them and gather enough knowledge to make a significant advance in their management and treatment, which can cause difficulties in the collection of clinical data that limit the discovery of new diagnostic tools as their natural history.

Other challenge of rare diseases is to avoid selection and reporting bias, the research needs to be multicentric and international to guarantee enough information and to generate quality data. The effort in recent years to improve and create advances in rare diseases has led to the creation of rare disease networks such as the European Reference Networks (ERN) that are virtual networks involving healthcare providers across Europe. Their aim is to tackle complex or rare diseases and conditions that require highly specialised treatment and a concentration of knowledge and resources. ERN focus on the need to establish reference centres for the diagnosis and management of rare diseases and establish multicentric databases that could accumulate experience in these conditions. In fact, there is one ERN dedicated of rare liver diseases centralized in the management of rare liver disease both in children and in adults. The Hospital Clínic is one ERN in rare liver diseases in Europe, fully dedicated to the management of patients with vascular liver disorders (VLD), that is helping to advance in their knowledge. In particular, in the context of hepatic diseases, RARE-Liver ERN has divided rare liver diseases into three main groups: metabolic diseases, autoimmune diseases, and vascular liver diseases. Vascular liver

diseases involve several disorders of the hepatic vasculature among which are congenital vascular malformations, sinusoidal obstruction syndrome,etc…(3).

The most common VLD are splanchnic vein thrombosis (SVT), that include Budd Chiari syndrome (BCS) and non-cirrhotic non-tumoral portal vein thrombosis (PVT) and porto-sinusoidal vascular liver disorder (PSVD), previously named as idiopathic non-cirrhotic portal hypertension (IPH)(3).

It is worth highlighting, that PVT in the setting of cirrhosis is not considered a rare disease and it is not in the scope of this thesis, since it has a different underlying pathophysiology.

Many of these VLDs could cause non-cirrhotic portal hypertension (PH). Particularly, BCS and PVT are mainly caused by prothrombotic conditions and could develop PH related complications such as variceal bleeding or ascites among others. On the other hand, PSVD includes a variety of histological alterations (obliterative portal venopathy, nodular regenerative hyperplasia or incomplete septal fibrosis) that either could develop or not PH (4,5). Prognosis mainly depends on PH development, being indeed the appearance of gastroesophageal varices and ascites a milestone in their natural history (6,7). This fact is relevant because usually they are young patients with an otherwise normal life expectancy that could be markedly reduced if not adequately treated. Hence the importance of expanding knowledge in their pathophysiology, natural history, diagnostic tools and potential treatments (3).

BCS and PVT are mainly caused by prothrombotic conditions and could develop portal hypertension related complications such as variceal bleeding or ascites among others. On the other hand, PSVD includes a variety of histological alterations (obliterative portal venopathy, nodular regenerative hyperplasia or incomplete septal fibrosis) that could develop or not portal hypertension (4,5).

This thesis will focus on two VLD: non-tumoral noncirrhotic SVT and porto-sinusoidal vascular liver disorder.

I.2 Splanchnic vein thrombosis in patients without underlying liver disease

Non cirrhotic splanchnic vein thrombosis (SVT) comprises as previously mentioned: PVT, BCS, superior mesenteric vein (SMV) and splenic vein (SV) thrombosis. Clinical consequences of SVT can be different depending on the affected territory, i.e. gastric varices may be developed more frequently after SV thrombosis or mesenteric ischemia after SMV (8). Nevertheless, the ultimate

consequence of splanchnic thromboses, if venous recanalization is not achieved, is the development of PH, which is common to all localizations.

I.2.1 Non-cirrhotic non-tumoral portal vein thrombosis

PVT is defined by the presence of a thrombus within the main portal vein trunk and/or in the intrahepatic portal branches. Sometimes, PVT can extend towards the SMV or the SV. When PVT is acute, it can manifest as abdominal pain and a systemic inflammatory response, although it is not uncommon that it causes only mild non-specific symptoms, making it difficult to recognize clinically. Indeed, in many patients PVT is initially asymptomatic and patients are diagnosed when they have already developed chronic PVT/portal cavernoma with PH manifestations. In fact, if the acute PVT is not recanalized, it may develop chronic PVT.

Regarding the etiology, in more than 40-50% of cases there is an underlying systemic acquired or hereditary prothrombotic disorder, in 20-30% there is a local infectious/inflammatory process while in the remaining 20-30% of cases, despite an exhaustive study, no underlying cause is found and they are considered as idiopathic.

It should be noted that the early identification and treatment of the underlying causes of PVT may have great influence in the outcome, hence, etiological work-up is a key pillar of the initial management of PVT. As a matter of example, diagnosis and adequate treatment of MPN have been shown to improve prognosis of patients with SVT. Additionally, early identification of an acute PVT, allowing the rapid initiation of anticoagulation, influences the probability of recanalization and therefore is usually associated with a better outcome. The duration of anticoagulation is not well established, although it seems that the probability of recanalization markedly decreases after 6 months of anticoagulation and is almost null after 12 months(9). Therefore, the current recommendation is, with the aim of achieving recanalization, to maintain anticoagulant therapy for at least six but preferentially 12 months. However, currently indefinite anticoagulation to prevent splanchnic rethrombosis or extrahepatic thrombosis is prescribed in those patients with a permanent underlying prothrombotic state, previous thrombotic events in other territories or recurrence of thrombosis when anticoagulation is stopped(10). Other situation in which it is currently recommended the use of indefinite anticoagulation is acute thrombosis when associated with severe mesenteric ischemia. However, recent data suggest that there are other subgroups of patients, different from the ones previously described that may benefit from long-term anticoagulation(11). Indeed, the risk of rethrombosis, although lower, is also present in patients without a recognized prothrombotic disorder and may justify

long-term anticoagulation could also be considered in patients without underlying prothrombotic state. However, we still do not know the real risk/benefit of prescribing anticoagulation these patients and further studies identifying predicting factors for rethrombosis are needed (12).

Once chronic PVT has developed, around 70.8% already have gastroesophageal varices at diagnosis (13). Of those without varices at diagnosis, this study showed that the probability of developing oesophageal and gastric varices requiring primary prophylaxis is 13%, 40% and 50% at 1, 3 and 5 years, respectively (13) and, as in cirrhosis, the risk of variceal bleeding is higher in large varices with red signs (14,15). Although other PH complications, such as ascites, hepatic encephalopathy (HE) and bacterial infections can occur, they are less frequent in chronic PVT than in cirrhosis(16).

I.2.2 Budd Chiari Syndrome

The thrombosis of the hepatic veins is known as Budd Chiari Syndrome (BCS), the obstruction can be located from the small hepatic venules to the entrance of the inferior vena cava and even in the right atrium (16). BCS can present clinically from asymptomatic to fulminant liver failure in 5% of cases due to sinusoidal congestion and secondary ischemia(17), being the most frequent clinical manifestation ascites (83% of patients) followed by hepatomegaly (67%), abdominal pain (60%), development of oesophageal varices (58%) and/or acute variceal bleeding (5%)(18). The heterogeneous range of manifestations probably correlates with the extent and the speed of establishment of thrombosis (16). Partial and/or slow progression of thrombosis could be compensated by the development of collaterals that would decompress the portal venous system, while in cases where the onset of thrombosis is rapid, acute liver failure or even death could occur (19).

The BCS therapeutic algorithm is based on a stepwise strategy(20), from the least invasive approach, including the treatment of a potential underlying disease together with long-term anticoagulation, to the most invasive (21), including transjugular intrahepatic portosystemic shunt (TIPS) or liver transplant if TIPS fails or in some cases of fulminant BCS. (21,22). Due to the potential severity of BCS and of a possible rethrombosis, patients with BCS should receive long-term anticoagulation although no thrombophilia risk factor could be identified (23). In addition, it is very important to initiate treatments to prevent PH–related complications and closely monitorization.

I.3. Porto-sinusoidal vascular liver disorder

Porto-sinusoidal vascular liver disorder (PSVD) (traditionally known as idiopathic portal hypertension) (24) is a rare entity, characterised by the absence of cirrhosis and the presence of histological alterations suggestive of this disease, with or without PH, and possibly within a clinical scenario of haematological, prothrombotic or autoimmune disease(24). Despite its rarity, recent advances in their study have conditioned a greater recognition of this disorder. Although the pathophysiology of PSVD remains largely unknown, this entity is frequently associated with underlying immunological, haematological, prothrombotic, toxic or genetic disorders (6,7). Laboratory test of patients with PSVD usually show a preserved liver function and a decreased in red blood cells, leukocytes and platelets counts secondary to the presence of splenomegaly, which makes PSVD´s diagnosis more difficult. Furthermore, PSVD does not have a specific diagnostic test and its diagnosis requires the exclusion of cirrhosis and of other causes of PH such as SVT among others. It is therefore an unmet need to find easy-to-use diagnostic tools such as blood markers to facilitate its diagnosis.

I.4 Vascular liver disorders diagnostic workout

The main big challenges in the VLDs field are the understanding of their pathophysiology and diagnosis of patients at early stages of the disease.

As previously mentioned, it is of special relevance to determine the underlying prothrombotic condition in patients with splanchnic vein thrombosis. Regardless of the availability of better recent diagnostic tools, in about 30% of cases it is not possible to identify the thrombosis etiology and these patients are still classified as idiopathic. However, the fact that we are not able to identify a prothrombotic condition does not necessarily mean that it does not exist, but that our current diagnostic tools could not be able to recognize them.

Regarding systemic conditions, myeloproliferative neoplasms (MPNs) are the most common etiological cause of non-cirrhotic PVT and BCS, being detected in 25–35% and 40–50% of patients, respectively (25,26). MPNs are chronic clonal hematopoietic stem cell disorders characterized by an overproduction of erythrocytes, granulocytes, and/or platelets. MPNs are the most common aetiological cause of non-cirrhotic PVT and BCS, being detected in 25–35% and 40–50% of patients, respectively (26). The diagnosis of MPNs was greatly facilitated by the discovery of the *Janus kinase 2 (JAK2) V617F* mutation (27,28), that is detected in in 90-95% of Polycythaemia Vera patients (29) and 60% of Essential Thrombocythemia patients and found in

45% of those with BCS and in 34% of those with PVT. This is of special relevance in patients with SVT, in whom the presence of PH by causing hypersplenism and volume expansion can mask the classical haematological findings of the MPN, challenging its diagnosis.

Subsequently, in 2014 a study by *Turon et al.* including 209 patients with SVT and a complete etiological diagnostic work-out in whom Calreticulin (*CALR*) mutations were screened, showed that 4 of the 209 patients (1.9%), previously considered to have idiopathic SVT, had CALR mutations identifying therefore a small additional number of patients with an underlying MPN.

As a result, in patients with SVT it is recommended to first evaluate possible mutations in the *JAK2 gen* and, if negative, *CALR* mutations should also be investigated.

Despite these advances, there are still patients with underlying MPN who remain undiagnosed and therefore not adequately treated with the underlying increased high risk of arterial and venous thrombotic events (30) among other complications. The knowledge that there are still patients with MPN that remain undiagnosed prompt physicians to request a high number of bone marrow biopsies to diagnose/discard an unrecognized MPN. This invasive procedure could be avoided by increasing our capacity to molecularly discard the presence of a MPN.

The most frequent local factors in patients with PVT (much less rare in patients with BCS) are intra-abdominal infectious processes or intra-abdominal surgeries. Abdominal inflammation or infection constitutes approximately 30% of cases, as activation of coagulation is a relevant response in the host's defense against infections as it prevents the spreading of microorganisms (16). In paediatric age, umbilical catheterization is the most frequent local factor(31).

SVT is usually the result of multiple risk factors, interestingly the concurrence of two or more prothrombotic factors is found in up to 10% of patients with PVT and 45% in patients with BCS(32). Likewise, an underlying prothrombotic factor can be found in up to 35% of cases that also present a local factor. Therefore, the finding of a single prothrombotic risk factor does not exclude the presence of other factors, whether local or systemic, and all patients should undergo an exhaustive etiological study.

On the other hand, PVSD does not have a specific diagnostic test, the lack of a positive test, or specific biomarkers makes that its diagnosis challenging. In early stages of the disease, still without or with mild manifestations of PH, the clinical scenario may be even difficult to differentiate from a patient with a healthy liver. That is why it is important to increase the awareness of PSVD, in order to avoid the misdiagnosis or a markedly delayed diagnosis.

This promotes frequent inadequate treatments, excess of complementary (some invasive) tests and/or inadequate use of screening tools. Also, it entails an increased health cost and reduction in patient´s quality of life. The improvement in diagnostic tools for PSVD would allow reducing unnecessary complementary explorations (reducing costs and discomfort for patients) and shortening the time required for the diagnosis and reducing misdiagnosis.

II. Justification, Hypothesis and Objectives

II.1 Justification and Hypothesis

The low prevalence of rare vascular liver disorders makes difficult the advance in their knowledge and there are several unmet areas (natural history, etiology, diagnostic strategies and therapeutic approaches) that deserve further research. Performing multicentre collaborative studies is crucial for a better understanding of these entities and to be able to collect a greater amount of data.

Study 1:

Previous studies have evaluated different risk factors that could be involved in the development of the SVT, however some patients are still considered as idiopathic and might have a misdiagnosed MPN (which is the most common cause) not detected with the current standard molecular techniques.

In this setting, if the MPN that requires long-life anticoagulation and cytoreductive treatment, is not detected the patients could have a misdiagnosed MPN and otherwise the risk of rethrombosis in the splanchnic territory and also in other vascular territories would be high. Therefore, it is important to accurately diagnose this type of patients because it allows a correct risk stratification and a proper management approach, and in this way to be able to make the decision with greater security of prescribing chronic treatment and monitoring by expert haematology service.

The hypothesis of our first study was to consider whether next generation sequencing (NGS) could be capable of detecting MPN not detected by conventional methods.

Study 2:

In the field of the MPN with splanchnic vein thromboses, the knowledge on the natural history and outcome of these patients has not been well studied. SVT is the initial presentation in only

a minority of MPN (27,33), then the rarity of SVT as a form of presentation of the MPNs and the prolonged clinical course of these patients has made it difficult to establish the natural history of the disease in such cases.

Therefore, since knowledge on the natural history and outcome of patients with both diseases is scarce we hypothesized that collecting a large amount of patients from different reference centres with SVT and MPN would enable a better understanding of the natural history of these conditions.

Study 3:

In the PSVD field, there is a need to better understand its pathophysiology and discover diagnostic tests to investigate the diagnostic implements that facilitate their diagnosis in clinical practice.

Its challenge detection may delay the diagnosis and despite the pathophysiology remains unknown immunological disorders have been suggested as etiological factors (34). Additionally, PSVD has been frequently associated with celiac disease, autoimmune disorders such as systemic lupus erythematous, systemic sclerosis, chronic thyroiditis, Raynaud´s phenomenon and scleroderma among other conditions, supporting the immunological involvement in the pathogenesis of the disease (35,36).

Also, several studies have shown the presence of different serum auto-antibodies (auto-Ab), such as antinuclear (ANA), or anti-smooth muscle antibodies (anti-SMA), in patients with PSVD. In addition, increased expression of Human Leukocyte Antigen – DR molecule (HLA-DR) in the endothelial cells (EC) of the smaller portal venous radicles, as well as a higher number of T helper-1 cells in peripheral and spleen lymphocytes has been found (37). Similarly, a pathophysiological role for anti-endothelial cell antibodies (AECA) has been suggested (38–40).

We hypothesize that they could promote endothelial-mesenchymal transition in the EC of the small portal veins promoting portal vein stenosis and collagen deposition in peripheral portal tracts(39). These findings could suggest a potential pathophysiological role of immunological alterations in the pathophysiology of PSVD.

II.2 Aims

Improving the current knowledge in rare liver disorders with the aim of enhancing the diagnostic capacity and therapeutic management of these disorders. Promoting a homogeneous approach among different centres, which will subsequently impact on the patients' life expectancy and quality of life. Simultaneously, we also aim at increasing awareness of rare liver disorders to further promote the creation of international research networks that facilitate the unbiased study of these disorders.

To develop these points, we have designed three studies with different specific objectives:

1) The aim of the present study was to evaluate the potential role of Next-generation sequencing in the diagnosis of splanchnic vein thrombosis.
2) The aim of the present study was to characterize the natural history of polycythemia vera and essential thrombocythemia patients presenting with splanchnic vein thrombosis.
3) The main aim of the study was to characterize the humoral autoimmune profile in patients with portosinusoidal vascular disorder, evaluating its potential role in its pathophysiology. The secondary aim was to assess the potential value of serum autoantibodies in the diagnosis of the disease.

III. Results

III.1 Study 1

Next-generation sequencing in the diagnosis of non-cirrhotic splanchnic vein thrombosis.

Marta Magaz , Alberto Alvarez-Larrán, Dolors Colomer, Mónica López-Guerra, M Ángeles García-Criado, Gabriel Mezzano, Ernest Belmonte, Pol Olivas, Guillem Soy, Francisco Cervantes, Anna Darnell, José Ferrusquía-Acosta, Anna Baiges, Fanny Turon, Virginia Hernández-Gea, Juan Carlos García-Pagán.

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Research Article
Cirrhosis and Liver Failure

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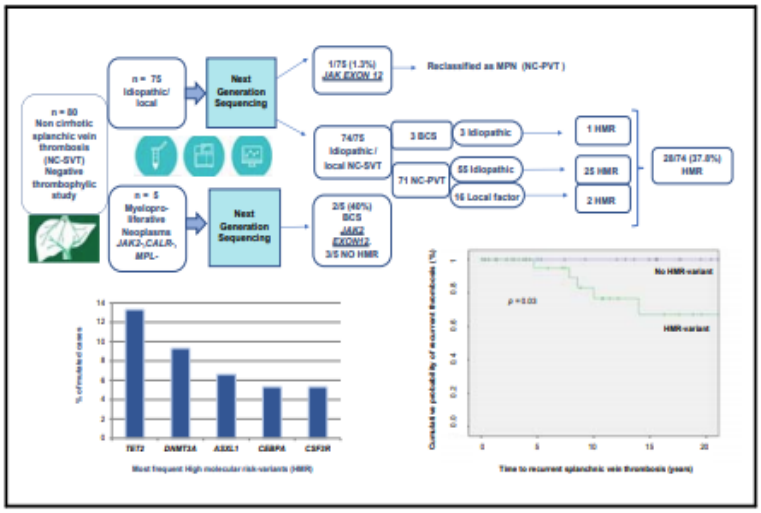
Next-generation sequencing in the diagnosis of non-cirrhotic splanchnic vein thrombosis

Graphical abstract



Highlights

- NGS allows the simultaneous evaluation of multiple genes associated with myeloproliferative neoplasms, even at low mutational levels.
- NGS could identify *JAK2*-exon12 mutations not previously detected by the conventional techniques.
- NGS also identified high-molecular-risk HMR variants associated with clonal haematopoiesis.
- Patients with idiopathic/local factor NC-SVT harbouring HRM variants seem to be at a higher risk of recurrent splanchnic thrombosis.

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Lay summary

Next-generation sequencing (NGS) performs massive sequencing of DNA allowing the simultaneous evaluation of multiple genes even at very low mutational levels. Application of this technique in a cohort of patients with non-cirrhotic non-tumoral portal vein thrombosis (NC-SVT) and a negative study for thrombophilic disorders was able to identify patients with a mutation in exon 12 not previously detected by conventional techniques. Moreover, NGS detected High Molecular Risk (HMR)-variants (Mutations involved in myeloid disorders different from *JAK2*, *CALR* and *MPL* genes) in approximately one third of patients. These patients appear to be at increased risk of rethrombosis. All these findings supports NGS as a potential useful tool in the management of NC-SVT.

Research Article
Cirrhosis and Liver Failure

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Next-generation sequencing in the diagnosis of non-cirrhotic splanchnic vein thrombosis

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Background & Aims: Myeloproliferative neoplasms (MPNs) are the most frequent cause of non-tumoural non-cirrhotic splanchnic vein thrombosis (NC-SVT). Diagnosis of MPN is based on blood cell count alterations, bone marrow histology, and detection of specific gene mutations. Next-generation sequencing (NGS) allows the simultaneous evaluation of multiple genes implicated in myeloid clonal pathology. The aim of this study was to evaluate the potential role of NGS in elucidating the aetiology of NC-SVT.

Methods: DNA samples from 80 patients (75 with idiopathic or exclusively local factor [Idiop/loc-NC-SVT] and 5 with MPN and NC-SVT [SVT-MPN] negative for Janus kinase 2 gene [*JAK2*] [*V617F* and exon 12], calreticulin gene [*CALR*], and thrombopoietin gene [*MPL*] mutations by classic techniques) were analysed by NGS. Mutations involved in myeloid disorders different from *JAK2*, *CALR*, and *MPL* genes were categorised as high-molecular-risk (HMR) variants or variants of unknown significance.

Results: In 2/5 triple-negative SVT-MPN cases (40%), a mutation in exon 12 of *JAK2* was identified. *JAK2*-exon 12 mutation was also identified in 1/75 patients with Idiop/loc-NC-SVT. Moreover, 28/74 (37.8%) of the remaining Idiop/loc-NC-SVT had at least 1 HMR variant. Sixty-two patients with Idiop/loc-NC-SVT were not receiving long-term anticoagulation and 5 of them (8.1%) had recurrent NC-SVT. This cumulative incidence was significantly higher in patients with HMR variants than in those without.

Conclusions: NGS identified *JAK2*-exon12 mutations not previously detected by conventional techniques. In addition, NGS detected HMR variants in approximately one-third of patients with Idiop/loc-NC-SVT. These patients seem to have a higher risk of splanchnic rethrombosis. NGS might be a useful diagnostic tool in NC-SVT.

Lay summary: Next-generation sequencing (NGS) performs massive sequencing of DNA allowing the simultaneous evaluation of multiple genes even at very low mutational levels. Application of this technique in a cohort of patients with non-cirrhotic non-tumoral portal vein thrombosis (NC-SVT) and a negative study for thrombophilic disorders was able to identify patients with a mutation in exon 12 not previously detected by conventional techniques. Moreover, NGS detected High Molecular Risk (HMR)-variants (Mutations involved in myeloid disorders different from *JAK2*, *CALR* and *MPL* genes) in approximately one third of patients. These patients appear to be at increased risk of rethrombosis. All these findings supports NGS as a potential useful tool in the management of NC-SVT.

Introduction

Non-tumoural non-cirrhotic splanchnic vein thrombosis (NC-SVT) includes Budd-Chiari syndrome (BCS) and non-tumoural non-cirrhotic portal vein thrombosis (NC-PVT); both are rare disorders with high morbi-mortality. BCS usually affects young patients, whereas NC-PVT usually affects middle-aged persons, and both reduce life expectancy.[1] In recent years, their prognosis has significantly improved, as a result in part of an earlier diagnosis and a better management of the underlying prothrombotic disorder.[2] The main aetiological factors for both BCS and NC-PVT are myeloproliferative neoplasms (MPNs). MPNs are clonal alterations of haematopoietic stem cells, characterised by an overproduction of functional and mature granulocytes, red blood cells, and/or platelets. The increases in platelet aggregation and/or thrombin generation associated with MPNs are precipitating causes of thrombotic events.[3,4] Therefore, the reason why MPNs should be actively sought is because these disorders confer a high risk of thrombosis recurrence and a potential risk of progression to myelofibrosis or even leukaemia.[5–7]

Nowadays, diagnosis of MPN relies on the alterations of the blood count, bone marrow histology, and/or the detection of the

Keywords: NGS (next-generation sequencing); Non-cirrhotic splanchnic vein thrombosis; Portal vein thrombosis; Budd-Chiari syndrome; Myeloproliferative neoplasms.
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classic haematological mutations in Janus kinase 2 gene (*JAK2*), calreticulin gene (*CALR*), and thrombopoietin gene (*MPL*).[8–10] However, patients with NC-SVT usually present hypersplenism and haemodilution caused by portal hypertension that masks the classical diagnostic findings in peripheral blood, which increases the difficulty of diagnosing MPN.

To date, *JAK2V617F* is the most frequent mutation found in patients with NC-SVT.[11] Screening of the other classical mutations at exon 12 of *JAK2*, exon 10 of *MPL*, or exon 9 of *CALR* may increase the number of patients with NC-SVT diagnosed with MPN.[8,12,13] Indeed, *CALR* mutation was found in up to 1.5% of patients previously considered to have idiopathic NC-SVT, identifying a small additional number of patients with an underlying MPN.[12,14] However, in a large cohort of 241 patients with NC-SVT, mutations at *JAK2* exon 12 or at exon 10 of *MPL* were not detected.[15]

In fact, despite a comprehensive prothrombotic diagnostic study, in more than 30% of patients with NC-SVT the study is negative and is considered as idiopathic NC-SVT.[1] In addition, local factors (inflammation, surgery, etc.) have been described as predisposing factors of NC-SVT, although in the available series up to one-third of the patients have a concomitant prothrombotic disorder. Whether local factor itself can provoke NC-SVT or act as a trigger of an underlying disease is still a matter of debate. Altogether, the presence of a local factor does not exclude the presence of other thrombophilic disorders, and a complete evaluation of all known aetiological factors should always be performed.[2,16,17]

Current clinical practice guidelines do recommend indefinite anticoagulation only when an underlying prothrombotic disorder is identified.[1,18] Therefore, clinicians should be aware of the possible presence of occult forms of MPN with the objective to initiate anticoagulation aimed to prevent recurrent phenomena of splanchnic thrombosis as well as thrombosis in other territories (both venous and arterial).[19]

Traditional techniques, employed to detect the classic haematological mutations, such as Sanger sequencing, can only analyse a single DNA fragment at a time. Next-generation sequencing (NGS) has the advantage of performing massive sequencing of DNA (being able to simultaneously test millions of fragments per run) with high sensitivity detecting gene mutations even at very low mutational loads.[20,21] In addition to the 3 classical mutations, NGS may detect other genes that have been currently grouped by the term 'CHIP' (clonal haematopoiesis of indeterminate potential) and that are related to a myeloid clonal pathology and/or pro-inflammatory state.[7,22] CHIP has been associated with ageing in people without haematological disease,[23] and implies that some haematopoietic clones carry recurrent somatic variants (most frequently loss-of-function alleles in the genes *ASXL1*, *DNMT3A*, and *TET2* among others).[22] The presence of such variants has been recently related with an increased risk of haematological cancer and coronary artery disease.[24]

We hypothesise that some patients with NC-SVT still considered as idiopathic or in whom only an isolated local factor was found (Idiop/loc-NC-SVT) might have a misdiagnosed MPN not detected with the current standard molecular techniques used to analyse the classical genetic mutations at *JAK2*, *CALR*, or *MPL*. In addition, these patients may have mutations in other genes implicated in myeloid clonal pathology and/or in generating a pro-inflammatory state.

The aim of the present study was to evaluate the potential role of NGS in the diagnosis of NC-SVT.

Materials and methods

Patients and samples

This study was conducted in accordance with the International Guidelines for Ethical Review of Epidemiological Studies and principles of the Declaration of Helsinki, and has the approval of our institution's ethics committee (Hospital Clinic in Barcelona HCB/2017/0270).

Since 2003 all consecutive patients with BCS or NC-PVT seen at our unit were asked for permission to obtain a blood sample for research purposes. Blood samples were stored at the Hospital Clinic Biobank facilities. All patients included in the study provided their written informed consent.

In all, 314 patients with NC-SVT and a complete aetiological diagnostic workout were identified in the vascular liver diseases registry of our unit (214 with NC-PVT and 100 with BCS). Patients with a known prothrombotic factor (including positivity for *JAK2*, *MPL*, and *CALR* mutations) that did not have blood stored at Biobank or that denied consent were excluded (Fig. 1). Thus, 80 patients with NC-SVT were included in the study: 75 with Idiop/loc-NC-SVT (with a complete negative aetiological study) and 5 with histological diagnosis of MPN but without any identified mutation (*JAK2*, *CALR*, and *MPL* negative). Fig. 1 shows the flow chart of the study. For each individual, laboratory, haematological, and clinical data were collected in a predesigned case report form.

Diagnosis of BCS and/or NC-PVT was performed according to the European Association for the Study of the Liver (EASL) guidelines.[1] Routine scheduled imaging follow-up studies to specifically evaluate recurrent splanchnic thrombosis are performed in our clinical practice every 6 months with Doppler-ultrasound when complete adequate visualisation of the venous segment is achieved. Contrast-enhanced computed tomography (CT) or contrast-enhanced magnetic resonance imaging (MR) was performed in cases of non-visualisation of the axis.[25] Splanchnic recurrent thrombosis was defined as the development of a thrombus in a segment of the portal venous axis not previously involved, or progression from partial to occlusive thrombosis. During the clinical follow-up, the occurrence of any extra-splanchnic thrombotic event (deep vein thrombosis, arterial thrombosis, myocardial infarction, stroke, etc.) was also registered.

Molecular studies

DNA was isolated from whole peripheral blood or granulocyte fraction using the QIAmp® DNA Mini Kit (QIAGEN, Hilden, Germany). Mutations in *JAK2* (V617F and exon 12), *CALR* (exon 9), and *MPL* (exon 10) were analysed in all 80 patients using the routine conventional techniques: *JAK2 V617F* mutation was analysed by allele-specific PCR.[26] *JAK2 exon 12* mutations and *MPL* were analysed by direct sequencing[13,27] and *CALR* mutations by PCR and fragment analysis.[9] *JAK2 V617F* was additionally rechecked in the negative cases by quantitative PCR.[28]

Targeted NGS was performed using the SOPHiA GENETICS (Boston, USA) Myeloid Tumor Solution Panel®, including the following genes: *ABL1*, *ASXL1*, *BRAF*, *CALR*, *CBL*, *CEBPA*, *CSF3R*, *CSNK1A1*, *DNMT3A*, *ETV6*, *EZH2*, *FLT3*, *HRAS*, *IDH1*, *IDH2*, *JAK2*, *KIT*, *KMT2A*, *KRAS*, *MPL*, *NPM1*, *NRAS*, *PTPN11*, *RUNX1*, *SETBP1*, *SF3B1*, *SRSF2*, *TET2*, *TP53*, *U2AF1*, *U2AF2*, *WT1*, and *ZRSR2*. A capture-

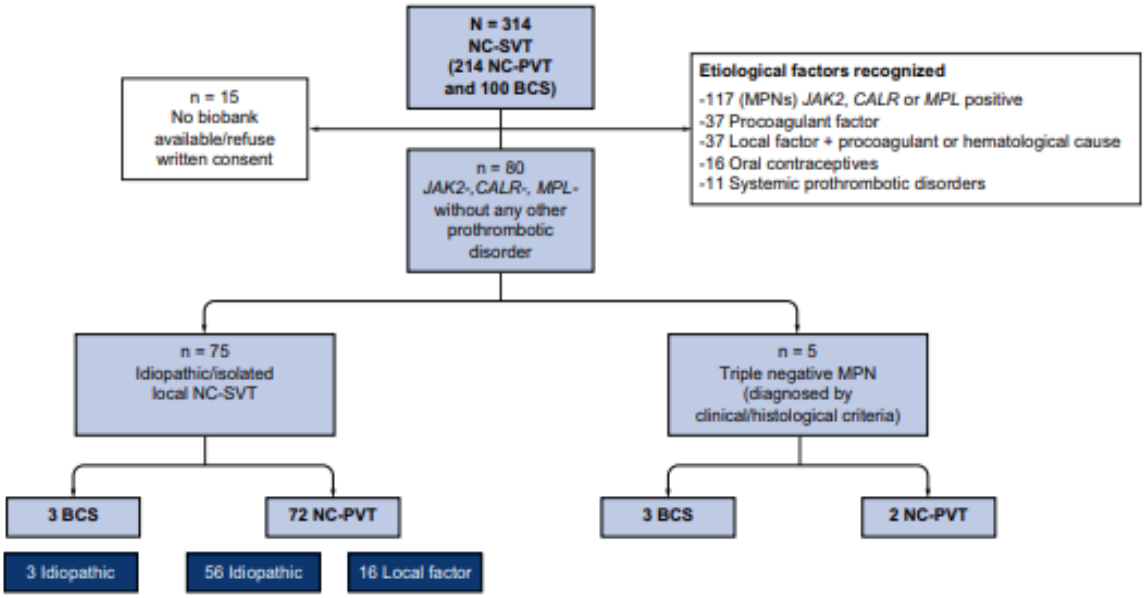

Fig. 1. Patient flow chart. BCS, Budd-Chiari syndrome; *CALR*, calreticulin gene; *JAK2*, Janus kinase 2 gene; *MPL*, thrombopoietin gene; MPN, myeloproliferative neoplasm; NC-PVT, non-cirrhotic portal vein thrombosis; NC-SVT, non-cirrhotic splanchnic vein thrombosis.

based target enrichment kit was used and the libraries were sequenced 2×300 bp on an Illumina MiSeq® (San Diego, USA) according to the manufacturer's protocols. The analysis of the detected variants was carried out using the SOPHiA DDM® software (SOPHiA GENETICS).[29] The software considered a minimum variant allele frequency (VAF) of ≥2%.[23] Synonymous, intronic, and polymorphic variants were discarded. According to the general guidelines, this software categorised the variants at different levels of pathogenicity, from highly pathogenic to benign: A, highly pathogenic (if the variant has been previously described as pathogenic in myeloid neoplasms); B, potentially pathogenic (if the variant has been previously described as pathogenic in non-myeloid neoplasms or solid tumours, or if the variant has not been described, but the predictive algorithms classify it as pathogenic); C, unknown significance (if the variant has been previously described, but with no sufficient evidence of its pathogenicity, or if the variant has not been previously described for which predictive algorithms are not conclusive); and D, likely benign (described as clinically insignificant).[30,31] A, B, and C variants were selected for further review using COSMIC (http://cancer.sanger.ac.uk) and ClinVar (www.ncbi.nlm.nih.gov/clinvar) databases and published studies. Variants were finally grouped, according to the current guidelines, as high-molecular-risk (HMR) variants if they were compatible with potentially/highly pathogenic variants (A and B) or as variants of unknown significance (VUS; C) in those with uncertain significance.

Statistical analysis

Quantitative data are expressed as median and range. Qualitative data are expressed as percentages. Variables were compared by *t* test, Mann-Whitney *U* test, or chi-square test, as appropriate.

The probability of recurrent thrombosis as a function of time was estimated using the Kaplan-Meier method by analysing the interval between the index thrombosis and a recurrent thrombotic event (uncensored observations) or the duration of time until the last image test (censored observations). The thrombosis recurrence-free survival was compared between the groups using the log-rank test (statistical significance threshold set at $p < 0.05$), and the relative risk of recurrence was estimated as a hazard ratio (HR) with a 95% confidence interval (CI). Cox regression was used to identify independent risk predictors (HR 95% CI) for splanchnic recurrent thrombosis.

Results

From the 80 patients with NC-SVT, 45 (56.3%) were males. Six patients had a BCS and 74 an NC-PVT. Table 1 summarises the clinical characteristics of patients. MPN diagnosis was established in 5 patients (3 BCS and 2 NC-PVT) by clinical and histological criteria with negative mutations by conventional methods for *JAK2* (*JAK2V617F* and exon 12), *CALR*, and *MPL*. Of the remaining 75 patients with a negative prothrombotic study, 16 (21.3%) had a local factor as a potential cause of thrombosis (3 inflammatory intra-abdominal lesions, 12 intra-abdominal surgery, and 1 umbilical vein catheterisation), while no potential cause was found in the remaining 59 (78.6%) patients (Fig. 1). This group of 75 patients is encompassed by the term Idiop/loc-NC-SVT.

NGS evaluation of MPN classical mutations

NGS analysis identified a mutation in the hotspot exon 12 region of *JAK2* in 2 of the 5 patients with triple-negative SVT-MPN (40%). These mutations were p.His538_Lys539delinsGln and p.Asn542_Glu543del with allele loads of 6% and 7%, respectively. Both patients had a BCS; the associated HMR variants are outlined in Table S1. No other mutations were detected in the other 3 SVT-MPN cases; only 1 patient had a VUS (*ASXL1* p.Ser1166Arg VAF 50.8%).

Among the 75 patients with Idiop/loc-NC-SVT, 1 case (1.3%) presented a mutation at exon 12 of *JAK2* (p.His538_Lys539delinsGln) that was detected by NGS with a 2.1% allele load. This

Table 1. Baseline characteristics: full cohort (80 patients).

Baseline characteristics at diagnosis (n = 80)	n (%) or mean ± range
Age at diagnosis	42 [13–79] years
Women	35 (43.8%)
Haemoglobin	13 [8.4–17.7] g/L
Platelets	216 [54–593] × 10^9/L*
Leucocytes	6.4 [1.2–12.5] × 10^9/L
INR	1.2 [0.7–2.7]%
ALT	47 [7–115] U/L
GGT	124 [6–546] U/L
Bilirubin	0.9 [0.2–3] mg/dl
Albumin	40 [25–49] g/L
Ascites	10 (12.5%)
Oesophagogastric varices	49 (61.2%)
NC-PVT/BCS	74/6
Idiopathic	59 (73.8%)
Isolated local factor	16 (20%)
Myeloproliferative neoplasm (*JAK2*, *CALR*, and *MPL*)	5 (6.2%)

ALT, alanine aminotransferase; BCS, Budd-Chiari syndrome; *CALR*, calreticulin gene; GGT, gamma glutamyl transferase; INR, international normalised ratio; *JAK2*, Janus kinase 2 gene; *MPL*, thrombopoietin gene; NC-PVT, non-cirrhotic portal vein thrombosis.
*In the cohort, there were a total of 8 splenectomised patients.

patient also had an HMR variant in *ZRSR2* p.Ser447_Arg448dup, with a VAF of 8.52%. Therefore, the patient previously considered to have an idiopathic NC-PVT was reclassified as an MPN, therefore leaving 74 patients as Idiop/Loc-NC-SVT. No mutations in *CALR* or *MPL* were detected.

NGS evaluation of other genes implicated in myeloid clonal pathology

Twenty-eight of the remaining 74 Idiop/loc-NC-SVT (37.8%) had at least 1 HMR variant (potentially/highly pathogenic variants detected by NGS), 73 had a PVT, and 1 a BCS. Eleven of the 28 patients with an HMR (39.2%) presented 2 HMR variants. The other 46 patients (62.2%) did not have any HMR variants. Eighteen of these 46 patients (39.1%) had a VUS, while no mutation at all (HMR or VUS) was found in the remaining 28 patients. The different variants found are listed in Table S1 and outlined in Fig. 2. There were no differences in baseline characteristics according to the presence or absence of HMR variants. Interestingly, patients with a local factor had significantly less frequently HMR variants (2/16; 12.5%) than those without a local factor (26/58; 44.8%) (p <0.02).

In the HMR variants group, 5 individual genes (*TET2*, *CEBPA*, *DNMT3A*, *CSF3R*, and *ASXL1*) were mutated in more than 5% of patients, being *TET2*, *DNMT3A*, and *ASXL1* (Fig. 2) the most frequently mutated. These 3 genes are the most frequently detected genes in clonal haematopoiesis.[32,33]

Presence of HMR variants and clinical outcome

Splanchnic recurrent thrombosis

According to current guidelines,[1,18] the 5 patients with SVT-MPN were treated with long-term anticoagulation. Of the remaining 75 patients, 13 were receiving long-term anticoagulation because of severe intestinal ischaemia at diagnosis, a previous non-splanchnic thrombotic event, or other conditions requiring anticoagulation. None of the 18 patients who were anticoagulated presented recurrent splanchnic thrombosis during follow-up. Sixty-two patients with Idiop/loc-NC-SVT did not receive long-term anticoagulation (26 patients had at least an HMR variant

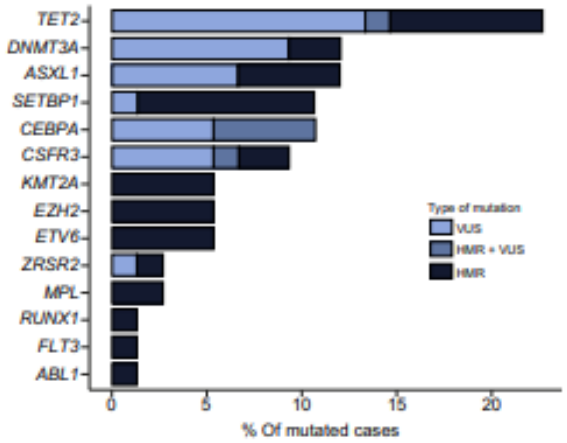

Fig. 2. Mutation frequency of all variants detected by NGS. HMR, high-molecular-risk variant; NGS, next-generation sequencing; VUS, variant of unknown significance.

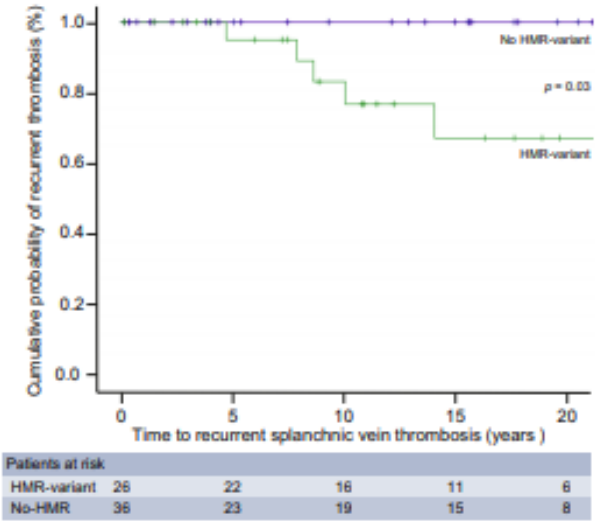

Fig. 3. Cumulative incidence of recurrent splanchnic vein thrombosis in 62 patients with chronic portal vein thrombosis of idiopathic/local aetiology not receiving long-term anticoagulation comparing those with and without HMR variants. The probability of recurrent thrombosis as a function of time was estimated using the Kaplan-Meier method. HMR, high-molecular-risk variant; VUS, variant of unknown significance.

and 36 patients did not present any HMR variant). Five of these 62 patients who were not anticoagulated (8.1%) presented recurrent splanchnic vein thrombosis after a median of 161 months (range 4–600). In 1 patient, recurrent thrombosis was diagnosed because of abdominal pain, while the remaining 4 were asymptomatic and detected at a scheduled imaging study. These 5 patients with splanchnic recurrent thrombosis are marked with an asterisk (*) in Table S1. All 5 patients with splanchnic rethrombosis had at least an HMR variant (5/26; 19.2%) *vs.* none of the 36 without HMR variants (0/36) (p = 0.03). Cumulative incidence of rethrombosis was significantly higher in patients with HMR mutation than in those without (Fig. 3). This higher risk remains after adjusting the re-thrombotic risk by patient's age.

Ten of the 26 (38.4%) patients who were not anticoagulated with HRM variants had 2 HRM variants. Interestingly, 3 of the 5 patients with splanchnic recurrent thrombosis had 2 HMR variants (3/10; 30%) *vs.* 2/16 with only 1 HMR variant (12.5%) ($p = 0.27$). Anticoagulation was initiated in all 5 patients with recurrent thrombosis, and they did not have further thrombotic events.

Extra-splanchnic thrombosis

Seven of the 62 patients (11.3%) that did not receive anticoagulation had an extra-splanchnic vein thrombosis (1 deep vein thrombosis; 1 inferior vena cava and a femoral vein thrombosis, 2 acute myocardial infarctions, and 3 ischaemic strokes) during follow-up. Three patients had HMR variants (3/26; 11.5%), while the remaining 4 occurred in the cohort of 36 patients without HMR variants (11.1%) (n.s.).

Discussion

The present study evaluates the diagnostic profitability of NGS in a large cohort of patients with Idiop/loc-NC-SVT. All these patients had, in their clinical evaluation, a comprehensive coagulation study, including the determination of classical mutations for MPN by conventional techniques. In addition, we analysed 5 patients with NC-SVT with an underlying MPN, diagnosed by histological criteria, but negative for *JAK2*, *CALR*, or *MPL*. The diagnosis of an underlying prothrombotic disorder in the setting of NC-SVT is relevant because it influences the decision to maintain patients in long-term anticoagulation.[1,18] This need is especially important for those highly prothrombotic states, such as anti-phospholipid syndrome or MPN.[1,34]

The identification of classical haematological mutations has represented an important progress facilitating the diagnosis of MPN. Despite this fact, there are patients with MPN that are negative for all the known mutations, and surprisingly, several large cohorts of patients with NC-SVT from Europe and Asia did not detect any case of *JAK2* exon 12 mutation.[15,35]

In the current study, NGS-based sequencing detected *JAK2* exon 12 mutation in 3 patients who were negative by conventional sequencing. BCS was present in 2 of the 3 triple-negative patients. However, the diagnosis of MPN in these 2 patients was already made on clinical and histological grounds. Thus, no further patients with BCS and an underlying MPN were diagnosed. These data emphasise that in patients with BCS if a thorough evaluation is performed, a potential underlying MPN will be finally identified. Nevertheless, NGS clearly would facilitate this task.

The sensitivity of traditional sequencing is recognised to be consistent from approximately 15% to 20% mutant allele frequency;[15,36] therefore, cases with low mutation loads could be misdiagnosed. Remarkably, the 3 patients of our cohort diagnosed by NGS had an allele load far below 10%. In addition, recent data show that *JAK2 V617F* allele burden is usually lower than 10% in MPN patients with associated NC-SVT and clearly below than that observed in MPN patients without NC-SVT (median 5% *vs.* 36.3%; $p = 0.019$).[37] Then, detection of *JAK2* at exon 12 led to the diagnosis of an underlying MPN in 3 of 122 patients with NC-SVT registered in our centre (2.5%) (Fig. 1). Our results suggest that NGS technology would facilitate and therefore increase the number of patients diagnosed with MPN in the setting of NC-SVT.

In addition, NGS identified HMR variants in more than one-third of our patients with Idiop/loc-NC-SVT. This percentage is much higher than the observed percentage in healthy individuals (2–3%) even those aged over 70 (around 10%),[38,39] strongly supporting a pathophysiological role of these variants in NC-SVT.[23,33] Indeed, and despite the low number of events, patients holding HMR variants seem to have a higher risk of splanchnic recurrent thrombosis, further suggesting a pathogenetic role for HMR variants facilitating NC-SVT. Importantly, this higher risk associated with HMR variants persisted after adjusting by patient age. There seems also to be a trend towards a higher risk of splanchnic recurrent thrombosis in those patients with 2 HRM variants compared with those with a single HRM variant, although it was not statistically significant, probably because of the low number of events. This fact would be in accordance with previous data that suggest that patients with 2 or more HMR variants present a worse prognosis.[20,40]

The majority of patients (both those who detected *JAK2* exon 12 and those in whom an HMR mutation was observed) did not present significant differences in the number of platelets or the size of the spleen compared with those patients who did not present these mutations. So, if the criteria based on this European group work would be applied to avoid unnecessary tests (platelets >200 [×109/L] and spleen ≥16 cm),[8] only in 1 patient of the SVT-MPN group could have been diagnosed. This fact would also support the use of NGS.

Interestingly, our study shows more representation for the presence of HMR variants in patients without any known cause of thrombosis (also without local factor as a potential causative for thrombosis), supporting the possible role of HMR variants in thrombosis development. In addition, these data support that looking for HMR variants would be of special interest in patients with NC-SVT in whom no local factors are identified. Nevertheless, it is not negligible to identify a potential prothrombotic factor in 12.5% of the subgroup of patients with a local factor that were negative for other prothrombotic factors after an exhaustive prothrombotic study.

HMR variants seemed not to influence the risk of developing extra-splanchnic thrombotic events. This may be explained by the mixed and heterogeneous extra-splanchnic thrombotic events and their different pathophysiological mechanisms.[41]

In our study and in agreement with previous MPN studies,[33,42] the HMR variants *TET2*, *DNMT3A*, and *ASXL1* were the most frequently mutated genes. Recently, it has been reported that these 3 genes (*TET2*, *DNMT3A*, and *ASXL1*) are associated with a higher thrombotic risk in *polycythemia vera*.[33] Some of these genes, as *TET2*, have essential functions that are independent of their enzymatic activity. In particular, *TET2* deficiency results in an increased pro-inflammatory phenotype in murine macrophages that could favour atherosclerosis development and thrombosis.[43] We also detected a not negligible prevalence of variants in *CEBPA* gene. *CEBPA* is a key factor in driving the development of myeloid cells and regulates *TET2* transcription.[44] Furthermore, it has been described that knock-in mice with *CEBPA* variants predispose mice to myeloproliferative disorders.[45]

Our data show that the introduction of NGS in the study of patients with NC-SVT may improve the diagnosis of MPN and it might even have prognostic implications.[23,33] Besides, this approach allows the simultaneous analysis of mutations at *JAK2* (*JAK2V617F* and exon 12), *MPL*, and *CALR* genes, and could reduce the need for additional studies and the number of invasive explorations, such as bone marrow biopsies, particularly in *polycythemia vera* patients. However, nowadays, the main limitation

for the implementation of an NGS-based strategy to study all NC-SVT patients is the spare availability of the method and its high cost. Given that *JAK2V617F* is the most frequent molecular alteration in patients with NC-SVT (around 35% in patients with BCS and 20% of patients with NC-PVT),[2,46–48] it could be more cost-effective to screen first for *JAK2V617F* and to perform NGS only in those patients that are *JAK2V617F* negative. NGS allows simultaneously determining the presence of HMR variants. Recent data suggest that patients with a *JAK2*-positive MPN that additionally hold an HMR variant had a worse prognosis than those MPN patients that only had the *JAK2* mutation.[33] If these data are confirmed, together with our results suggesting that the exclusive presence of an HMR variant may be a prothrombotic risk, NGS could become the initial technique to assess patients with NC-SVT in selected patients.

Abbreviations

BCS, Budd-Chiari syndrome; *CALR*, calreticulin gene; CHIP, clonal haematopoiesis of indeterminate potential; HMR, high molecular risk; Idiop/Ioc-NC-SVT, idiopathic/exclusively isolated local factor non-tumoural non-cirrhotic splanchnic vein thrombosis; *JAK2*, Janus kinase 2 gene; *MPL*, thrombopoietin gene; MPN, myeloproliferative neoplasm; NC-PVT, non-tumoural non-cirrhotic portal vein thrombosis; NC-SVT, non-tumoural non-cirrhotic splanchnic vein thrombosis; NGS, next-generation sequencing; SVT-MPN, splanchnic vein thrombosis associated to a myeloproliferative neoplasm; VAF, variant allele frequency; VUS, variant of unknown significance.

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Conflicts of interest

Virginia Hernández-Gea receives speaker fees from Gore. Juan Carlos García-Pagán reports from W. L. Gore and Associates, Cook Medical, Shionogi, and Vifor Pharma, and receives grants from Conatus Pharmaceuticals, Theravance Biopharma, Novartis, and Exalenz Bioscience outside the submitted work. Fanny Turon reports from W. L. Gore and Associates outside the submitted work. The remaining authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Marta Magaz, Alberto Alvarez-Larrán, Francisco Cervantes, Gabriel Mezzano, Virginia Hernández-Gea, and Juan Carlos García-Pagán designed the research, analysed data, and wrote the paper. Dolors Colomer and Mónica López-Guerra performed the molecular analysis and wrote the paper. M. Ángeles García-Criado, Ernest Belmonte, and Anna Darnell interpreted the imaging studies and drafted the paper. Marta Magaz, Fanny Turon, Anna Baiges, José Ferrusquía, Pol Olivas, and Guillem Soy collected and reviewed the data.

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Supplementary data

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Author names in bold designate shared co-first authorship

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- **Supplementary Table 1.** Mutations detected by NGS.

Abbreviations Supplementary Table 1: AA, Aminoacid; VAF, Variant allele frequency; HMR, High molecular risk-variant; VUS, Variant of unknown significance.

ID	*Gene*	Variant (AA)	VAF (%)	Classification
1	*ASXL1*	p.Glu635Argfs15	6	HMR
1	*TET2*	p.Leu1721Trp	48.6	HMR
2	*ASXL1*	p.Glu1102Asp	19.5	HMR (*)
2	*TET2*	p.Leu476Ile	19.9	HMR
2	*SETBP1*	p.Pro1130Thr	27.4	VUS
3	*ASXL1*	p.Glu1102Asp	34.9	HMR
3	*TET2*	p.Leu476Ile	37.2	HMR
3	*SETBP1*	p.Val1101Ile	38	VUS
4	*CSF3R*	p.Met696Thr	14.3	HMR (*)
4	*ASXL1*	p.Trp583Arg	2.4	HMR
4	*EZH2*	p.Asp185His	14.1	VUS
5	*CSF3R*	p.Met696Thr	49.1	HMR (*)
5	*CSF3R*	p.Asp510His	48.8	HMR
5	*EZH2*	p.Asp185His	50.9	VUS
6	*CSF3R*	p.Glu808Lys	34.1	HMR
6	*TET2*	p.Leu1667Met	14.3	HMR
6	*CSF3R*	p.Glu405Lys	32.2	VUS
7	*DNMT3A*	p.Thr671Argfs34	6.3	HMR
7	*DNMT3A*	p.Pro799Ala	4.1	HMR
7	*EZH2*	p.Asp185His	53.2	VUS

8	*DNMT3A*	p.(?)	2	HMR
8	*CSF3R*	p.Pro691Leu	50	HMR
8	*ETV6*	p.Leu201Pro	48	VUS
9	*DNMT3A*	p.Arg301Trp	4.7	HMR
9	*TET2*	p.Pro1723Ser	48.2	HMR
9	*RUNX1*	p.Leu29Ser	53.8	VUS
10	*TET2*	p.His1817Asn	52.1	HMR
10	*ASXL1*	p.Asp799Tyr	47.5	HMR
10	*TET2*	p.His1778Arg	48.2	VUS
11	*ZRSR2*	p.Ser447_Arg448	24.5	HMR
11	*TET2*	p.Pro363Leu	38.2	HMR
11	*ETV6*	p.Leu201Pro	32.6	VUS
12	*CEBPA*	p.Ser190Pro	3.6	HMR (*)
12	*ASXL1*	p.Gly652Ser	46.7	VUS
12	*CEBPA*	p.His195_Pro196dup	37.8	Polymorphism
13	*CEBPA*	p.Ser190Pro	2.6	HMR (*)
13	*CSF3R*	p.Gly683Arg	48.2	VUS
13	*CEBPA*	p.His195_Pro196	33.8	Polymorphism
14	*CEBPA*	p.Ser190Pro	2.6	HMR
14	*EZH2*	p.Asp185His	51.4	VUS
14	*CEBPA*	p.His195_Pro196	38.5	Polymorphism
15	*CEBPA*	p.Ser190Pro	3.2	HMR

15	*DNMT3A*	p.Asp765Gly	2.6	VUS
15	*CEBPA*	p.His195_Pro196	35.5	Polymorphism
16	*CEBPA*	p.Ser190Pro	2.1	HMR
17	*CEBPA*	p.Ser190Pro	2.4	HMR
18	*CEBPA*	p.Gly104del	2.2	HMR
18	*SETBP1*	p.Val610Ile	22.1	VUS
18	*KMT2A*	p.Ile3440Val	50.5	VUS
19	*CEBPA*	p.Gly104del	2.1	HMR
19	*SETBP1*	p.His917Leu	3.7	VUS
19	*CSF3R*	p.Gln346Arg	46.1	VUS
20	*CSF3R*	p.Arg698Cys	48.1	HMR
21	*DNMT3A*	p. (?)	2.5	HMR
21	*ETV6*	p.Leu201Pro	45.5	VUS
22	*DNMT3A*	p.Arg326Cys	3.6	HMR
23	*DNMT3A*	p.Val636Leu	20.9	HMR
24	*DNMT3A*	p.Leu888Pro	2.1	HMR
25	*TET2*	p.Thr1114Ile	52.9	HMR
26	*TET2*	p.Ala457Val	49.5	HMR
26	*SETBP1*	p.Val1101Ile	47	VUS
27	*TET2*	p.Gly355Asp	50.1	HMR
27	*MPL*	p.His308Asp	50.3	VUS
28	*TET2*	p.Tyr867His	48.3	HMR

28	*SETBP1*	p.Val231Leu	48.3	VUS
29	*ASXL1*	p.Trp583Arg	2.1	VUS
30	*ASXL1*	p.Ser1231Phe	49.1	VUS
31	*ASXL1*	p.Glu1102Asp	50.3	VUS
32	*DNMT3A*	p.Glu30Ala	52.6	VUS
33	*ETV6*	p.Leu201Pro	47.7	VUS
34	*FLT3*	p.Phe594Ser	52.2	VUS
35	*KMT2A*	p.Ala30Gly	48.5	VUS
36	*KMT2A*	p.Glu502Lys	50.3	VUS
37	*KMT2A*	p.Ser2395Pro	49.8	VUS
37	*ABL1*	p.Leu201Pro	51.3	VUS
38	*MPL*	p.His353Arg	50.8	VUS
39	*SETBP1*	p.Val1101Ile	48	VUS
40	*TET2*	p.His924Arg	49.2	VUS
41	*TET2*	p.Leu1721Trp	48.9	VUS
42	*TET2*	p.Pro363Leu	50.2	VUS
43	*TET2*	p.Leu1667Met	47.9	VUS
44	*TET2*	p.Met1701Ile	51.9	VUS
45	*TET2*	p.Met1701Ile	47.1	VUS
45	*SETBP1*	p.His1100Arg	52.7	VUS
46	*ZRSR2*	p.Ser447_Arg448dup	32.8	VUS

III.1 Study 2

Natural history of Polycythaemia vera and essential thrombocythemia presenting with splanchnic vein thrombosis.

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ORIGINAL ARTICLE

Natural history of polycythemia vera and essential thrombocythemia presenting with splanchnic vein thrombosis

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Abstract

Patients with polycythemia vera (PV) or essential thrombocythemia (ET) presenting with splanchnic vein thrombosis (SVT) might have a specific clinico-biological profile. To investigate this hypothesis, 3705 PV/ET patients from three national registers, 118 of them presenting with SVT, were reviewed. After correction for age and sex, PV/ET patients with SVT showed an increased risk of death (HR 2.47, 95% CI 1.5–4.01, $p < 0.001$), venous thrombosis (IRR 3.4, 95%CI 2.1–5.5, $p < 0.001$), major bleeding (IRR 3.6, 95%CI 2.3–5.5, $p < 0.001$), and second cancer (IRR 2.37, 95%CI 1.4–4.1, $p = 0.002$). No case of acute leukemia was documented among patients with PV/ET presenting with SVT and seven of them (6%) progressed to myelofibrosis. SVT was not associated with lower risk of MF after correction by age and sex. Patients with SVT more frequently died from complications related to hepatic disease, major bleeding, or second cancer, resulting in a 5-year reduction of age- and sex-adjusted median survival. In conclusion, PV and ET patients presenting with SVT have shorter survival than patients with PV and ET of the same age and sex. This excess mortality is related to liver disease, major bleeding, and second cancer rather than to the natural evolution of the MPN.

Keywords Polycythemia vera · Essential thrombocythemia · Splanchnic vein thrombosis · Myeloproliferative neoplasms

Introduction

Polycythemia vera (PV) and essential thrombocythemia (ET) are myeloproliferative neoplasms (MPN) characterized by an increased risk of thrombosis and bleeding and, in the long term, disease progression to myelofibrosis (MF) and/or acute myeloid leukemia (AML) [1–3]. These events are especially relevant for young patients with PV and ET, in whom

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cardiovascular complications and disease progression result in a reduction of the expected survival [2–5].

Splanchnic vein thrombosis (SVT) is the initial presentation in a minority of MPN patients with PV being the most frequent underlying disease [6, 7]. The younger age at diagnosis of these patients, together with the low mutational load of *JAK2*V617F, suggests that PV/ET patients presenting with SVT would have an MPN with different biological characteristics [8, 9]. However, the rarity of SVT as a form of presentation of the MPNs and the prolonged clinical course of these patients has made difficult to establish the natural history of the disease in such cases.

The aim of the present study was to characterize the natural history of PV and ET patients presenting with SVT.

Patients and methods

Study design

A search of PV and ET patients presenting with SVT as the first manifestation of their disease was performed in three multicentric registries including the Spanish Registry of Polycythemia Vera ($n = 1831$), the Spanish Registry of Essential Thrombocythemia ($n = 1304$), and the Spanish Registry of Hepatic Vascular diseases (REHEVASC). The latter contains information of patients with SVT from any etiology, including 68 cases diagnosed with MPN. We also queried the MPN database of the Hospital Clínic of Barcelona ($n = 926$) since this Hospital is a National Reference centre for SVT. The Spanish Registry of PV is a retrospective/prospective study started on January 2011 and including patients diagnosed after January 1 2000 from 57 recruiting centers. The Spanish Registry of ET is a prospective study started on January 2015 and including patients diagnosed after January 1, 2000 from 36 centers. The REHEVASC is a prospective study started on January 2011 and including patients from 21 centers. The Hospital Clínic database included all MPNs diagnosed after 1980. The study was restricted to patients with PV and ET or with a mild MPN without fibrosis; the latter were categorized as MPN unclassifiable without myelofibrosis.

We identified a total of 227 patients with SVT, with 118 of them displaying SVT as the first manifestation of the MPN (plus/minus 3 months). These 118 patients constituted the object of the present study and were defined as cases. Patients in whom the SVT preceded the diagnosis of MPN were excluded from further analysis to avoid any influence in the different outcomes as a result of the delay in MPN diagnosis. Alternatively, those patients experiencing a first SVT event after MPN diagnosis were included in the control group along with the remainder of patients without SVT ($n = 3587$) since the objective of the study was to assess specifically the natural history of PV and ET patients in whom the SVT was the first manifestation of the disease.

MPN diagnosis was established at the hospital of origin using the World Health Organization valid at time of diagnosis excepting for patients diagnosed before year 2000 in whom PVSG criteria were employed. Regarding the high frequency of masked PV among patients with SVT, isotopic red cell mass measurement was routinely performed in cases with SVT. Informed consent for the scientific use of the patients' clinicohematological data was obtained, and the study was approved by the Hospital Clínic ethics' committee. Main data at presentation of the MPN were collected, including age, sex, cardiovascular risk factors, hemoglobin (Hb) values, leukocyte and platelet counts, and *JAK2*V617F and *CALR* mutational status. Further data included the occurrence of thrombosis and bleeding, development of MF or acute leukemia, and the cause of death.

Outcomes

Survival was the main study outcome. Secondary outcomes included thrombosis, bleeding, disease progression, and second cancer. In the above-mentioned registries, thrombosis was defined as arterial (coronary artery disease, transient ischemic attack/stroke, or peripheral artery disease) or venous (splanchnic vein thrombosis, deep venous thrombosis/pulmonary embolism, superficial thrombophlebitis, and other). Splanchnic vein thrombosis included Budd-Chiari syndrome and thrombosis of the portal, splenic, or mesenteric veins. All cases with thrombosis were centrally reviewed by principal investigator in order to verify adequate allocation. Severe hemorrhage was defined as a symptomatic bleeding in a critical organ or an overt hemorrhage requiring transfusion or associated with a hemoglobin decrease > 20 g/L without transfusion.

Statistical analysis

Survival was calculated by the Kaplan-Meier method, and the incidence rate method was employed to estimate the risk of thrombosis, bleeding, and second cancer. Patients in the control group in whom SVT appeared long after diagnosis were censored at that time with regard to survival and the incidence of further complications. A Cox regression model adjusted for age, sex, and presence of SVT at MPN diagnosis was employed for assessing risk factors associated with shorter survival. Multivariate analyses of factors influencing the incidence rate of thrombosis and bleeding were performed by Poisson regression. The cumulative incidence of disease progression (AML/MDS or MF) was calculated by taking death as a competing risk. Multivariate analyses of factors predicting the hematologic transformation were done within the framework of competing risks by the method of Fine and Gray in which the interpretation of the subdistribution hazard

ratio (SHR) is similar to that of the Cox model. All the statistical analyses were performed with Stata, version 11 (www.stata.com). The time-span splitting method was used to calculate the incidence rates and fitting the Poisson models.

Results

Patient characteristics

The main clinical and hematological characteristics of the patients at diagnosis are shown in Table 1. Patients presenting with SVT were significantly younger, were more frequently diagnosed with PV, and had a lower prevalence of conventional cardiovascular risk factors (diabetes, smoking, arterial hypertension, and hypercholesterolemia), which persisted after adjustment for age and sex (data not shown). Anatomical location of SVT included the suprahepatic veins in 25 cases, the espleno-portal-mesenteric axis in 74, both locations in three, and it was not specified in 16.

The MPN genotype was available in 105 (89%) out of the 118 cases and in 2957 controls (87%). In cases, mutations in *JAK2* and *CALR* were detected in 102 (97%) and two (2%) patients, respectively. Among controls, 2570 (87%) were *JAK2*V617F mutated, 265 (9%) *CALR* mutated, 48 (1.5%) had *MPL* mutations, whereas 74 (2.5%) were triple negative.

Patients with SVT at diagnosis were treated with anticoagulants and double therapy (anticoagulants *plus* antiplatelet agents) more frequently than controls (67% vs. 3%, $p < 0.001$, and 14% vs. 2%, $p < 0.001$, respectively). There was no difference between both groups with regard to exposure to cytoreductive agents (77% vs. 72%, $p = 0.3$).

Survival

After a median follow-up of 5.8 years, 546 patients had died. The projected median survival was 24 (95% CI 20-not reached) and 22 years (95% CI 21–24) for cases and controls, respectively ($p = 0.2$). Causes of death are shown in Table 2. Cases more frequently died from complications related to hepatic disease, major bleeding, or second cancer (Table 2). Death due to disease progression was rare in patients with SVT at diagnosis. Multivariate analysis showed a higher risk of death in MPN patients presenting with SVT (HR 2.47, 95% CI 1.5–4.01, $p < 0.0001$) after adjusting for age (HR 1.090; 95%CI 1.08–1.098, $p < 0.0001$) and male sex (HR 1.57 95% 1.33–1.86, $p < 0.0001$). SVT resulted in a 5-year reduction in the expected median survival as compared with PV or ET patients of the same age and sex without SVT at diagnosis (Fig. 1). This higher risk of death was similar in patients diagnosed in the periods 1980–2000 (HR 2.50, 95%CI 1.33–4.70, $p = 0.004$) and 2001–2018 (HR 2.46, 95%CI 1.15–5.26, $p = 0.02$).

Thrombosis and bleeding

A total of 316 arterial and 231 venous events were registered during the observation period in 123 and 102 patients, respectively. Rates of arterial and venous thrombosis are shown in Table 3. Cases showed a significantly higher incidence rate of

Table 1 Main clinicohematological characteristics of 3705 patients with PV and ET according to the presence or not of splanchnic vein thrombosis (SVT) at diagnosis

	PV/ET with SVT $N = 118$	PV/ET without SVT $N = 3587$	p value
Age[a]	42 (35–55)	65 (52–74)	0.0001
Female sex, *n* (%)	72 (61)	1942 (54)	0.1
Type of MPN			< 0.0001
PV, *n* (%)	76 (64.4)	1852 (51.6)	
ET, *n* (%)	36 (30.5)	1732 (48.3)	
MPNu, *n* (%)	6 (5.1)	3 (0.1)	
Cardiovascular risk factors	35%	66%	< 0.0001
History of arterial thrombosis	3%	13%	0.003
History of other venous thrombosis	3%	4%	0.62
Hemoglobin level g/L	146 (134–166)	158 (14–177)	0.0004
Leukocyte count × 10^9/L	9.9 (7–12.4)	9.5 (7.6–11.9)	0.0004
Platelet count × 10^9/L	469 (324–594)	618 (465–804)	0.0004

ET essential thrombocythemia, *PV* polycythemia vera, *MPN* myeloproliferative neoplasm, *MPNu* myeloproliferative neoplasm unclassifiable without MF

[a] Median (Interquartile range)

Table 2 Causes of death in 3705 patients with PV and ET according to the presence or not of splanchnic vein thrombosis (SVT) at diagnosis

	PV/ET with SVT $N=118$	PV/ET without SVT $N=3587$	Total
Cardiovascular	4 (22.2)[b]	89 (17.0)	93 (17.2)
Second neoplasm	4 (22.2)[c]	73 (13.9)	77(14.2)
Infection	2 (11.1)[a]	73 (13.9)	75 (13.8)
Acute leukemia	0 (0)	97 (18.5)	97 (17.9)
Other	4 (22.2)[d]	56 (10.7)	60 (11.1)
Unknown	4 (22.2)	136 (26.0)	140 (25.8)
Total	18 (100)	524 (100)	542 (100)

ET essential thrombocythemia, *PV* polycythemia vera

[a] Infection due to liver transplantation and advanced hepatic disease, one case each

[b] Upper digestive bleeding, brain hemorrhage, hemorrhagic stroke, and peripheral artery disease, one case each

[c] Colon adenocarcinoma $n=2$, lung adenocarcinoma $n=1$, endometrium adenocarcinoma $n=1$

[d] Liver failure $n=3$, myelofibrosis $n=1$

venous thrombosis than controls, whereas no significant differences were observed regarding arterial thrombosis. In cases, most venous events occurred in the splanchnic territory ($n=21$), whereas deep venous thrombosis/pulmonary embolism (DVT/PE) and superficial thrombophlebitis occurred in six and one case, respectively. In controls, the corresponding figures were 34, 131, 29, and nine events for SVT, DVT/PE, superficial thrombophlebitis, and others, respectively. At multivariate analysis, SVT at PV/ET diagnosis was associated with an increased incidence of venous thrombosis after adjusting for other risk factors (Table 4). Besides SVT, the presence of prior venous thrombosis and female sex was also associated with increased frequency of venous thrombosis during follow-up. Advanced age, male sex, presence of cardiovascular risk factors, and prior arterial events were associated with a higher incidence rate of arterial thrombosis (Table 4).

A total of 164 episodes of major bleeding occurred in 126 patients. The incidence rate of major bleeding was significantly higher in cases than in controls (Table 3). At multivariate analysis, SVT at MPN diagnosis was associated with a 3.6-fold increased risk of developing a major bleeding event during follow-up (Table 4).

Disease transformation and second neoplasms

Progression to MF was documented in 214 patients, seven of them with SVT at MPN diagnosis. The probability of MF transformation at 10 years, taking death as a competing event, was 5.9% (95%CI 4.9–7.1) in the whole cohort of patients. SVT at PV/ET diagnosis was not associated with higher risk of MF after correction for age and sex (SHR 0.67, 95%CI 0.31–1.46, $p=0.3$) (Fig. 2). Ninety-eight progressions to MDS/AML were recorded, with none of them occurring in patients with SVT at diagnosis. The probability of MDS/AML transformation at 10 years in the whole series was 2.31% (95%CI 1.63–2.88). The estimated probability of progression to MDS/AML adjusted for age and sex is shown in Fig. 3.

A total of 288 s neoplasms were diagnosed after MPN diagnosis, 15 of them in patients with SVT at diagnosis. The incidence rate of second cancer was 1.35% and 1.05% per year in cases and controls, respectively. Eighty-two neoplasms corresponded to non-melanoma skin cancer, two in cases, and the rest in controls ($p=0.8$, Table 5). Patients with SVT at PV/

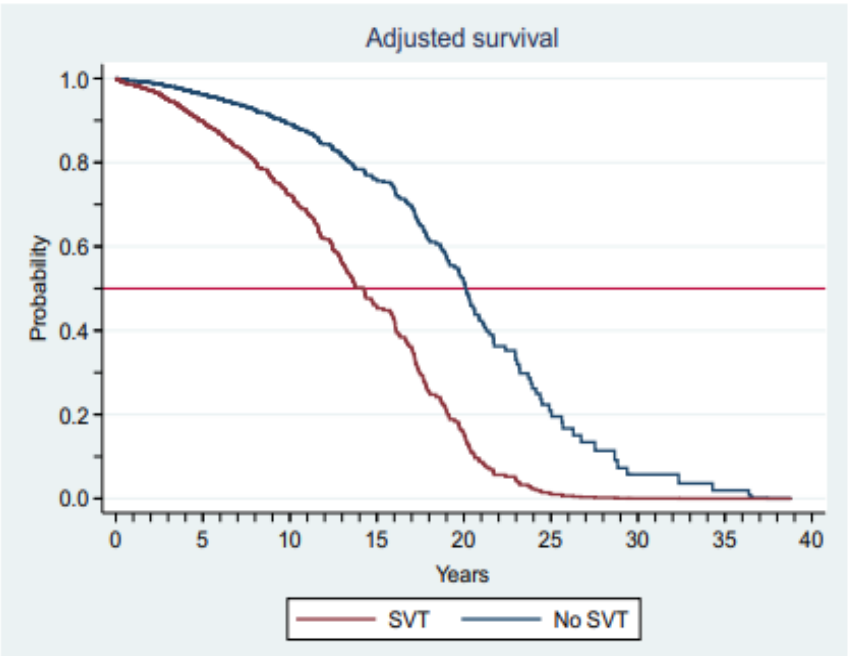

Fig. 1 Estimated survival adjusted by age and sex in polycythemia vera and essential thrombocythemia with splanchnic vein thrombosis (SVT) at diagnosis (red line) or not (blue line) $p=0.001$

Table 3 Incidence of thrombosis and major bleeding in 3705 patients with polycythemia vera or essential thrombocythemia according to the presence of splanchnic vein thrombosis (SVT) at diagnosis

	SVT at diagnosis (1110 person-years)		Remaining patients (26,106 person-years)		
	No events	Incidence rate[a]	No events	Incidence rate[a]	*p* value
Arterial thrombosis	10	0.90	306	1.18	0.4
Venous thrombosis	28	2.55	203	0.78	< 0.0001
Major bleeding	27	2.43	137	0.53	< 0.0001

[a] Events × 100 person-years

ET diagnosis showed a higher risk of developing a second cancer after adjustment for age and sex, even when non-melanoma skin cancer was excluded (Table 5).

Discussion

We have analyzed the natural history of patients with PV and ET in whom SVT was the first manifestation of disease. The main observation of the present study was the reduction in the expected survival in this subset of patients. This excess of mortality was attributable to a higher frequency of fatal hemorrhage, liver failure, and second malignancies, indicating that the outcome of these patients is mostly determined by complications other than MPN progression.

The abovementioned observations might appear in contradiction with previous studies reporting a favorable outcome in PV and ET patients with SVT [8–12]. However, it should be noted that the reduction in survival was only uncovered after correction for age, an aspect generally not explored in the literature [12]. Second, most previous studies also included patients with MF, which may contribute to the general perception that the natural history of SVT is mainly determined by the type of MPN [10, 12]. Third, we have selected only cases in which SVT was the initial manifestation of PV or ET, allowing us to analyze a homogeneous cohort of patients. Finally, we have used a large control group of patients without SVT at presentation included in the Spanish PV and ET registries, who are representative of the current clinical practice in our country.

It has been suggested that MPNs presenting with SVT are biologically different entities because they usually show less altered blood counts, low *JAK2*V617F allele burden load, and a low rate of disease progression [8, 9, 12]. Accordingly, in the present series, no case of transformation to acute leukemia has been documented and only seven patients progressed to MF after a median follow-up of 6 years. However, these results are consistent with previous studies reporting the rarity of progression in young patients during the first decade of the disease [4, 13]. On the other hand, the excess mortality due to liver disease may compete with disease progression in a group of young patients that has a low transformation rate by itself.

Patients with SVT have a high rate of recurrent venous thrombosis [12, 14, 15]. Thus, in the largest studies on the topic, it has been reported an incidence rate of 2.5% and 1.6% venous thromboses and SVT recurrences, respectively [12, 15]. In our series, with a longer follow-up, the rate of recurrence was exactly the same (2.55% per year) and 3-fold higher than the one observed in the control group (0.79% per year). Of note, the rate of venous thrombosis in the control group was similar to that reported in other studies including unselected patients with PV and ET [16, 17].

Table 4 Multivariate analysis of risk factors for arterial thrombosis, venous thrombosis, and major bleeding in 3705 patients with PV or ET

	Arterial thrombosis		Venous thrombosis		Major bleeding	
	IRR (95%CI)	*p*	IRR (95%CI)	*p*	IRR (95%CI)	*p*
Age	1.1 (1.01–1.2)	< 0.001	1.04 (1.0–1.1)	0.1	1.1 (1.01–1.3)	0.008
Male sex	1.5 (1.20–1.91)	0.001	0.7 (0.5–0.9)	0.03	1.0 (0.8–1.2)	0.7
SVT at diagnosis	1.3 (0.7–2.5)	0.4	3.4 (2.1–5.5)	< 0.001	3.6 (2.3–5.5)	< 0.001
CVRF	1.8 (1.4–2.4)	> 0.001	–	–	–	–
Prior arterial thrombosis	1.7 (1.3–2.3)	0.001	–	–	–	–
Prior venous thrombosis	–	–	3.2 (2.02–5.1)	< 0.001	–	–
Prior major bleeding	–	–	–	–	2.0 (1.5–2.6)	< 0.001

PV polycythemia vera, *ET* essential thrombocythemia, *SVT* splanchnic vein thrombosis, *IRR* incidence rate ratio, *CVRF* cardiovascular risk factors

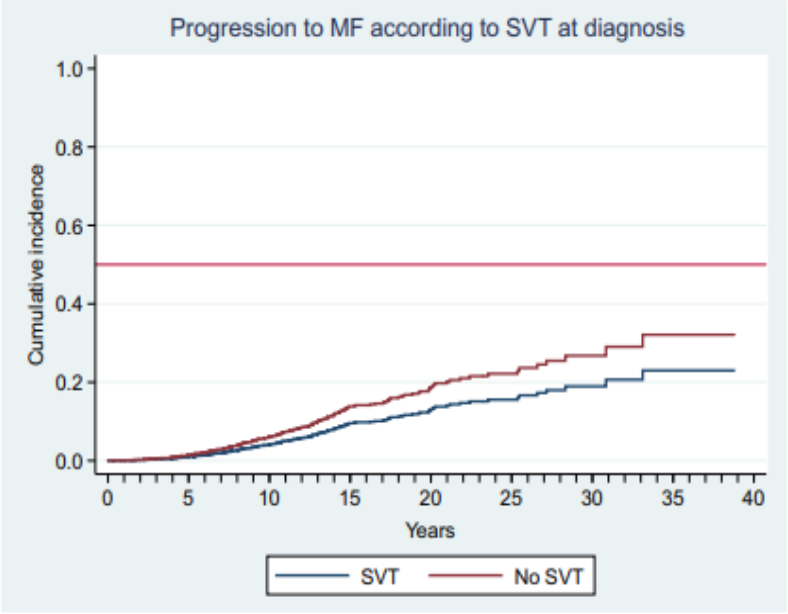

Fig. 2 Estimated probability of progression to myelofibrosis (MF) adjusted by age and sex in polycythemia vera and essential thrombocythemia patients with splanchnic vein thrombosis (SVT) at diagnosis (blue line) or not (red line) p not significant

Another remarkable aspect of the present study is the increased risk of major bleeding in patients with SVT [6, 11, 18]. De Stefano et al. have reported an incidence of severe bleeding of 2.1% per year, very similar to the 2.4% rate observed in our series [15]. The increased risk of bleeding can be explained by a wider use of anticoagulant agents, often associated with antiplatelet agents and the coexistence of esophageal varices with the latter being the main risk factor for major bleeding in a series including 518 MPN-SVT patients [11, 12].

Recently, a higher incidence of second cancer has been reported in patients with MPN [19, 20]. Moreover, exposure to hydroxyurea, pipobroman, or ruxolitinib has been significantly associated with non-melanoma skin cancer [19, 20]. However, and despite the frequent use of hydroxyurea, we did not observe a higher incidence of non-melanoma skin cancer in PV/ET cases presenting with SVT. This could be explained by the young age of these patients, because age and duration of sun exposure are essential risk factors for the development of non-melanoma skin cancer. Surprisingly, when non-melanoma skin cancer was excluded from the analysis, a higher incidence of second cancer was observed in PV and ET patients presenting with SVT in comparison with their age- and sex-matched controls. To the best of our knowledge, this is the first study reporting this

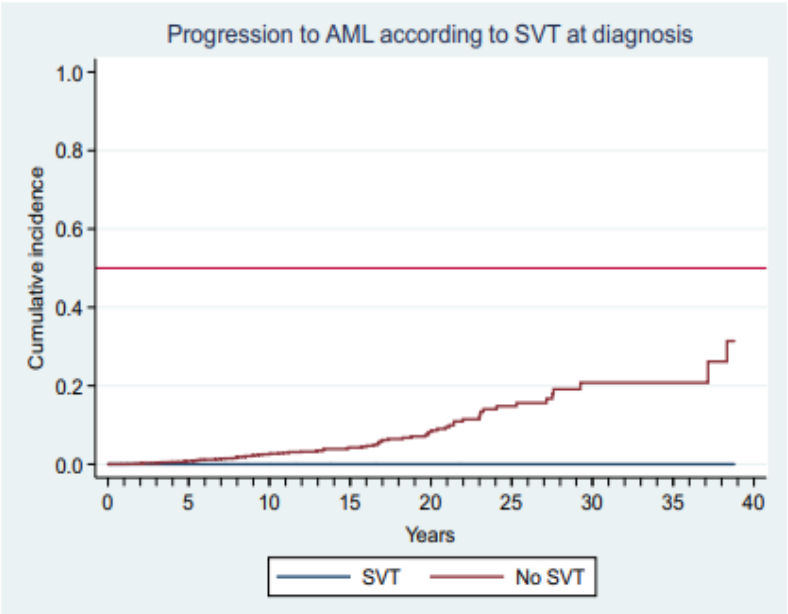

Fig. 3 Estimated probability of progression to myelodysplastic syndrome/acute myeloid leukemia (MDS/AML) adjusted by age and sex in polycythemia vera and essential thrombocythemia patients with splanchnic vein thrombosis (SVT) at diagnosis (blue line) or not (red line) p not significant

Table 5 Multivariate analysis of risk factors for developing second cancers in 3705 patients with PV or ET

	Total cancer		Non-melanoma skin cancer		Cancer (skin cancer excluded)	
	IRR (95%CI)	*p*	IRR (95%CI)	*p*	IRR (95%CI)	*p*
Age	1.19 (1.14–1.24)	< 0.001	1.30 (1.19–1.42)	< 0.001	1.15 (1.11–1.20)	< 0.001
Male sex	1.95 (1.25–3.71)	< 0.001	2.97 (1.80–4.98)	< 0.001	1.67 (1.27–2.17)	< 0.001
SVT at diagnosis	2.15 (1.25–3.71)	0.006	1.31 (0.33–5.23)	0.7	2.37 (1.37–4.11)	0.002

PV polycythemia vera, *ET* essential thrombocythemia, *SVT* splanchnic vein thrombosis, *IRR* incidence rate ratio

association, which calls for additional studies to confirm this observation.

Data collected from registries may be prone to some biases, and there is a non-trivial chance of potentially missing data, what might have influenced on the estimated frequency of second neoplasms. In addition, it can be argued that including small centers may give rise additional issues such as accurate diagnosis and managing of challenging situations, SVT particularly. However, it must be pointed out that the majority of cases with SVT and controls included in the present study were registered from large reference hospitals. Finally, accurate diagnosis of MPN subtype may be particularly challenging in patients with SVT; for this reason, in Spain, isotopic red cell mass measurement is routinely performed in cases with SVT in whom MPN is suspected.

In conclusion, patients with PV and ET presenting with SVT have a reduced survival compared to other patients with PV and ET of the same age and sex. This excess mortality seems to be more related to liver disease and second malignancies than to the natural evolution of the MPN.

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Author's contributions AAL designed the study, collected the data, performed the statistical analysis, analyzed and interpreted the results, and wrote the paper. AP designed the study, performed the statistical analysis, analyzed and interpreted the results, and wrote the paper. MM collected the data, interpreted the results, and wrote the paper. JCHB and FC interpreted the results and wrote the paper. JGP designed the study, interpreted the results, and wrote the paper. MG, BC, FFM, MTGC, VGG, MIMV, FT, and VHG collected the data and approved the final version. A complete list of investigators participating in GEMFIN and REHEVASC studies is provided in supplementary appendix.

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Compliance with ethical standards

Informed consent for the scientific use of the patients' clinicohematological data was obtained, and the study was approved by the Hospital Clínic ethics' committee.

Conflict of interest The authors declare that they have no conflict of interest.

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III.1 Study 3

Autoimmune biomarkers in porto-sinusoidal vascular disease: Potential role in its diagnosis and pathophysiology.

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Autoimmune biomarkers in porto-sinusoidal vascular disease: Potential role in its diagnosis and pathophysiology

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Abstract

Background and aims: Porto-sinusoidal vascular disease (PSVD) is a rare disease that requires excluding cirrhosis and other causes of portal hypertension for its diagnosis because it lacks a specific diagnostical test. Although it has been occasionally associated with autoimmune diseases, the pathophysiology of PSVD remains unknown. The aim of this study was to evaluate the potential role of autoimmunity in the pathophysiology and diagnosis of PSVD.

Methods: Thirty-seven consecutive patients with PSVD and 39 with cirrhosis matched by gender, signs of portal hypertension and liver function were included (training set). By using Indirect Immunofluorescence, ELISA and slot-blot methods data 22 autoantibodies were identified in patients with PSVD and cirrhosis. Presence of anti-endothelial cells antibodies (AECA) was assayed by a cell-based ELISA. Thirty-one

Abbreviations: AECA, anti-endothelial cells antibody; ANA, anti-nuclear antibody; anti-SMA, anti-smooth muscle antibody; anti-TPO, anti-ThyroPerOxidase Ab; anti-tTG, anti-tissue transglutaminase Ab; autoAb, auto-antibody; CI, confidence interval; CTGF, connective tissue growth factor; EC, endothelial cells; ELISA, enzyme-linked immunosorbent assay; HeD, haematological disease patients with splenomegaly; HNL, histological normal liver; HRP, horse radish peroxidase; HSP60, heat shock protein 60; HSP72, heat shock protein 72; HSPs, heat shock proteins; HVPG, hepatic venous pressure gradient; IIF, indirect immunofluorescence; INCPH, idiopathic non-cirrhotic portal hypertension; LR, likelihood ratio; LT, liver transplant; NASH, non-alcoholic steatosis hepatitis; NPV, negative predictive value; OD, optical density; PH, portal hypertension; PPV, positive predictive value; PSVD, porto-sinusoidal vascular disease; PVT, portal vein thrombosis; SD, standard deviation; SEC, sinusoidal endothelial cells; SLE, systemic lupus erythematous; VCAM-1, vascular cell adhesion molecule-1; WB, Western Blotting.

Eira Cerda Reyes, Europa Azucena González-Navarro and Marta Magaz Share first co-authorship

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PSVD, 40 cirrhosis patients, 15 patients with splenomegaly associated with haematological disease and 14 healthy donors were included in a validation set.

Findings: The proportion of patients with at least one positive antibody was statistically significantly higher in patients with PSVD compared with cirrhosis (92% vs 56%; $P < .01$). Specifically, AECA were significantly more frequent in PSVD than in cirrhosis (38% vs 15%; $P = .013$). Results were confirmed in the validation set. In the overall population, presence of AECA had a 63% positive predictive value for diagnosing PSVD and a 71% negative predictive value, with a specificity of 94% when the 1/16 level is used as cut-off. AECA positive serum samples react with a 68-72 kDa protein of human liver endothelial sinusoidal cells.

KEYWORDS

AECA, idiopathic non-cirrhotic portal hypertension, INCPH, porto-sinusoidal vascular disease, PSVD immunological biomarkers

1 | INTRODUCTION

Porto-sinusoidal vascular disease (PSVD), formerly known as Idiopathic non-cirrhotic portal hypertension (INCPH) is a rare disease frequently characterised by the presence of intrahepatic portal hypertension.[1] PSVD does not have a specific diagnostic test and its diagnosis requires the exclusion of cirrhosis and of other causes of portal hypertension (PH) such as splanchnic vein thrombosis among others. This challenges its detection and slow down its diagnosis and many cases remain undetected.[2-4] Although pathophysiology remains largely unknown, immunological disorders, recurrent infections, drug treatments and underlying prothrombotic disorders have been suggested as ethnological factors.[3,5,6]

PSVD has been frequently associated with autoimmune disorders such as systemic lupus erythematous (SLE), progressive systemic sclerosis, chronic thyroiditis, Raynaud´s phenomenon, scleroderma and celiac disease among others conditions, supporting the immunological involvement in the pathogenesis of the disease[7-11]. Furthermore, several studies have shown the presence of different serum auto-antibodies (auto-Ab), such as antinuclear (ANA), or anti-smooth muscle antibodies (anti-SMA), in patients with PSVD.[8,12] In addition, increased expression of human leukocyte antigen—DR molecule (HLA-DR) in the endothelial cells (EC) of the smaller portal venous radicles, as well as a higher number of T-helper 1 cells in peripheral and spleen lymphocytes has been found.[13] Similarly, a pathophysiological role for anti-endothelial cell antibodies (AECA) has been suggested[14-16] hypothesizing that they could promote endothelial-mesenchymal transition in the EC of the small portal veins promoting portal vein stenosis and collagen deposition in peripheral portal tracts.[15] All these findings suggest a potential pathophysiological role of immunological alterations in the pathophysiology of PSVD. However, the potential diagnostical or pathophysiological role of autoimmunity has never been extensively evaluated in patients with PSVD.

The primary aim of our study was to characterise the humoral autoimmune profile in patients with PSVD, evaluating its potential role in its pathophysiology. The secondary aim was to assess the potential value of serum autoAb in the diagnosis of the disease.

2 | PATIENTS AND METHODS

2.1 | Training set

Thirty-seven consecutive patients diagnosed at our unit, between December 1995 and December 2012, with PSVD and PH were included. PSVD diagnosis was based on[2]: (a) presence of unequivocal signs of portal hypertension; (b) discarding cirrhosis, advanced fibrosis or other causes of chronic liver diseases by appropriate serological, biochemical tests and by liver biopsy (performed in all patients); and (c) absence of thrombosis of the hepatic veins or of the portal vein at imaging studies performed at diagnosis. Patients with early (without portal hypertension) or late (with portal vein thrombosis) PSVD stages were not included in this study.[1] Liver biopsies were performed by transjugular[17] or percutaneous route and were re-evaluated by one experienced pathologist (AD) for the purpose of the study.

Patients were followed up every 6 months, and clinical events during follow-up were recorded until, liver transplant (LT), death or September 2019, whichever occurred first. Since 1995, patients with vascular disorders of the liver visited at our unit are prospectively registered (after written informed consent) and a blood sample stored at the IDIBAPS Biobank.

Thirty-nine patients with cirrhosis and PH matched by age, gender, clinical signs of portal hypertension and liver function were included as control groups. In patients with cirrhosis, PH was defined as a hepatic venous pressure gradient (HVPG) ≥10 mm Hg and/or presence of gastroesophageal varices or ascites. Presence of

TABLE 1 Baseline characteristics of patients with porto-sinusoidal vascular disease and with cirrhosis in the training set

	PSVD (n = 37)	Cirrhosis (n = 39)	*P* value
Gender (male), n (%)	25 (67)	26 (67)	.000
Age at diagnosis (y)	41.30 ± 21.30	51.90 ± 8.30	.006
Aetiology cirrhosis			
Alcohol/other	ND	35/4	
MELD	12.00 ± 4.70	11.50 ± 5.70	.864
Child-Pugh (A/B/C)	30/7/0	27/9/3	.444
HVPG (mm Hg)	7.90 ± 4.30	15.40 ± 3.50	.000
Oesophageal varices	33 (89)	34 (87)	1.000
Ascites	5 (13.50)	10 (26)	.184
Splenomegaly	30 (81.10)	27 (69)	.293
Haemoglobin (g/dL)	12.80 ± 1.90	13.10 ± 2.40	.577
Platelet ($\times 10^9$/L)	112 ± 80	140 ± 65	.013
Prothrombin time (%)	71.50 ± 21.30	73.10 ± 14.20	.712
Total bilirubin (mg/dL)	1.80 ± 2.00	1.40 ± 0.80	.186
Creatinine (mg/dL)	0.80 ± 0.60	0.80 ± 0.30	.877
Albumin (g/L)	38.50 ± 4.40	38.30 ± 5.80	.880

Note: Results were expressed as n (%) and means (±SD).

Abbreviation: HVPG, hepatic venous pressure gradient; MELD, model for end-stage liver disease; ND, non defined; PSVD, porto-sinusoidal vascular disease.

TABLE 2 Associated disorders of patients with porto-sinusoidal vascular disease (PSVD) and with cirrhosis in the training set

	PSVD (n = 37)	Cirrhosis (n = 39)
Cardiovascular disorders	**n = 5**	**n = 8**
AH	4[a]	7[a]
Ischaemic heart disease	1[a]	1[a]
Endocrine disorders	**n = 4**	**n = 10**
T2DM	2[a]	7[a]
Hypothyroidism	0	1
Subclinical hypothyroidism	2	2
Neurological disorders	**n = 1**	**n = 0**
Parkinson	1	0
Prothrombotic disorders	**n = 3**	**n = 0**
Protein S deficiency	2[a]	0
ITP	1[a]	0
Lung disorders	**n = 1**	**n = 2**
Pulmonar hypertension	1	1[a]
COPD	0	1
Immunological disorders	**n = 5**	**n = 0**
T1DM	1	0
SLE	1[a]	0
Rheumatoid arthritis	1[a]	0
Graves' disease	1[a]	0
Systemic sclerosis	1[a]	0
Haematological disorders	**n = 4**	**n = 0**
Marginal B cell lymphoma,	1[a]	0
Medullar aplasia	1	0
IgG-monoclonal gammopathy	1[a]	0
Polyclonal B lymphocytosis	1	0
Neoplasia	**n = 1**	**n = 1**
Breast ductal cancer	1	1[a]
HIV Infectious	**n = 12**	**n = 0**
Others	**n = 1**	**n = 0**
Ramsay hunt syndrome	1	0

Abbreviation: AH, arterial hypertension; COPD, chronic obstructive pulmonary disease; HIV, human immunodeficiency virus; ITP, idiopathic thrombocytopenic purpura; SLE, systemic lupus erytematosus; T1DM, diabetes mellitus type 1; T2DM, diabetes mellitus type 2.

[a]PSVD group: one patient had protein S deficiency and ITP, one with ischaemic heart disease, T2DM and AH, one with T2DM and AH, one with rheumatoid arthritis and SLE, one with marginal B-cell lymphoma and systemic sclerosis. Cirrhosis group: one patient had breast ductal cancer and T2DM, one pulmonary hypertension and ischaemic heart disease, one T2DM and AH.

3.2 | Autoantibody determination

Twenty-two different autoantibodies were analysed (Table 3). A significantly higher number of patients with PSVD had at least one autoantibody [34/37 (92%) vs 22/39 (56%) of patients with cirrhosis; $P = .002$]. This difference also remained after moving from the analysis the four patients with PSVD associated with an immune disorder [31/33; 94% vs 56% in cirrhosis; $P < .001$]. The higher rate of autoantibodies was mainly due to a higher prevalence of positive ANA (titer range, 1/80 to 1/640), anti-SMA (>1/80), anti-tTG-IgA, anti-TPO and AECA (titer range, 1/4 to 1/256). However, only AECA were significantly increased (38% in PSVD vs 15% in cirrhosis; $P = .037$). This difference was stronger when the cut-off for AECA was set at 1/16 (22% vs 3%; $P = .0013$). These results were confirmed in the validation set where AECA were positive in 58% of PSVD patients versus 30% of cirrhosis; $P = .029$. If the cut-off was established at 1/16, the respective values were 35.5% versus 12.5%; $P = .043$. AECA were also tested in 15 haematological patients with splenomegaly with any AECA positive and 14 healthy subjects being positive in only one at 1/16 level.

3.3 | Potential diagnostical role of AECA in PSVD

Owing to the different prevalence of AECA between patients with PSVD and cirrhosis, we evaluated the potential diagnostical value of these autoAbs in the overall cohort of patients from the training and the validation set (68 PSVD and 108 control patients, 79 cirrhosis + 15 haematological patients with splenomegaly and 14 healthy subjects; Table 4). Presence of AECA (≥1/4 level) had a 63% PPV and a 71% NPV, with a sensitivity and specificity of 47% and 82%

TABLE 3 Comparison of antibodies between patients with porto-sinusoidal vascular disease and with cirrhosis in the training set

Antibodies	PSVS (n = 37)	cirrhosis (n = 39)	*P* value
At least one	34 (92)	22 (56)	**.002**
ANA > 1/80	11 (30)	5 (13)	.092
AMA > 1/80	0 (0)	3 (8)	.240
Anti-PCA > 1/80	5 (13)	2 (5)	.431
Anti-SMA > 1/80	15 (41)	9 (23)	.141
Anti-LKM > 1/80	0	4 (10)	NA
Anti-M2	1 (3)	0	1.000
Anti LC1	0	0	NA
Anti-SLA	0	1 (3)	1.000
Anti-LKM1	3 (8)	0	.240
Anti-F-actin	2 (5)	0	.494
Anti-U1RNP	0	0	NA
Anti-Scl70	1 (3)	0	1.000
Anti-Ro	1 (3)	0	1.000
Anti-La	0	0	NA
Anti-tTG-IgA	6 (16)	2 (5)	.148
Anti-TPO	10 (27)	4 (10)	.138
Anti-RF	3 (8)	1 (3)	.615
Anti-Sm	0	1 (3)	1.000
Anti-Lupus AC	0	1 (3)	.494
ACA-IgG	2 (5)	3 (8)	.675
ACA-IgM	1 (3)	2 (5)	.605
AECA 1/4	14 (38)	6 (15)	**.037**
AECA 1/16	8 (22)	1 (3)	**.013**

Note: Results expressed in n (%).

Abbreviation: NA, not applicable.

respectively with a positive and a negative LR of 2.7 and 0.6 respectively for the diagnosis of PSVD. If the cut-off for AECA is increased to 1/16, PPV, NPV, sensitivity, specificity, LR+ and LR− being 73%, 67%, 28%, 94%, 4.3 and 0.8 respectively.

3.4 | Potential role of AECA in the pathophysiology of PSVD

Double IFF staining of HNL with serum from patients with positive AECA (either with PSVD or cirrhosis) showed positive staining in vessels within the portal tracts (Figure 1). To identify the antigen which reacts with AECA, sera from patients with AECA positive (8 PSVD and 1 cirrhosis) and from 12 AECA negative patients (8 PSVD and 4 cirrhosis) were incubated with EA.hy926 cell lysate (primary endothelial source) in a WB assay. A reactive band directed against a 68-72-kDa protein of EA.hy926 cell was identified in all nine sera from AECA positive patients (Figure 2) and only in 3 of the 12 AECA negative patients.

3.5 | AECA positivity and clinical outcome in patients with PSVD

Regarding clinical outcome the time of diagnosis of PSVD was considered as the time 'zero' and patients were followed up until death, LT, or September 2019. Median follow-up was 7.45 ± 7.93 [0.28-38.97] years. Thirty-three of the 68 patients with PSVD (48.5%) had one or more clinical decompensations during follow-up (31 patients variceal bleeding, 10 ascites and 3 hepatic encephalopathy). Three patients died (1 due to a larynx neoplasia, 1 due to variceal bleeding and 1 due to septic shock). LT was successfully done in one patient who had chronic hepatic encephalopathy. The overall cumulative probability of decompensation development was 19%, 21% and 31% at 1, 2 and 5 years of follow-up. AECA presence (either at 1/4 or at 1/16 levels) was not associated with a higher probability of developing clinical decompensation (data not shown).

In addition, 24 (35.3%) patients developed portal vein thrombosis (PVT) during follow-up. Overall cumulative probability of developing PVT was 12%, 28%, and 30% at 1, 2 and 5 years respectively. AECA positivity was not associated with PVT development (data not shown).

4 | DISCUSSION

Porto-sinusoidal vascular disease is a rare cause of non-cirrhotic PH with an unknown aetiology. The results of this study support, as previously suggested, a potential role for an immunological-mediated mechanism in the pathophysiology for at least a subgroup of PSVD patients. In addition, it demonstrates that the finding of different autoantibodies is frequent in patients with PSVD and therefore its presence should not discard the diagnosis of PSVD or being a definite reason to diagnose an autoimmune liver disorder. On the contrary, our study shows that the presence of auto-Abs is significantly higher in patients with PSVD than in patients with cirrhosis, despite non-alcoholic steatosis hepatitis (NASH) patients were also included in the cirrhotic cohort and ANA have been shown to be positive in

TABLE 4 Comparison of anti-endothelial cells antibodies (AECA) in the overall cohort

	PSVD (n = 68)		Control group (n = 108)		
	Positive	Negative	Positive	Negative	*P* value
1/4 AECA	32	36	19	89	<.001
1/16 AECA	19	49	7	101	<.001

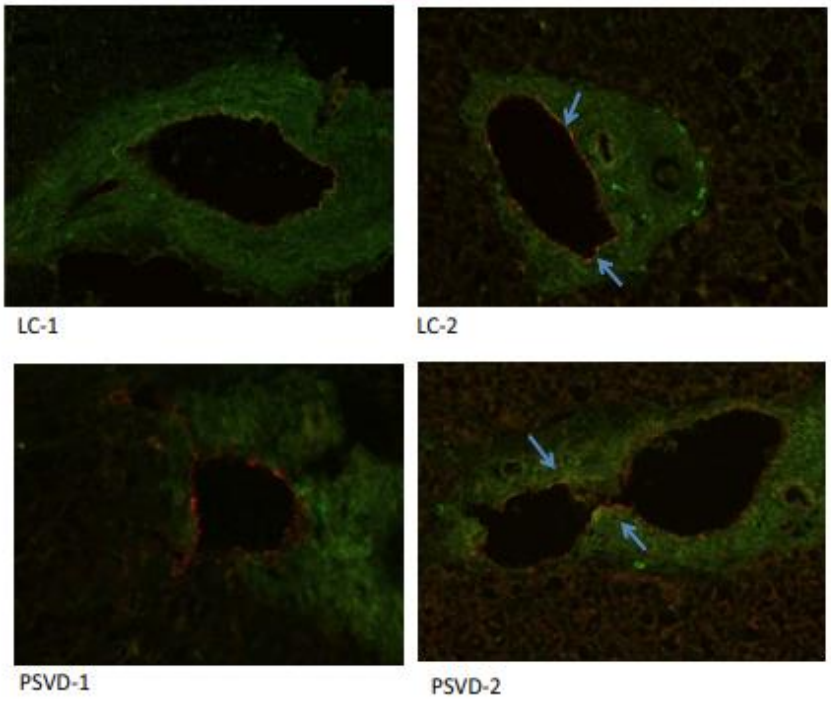

FIGURE 1 Double immunofluorescence (IF) staining of histological normal livers with serum from patients with negative (PSVD-1 and Cirrhosis-1) and positive AECA (PSVD-2 and Cirrhosis-2). A positive stained vessel at peripheral portal tracts was observed in patients with positive AECA (green). Anti-human von Willebrand Factor was used to identify endothelial cells (red). Semi-quantitative analysis demonstrated a relation between intensity of IF and serum autoantibody titers. AECA, anti-endothelial cells autoantibody; PSVD, porto-sinusoidal vascular disease

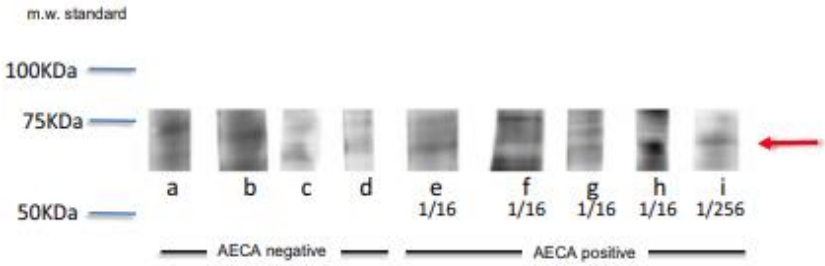

FIGURE 2 Representative Western blot for detection of antigen which anti-endothelial cells antibodies (AECA) are reactive. A band in the region of 68-72 kDa was found

NASH cirrhosis in absence of autoimmune hepatitis.[20] When focusing in the specific autoAbs found in PSVD patients, although there was a trend for increased reactivity for ANA, anti-SMA and anti-tTG-IgA, the only autoAb that were significantly increased were AECA. Although Anti-SMA Ab was also highly prevalent, autoimmune hepatitis was disregarded because the biopsies did not have characteristic data of plasma cells, interphase hepatitis or hepatocyte rosetting, in addition to performing the simplified criteria for the diagnosis of AIH,[21] the scale was <6 points in each patient.

It is worth noting that the presence of AECA could facilitate the diagnosis of patients with PSVD. Unfortunately, the diagnostic capacity of AECA was modest. However, AECA positivity, especially at a titer ≥1/16 in a patient with a chronic liver disease may become, together with low elastography or low HVPG values, an additional parameter increasing the probability that the patient has a PSVD. By contrast, AECA negativity, although not discarding, will not reinforce PSVD suspicion.

In addition, the results of our study also suggest that AECA may have a pathophysiological role in PSVD. AECA have been identified as circulating autoAbs targeting the EC.[14] The pathogenic implication of AECA has already been defined in other diseases such as systemic sclerosis.[22] Targeted antigens of AECA may be different among various pathological conditions and even in the same disease.[23] Although AECA have been previously described in patients with PSVD,[15] its significance and targeted protein in PSVD is largely unknown. The definition of AECA is usually restricted to autoAbs targeting the antigens expressed on the EC surface.[23] Our indirect IFF analysis in HNL tissue clarified the binding capacity of serum AECA to vascular EC of the liver. However, CytoELISA could detect some kinds of antibodies against cytoplasmic antigens and because of that; we aimed to identify the antigens to which the serum of patients with positive AECA was reacting. We observed a band directed against a 68-72-kDa protein at WB. Several heat shock proteins (HSPs) with a molecular weight in the range of 68-72 kDa had been described in the literature.[24] Interestingly, HSP72 (within the HSP family) increases its endothelial expression in different stress situations[25] and it is an early and sensitive indicator of hepatotoxicity and hepatic injury.[26,27] In addition, several studies show that ischaemia-reperfusion and hypoxia induce the expression of HSP72 in rat livers.[28-30] On the other hand, AECA have been suggested to contribute to the pathogenesis

of systemic vasculitis and vasculitis-associated diseases,[31] specifically through binding in the membrane of EC to phospholipids or HSP60, among others, activating and inducing EC apoptosis.[14,23,29,30] EC apoptosis could further induce the expression of HSPs. Moreover, Motoyama et al[32] suggested that sinusoidal endothelial cells (SEC) are the more sensitive liver cells to HSP72 expression on injury, demonstrating indeed that all apoptotic SECs express HSP72. All these data suggest that AECA could play an active role in PSVD pathogenesis via an antibody-mediated immune mechanism.

Remarkably, apoptotic EC have an increased secretion of connective tissue growth factor (CTGF) that is able to promote fibrosis.[33] In that regard, levels of CTGF in sera and in liver tissue are known to be up-regulated in patients with PSVD,[34] which is in keeping with a potential role of vascular endothelial cell apoptosis in the pathogenesis of PSVD.[35] Sera from patients with SLE containing IgG AECA induce in cultured EC the expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1).[36] Interestingly, soluble VCAM-1 serum levels are also elevated in PSVD patients, and VCAM-1 is expressed in vascular endothelial cells in liver of patients with PSVD.[37] All these findings together support the potential contribution of AECA in the pathogenesis of PSVD.[15] Thus, we hypothesise that damage to EC, arising from different etiological insults, could be the first step in the pathophysiology of PSVD. Furthermore, the expression of the HLA-DR molecule on EC of portal venous radicles, secondary to portal venous damage [13] could facilitate the development of other autoimmune mechanisms, mainly cell mediated. All these immunological mechanisms (cell and antibody mediated) are not exclusive, and probably they can be present simultaneously. It is likely that an immune-mediated mechanism is only present in part of the patients with PSVD. Indeed, several aetiological factors have been suggested for PSVD. It may be that some of these may cause a similar or very close clinical disease through non-immune-mediated mechanisms. This would be in relation to our finding that only around a quarter of patients with PSVD exhibit AECA.

In conclusion, our findings suggest that AECA determination may be an additional tool to facilitate the diagnosis of PSVD. In addition, our study provides evidence that supports the contribution of humoral autoimmunity in a subgroup of patients with PSVD. AECA may activate EC inducing endothelial apoptosis contributing to PSVD lesions.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

ECR and EAG-N.: study concept and design, acquisition of data; WB: analysis, interpretation of data and drafting of the manuscript; GM-S., GS-J. and SS: statistical analysis and drafting of the manuscript; LR and AD: interpretation of imaging studies; SS, FT, AT, AB, MM, GC, VPC, and EL: study concept and design, analysis and interpretation of data, drafting of the manuscript; VH-G. and AA-L.: critical revision of the manuscript for important intellectual; MJ and JCG-P.: study concept and design, analysis and interpretation of data, statistical analysis, drafting of the manuscript, obtained funding and study supervision. All the authors discussed and critically revised the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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IV. Discussion

The understanding of rare liver vascular disorders requires special considerations based on a multidisciplinary approach and cooperation among referral centres specialized in this kind of disorders, being essential coordinating efforts to gather knowledge, in order to reach an accurate diagnosis and search targeted treatments. Although liver vascular disorders are rare disorders, they represent a major health problem in the hepatology field. These liver vascular disorders share a widespread variety of diseases that present the common characteristic in that they could cause non-cirrhotic PH and develop PH complications with the consequent morbidity and mortality.

Besides, it is worth highlighting that these disorders affect usually young people, with an otherwise normal life expectancy that can be significantly shortened if there are not properly managed. These diseases progress in the absence of treatment capable of modifying the natural history and therefore treatment is currently symptomatic. In this context, our second study summarizes the common efforts of several specialized referral centres to optimize the clinical approach of two different rare disorders. The present thesis highlights that the collaboration among reference centers allows the accumulation of patients and the feasibility of this kind of studies with the aim of optimizing the clinical approach of non-cirrhotic SVT and PSVD.

The first study of this thesis focuses in the field of non-cirrhotic SVT. Many previous studies have addressed the clinical impact of this disease, being especially remarkable the development of PH-related complications and the complications derived from each precise thrombosis' localization. The etiology of SVT has been classified into thrombophilic disorders that include congenital and acquired thrombophilia and local inflammatory processes (local factors) that are able to trigger the development of thrombosis. These two causes are not mutually exclusive and it is fair to suspect that their combination could increase the risk of developing thrombotic events.

Remarkably, when no underlying prothrombotic cause is identified (approximately in 30% of cases), current guidelines recommend a different clinical management as the long-term anticoagulation is not recommended. Thereupon, it is relevant to identify the SVT prothrombotic factors in order to get an adequate approach and to avoid further complications because the prognostic changes as well as the way of treating these patients. Taking this into account, to perform a complete thrombophilia study in this population would be justified.

Specifically, in our first study all the patients included had a comprehensive coagulation study, including the determination of classical mutations for MPN (*JAK2, CALR,* or *MPL)* by conventional techniques. In addition, we analysed 5 patients with SVT with an underlying MPN, diagnosed by histological criteria, but negative for the three classical mutations. As we previously mentioned, the diagnosis of an underlying prothrombotic disorder in SVT is key because it influences the decision to maintain the long-term anticoagulation. The identification of the classical haematological mutations has represented an important progress, facilitating the diagnosis of MPN. Despite this fact, there are patients with MPN that are negative for these three mutations, and surprisingly, several large cohorts of patients with SVT from Europe and Asia did not detect any case of *JAK2 exon 12* mutation(27,41).

In our first study NGS-based sequencing was able to detect *JAK2 exon 12* mutation in 3 patients who were negative by conventional sequencing. BCS was present in 2 of the 3 triple-negative patients. Nevertheless, the diagnosis of MPN in these two patients was already performed based on clinical and histological criteria. Thus, no further patients with BCS and an underlying MPN were diagnosed. These data emphasise that in patients with BCS, if an extensive evaluation is performed, a potential underlying MPN will be finally identified.

However, NGS clearly would facilitate this task. The sensitivity of traditional sequencing is recognised to be consistent from approximately 15% to 20% mutant allele frequency (27,42); therefore, cases with low mutation loads could be misdiagnosed. Remarkably, the three patients of our cohort diagnosed by NGS had an allele load far below 10%. Additionally, it has been shown that *JAK2 V617F* allele burden is usually < 10% in MPN patients with associated SVT and clearly below than that observed in MPN patients without SVT (median 5% vs. 36.3%; p = 0.019) (43).

Then, detection of *JAK2 at exon 12* led to the diagnosis of an underlying MPN in 3 of 122 (2.5%) patients with SVT in our cohort. These results suggest that NGS technology would facilitate and increase the number of detections of MPNs in the context of SVT.

In addition, NGS identified HMR-variants in more than one third of our patients with Idiop/loc-SVT. HMR-variants are mutations involved in myeloid disorders different from *JAK2, CALR and MPL* that have been related with clonal haematopoiesis and that are related to a myeloid clonal pathology and/or pro-inflammatory state (44), already described in other diseases as coronary artery disease for instance(45). This percentage is markedly higher than the observed in healthy individuals (2-3%) even those aged over 70 (around 10%) (46,47), strongly supporting a pathophysiological role of these variants in SVT (48,49).

Despite the low number of events, patients holding HMR-variants seem to present a greater risk of splanchnic recurrent thrombosis, suggesting a pathogenetic role for HMR-variants that could promote SVT. Importantly, this higher risk associated with HMR-variants persisted after adjusting patients by age. There seems also to be a trend towards a higher risk of splanchnic recurrent thrombosis in those patients with two HRM-variants compared with those with a single HRM-variant, although it was not statistically significant, probably because of the low number of events. This fact would be in accordance with previous data that suggest that patients with two or more HMR variants could present more complications or a worse prognosis, in particular in Polycythaemia vera where the presence of ≥1 mutations significantly increased the risk of a thrombotic event(49)and in primary myelofibrosis were associated with shortened leukaemia-free survival(50,51).

Most patients (both those who detected *JAK2 exon 12* and those in whom an HMR-variant was observed) did not present significant differences in the number of platelets or the size of the spleen compared with those patients who did not present these mutations. Then, if we applied the criteria based on the European group work to avoid unnecessary tests (platelets >200 [$\times 10^9$/L] and spleen $\geq$16 cm) (52), only in 1 patient of the SVT-MPN group could have been diagnosed. This fact would be one more reason to support the employment of NGS.

Interestingly, our study showed more representation of HMR-variants in patients without any known cause of thrombosis, what supports the possible role of HMR-variants in the thrombosis development. These data encourage looking for HMR-variants, because it would be of special interest in patients with SVT in whom no local factors are identified. Also, it was observed that 7.1% (2/28) of patients with an HMR-variant had a local factor, therefore although it seems more cost effective to perform NGS in idiopathic patients with respect to an exclusive local factor, it is not negligible in 7% of patients in whom the therapeutic attitude would change and anticoagulant treatment should be maintained.

HMR-variants seemed not to influence the risk of developing extra-splanchnic thrombotic events. This may be explained by the mixed and heterogeneous extra-splanchnic thrombotic events and their different pathophysiological mechanisms.

In our study and in agreement with previous MPN studies (49,53), the *HMR-variants TET2, DNMT3A, and ASXL1* were the most frequently mutated genes. Recently, it has been reported that these 3 genes (*TET2, DNMT3A, and ASXL1*) are associated with a higher thrombotic risk in Polycythaemia vera (53). Some of these genes, as *TET2*, have essential functions that are independent of their enzymatic activity. In particular, *TET2* deficiency results in an increased

pro-inflammatory phenotype in murine macrophages that could favour atherosclerosis development and thrombosis (54). We also detected a not despicable prevalence of variants in *CEBPA* gene. *CEBPA* is a relevant factor in driving the development of myeloid cells and regulates *TET2* transcription (55).

Furthermore, it has been described that knock-in mice with *CEBPA* variants predispose mice to myeloproliferative disorders. Our data show that the introduction of NGS in the study of patients with SVT may improve the diagnosis of MPN and it might even have prognostic implications (44). Besides, this approach allows the simultaneous analysis of mutations at *JAK2 (JAK2V617F and exon 12), MPL,* and *CALR* genes, and could reduce the need for additional studies and the number of invasive explorations, such as bone marrow biopsies, particularly in patients with suspected Polycythaemia vera. Nowadays, the main limitation for the implementation of an NGS-based strategy to study all SVT patients is its spare availability and its high cost.

Given that *JAK2V617F* is the most frequent molecular alteration in patients with SVT (around 35% in patients with BCS and 20% of patients with NC-PVT) (22,56,57), it could be more cost-effective to screen first for *JAK2V617F* and to perform NGS only in those patients that are *JAK2V617F* negative. Although it is certain that NGS allows simultaneously determining the presence of HMR-variants and recent data suggest that patients with a *JAK2-positive* MPN that additionally hold an HMR-variant had a worse prognosis than those MPN patients that only had the *JAK2* mutation (49). If these data are confirmed, together with our results suggesting that the exclusive presence of an HMR-variant may be a prothrombotic risk, NGS could become the initial technique to assess patients with SVT in selected patients.

Accordingly with our results, we have proposed an easy clinical management algorithm to guide evaluation in patients with SVT.

The second study analyses the natural history of patients with SVT and an underlying MPN. This has been scarcely studied. Although the previous reports and series have enabled the understanding of these diseases, the limited and insufficient data make it necessary to further explore these conditions. Subsequently, we conducted this retrospective/prospective multicentric study, reporting one of the largest and best characterized series. The first remarkable fact is that the collaboration of 36 different hospitals was required to gather this large cohort of patients, which emphasizes the difficulty of bringing together so many patients with two coexistent rare disorders.

We explored the natural history of patients with PV and ET in whom SVT was the first manifestation of disease. The main observation of the present study was the reduction in the

expected survival in this subgroup of patients. This excess of mortality was attributable to a higher frequency of haemorrhage, liver failure, and second malignancies, indicating that the outcome of these patients may be mostly determined by complications other than MPN progression. The abovementioned observations might seem in contradiction with previous studies reporting a favourable outcome in PV and ET patients with SVT (43,58,59). Nevertheless, it should be highlighted that the reduction in survival was only uncovered after correction for age, an aspect generally not explored in previous studies (60).

Secondly, most previous studies also included patients with MF, which is the most serious MPN and has the worst prognosis, so it might have contributed to the general perception that the natural history of SVT is mainly determined by the type of MPN (60,61).

Third, we only selected patients in which SVT was the initial manifestation of PV or ET, making it possible to analyse a more homogeneous cohort of patients. Finally, we used a large control group of patients without SVT at presentation included in the Spanish PV and ET registries, who are representative of our current clinical practice. MPNs presenting with SVT have been suggested to be biologically different entities because they usually show low *JAK2V617F* allele burden load, less altered blood counts and a lower rate of disease progression. Accordingly, in the present series, no case of transformation to acute leukaemia was documented and only seven patients progressed to MF after a median follow-up of 6 years. However, these results are consistent with previous studies reporting the rarity of progression in young patients during the first decade of the disease (62).

Moreover, the excess mortality due to liver disease may compete with disease progression in a group of young patients that has a low transformation rate by itself. Patients with SVT have a high rate of recurrent venous thrombosis (63). Thus, in the largest studies on this topic, it has been reported an incidence rate of 2.5% and 1.6% venous thromboses and SVT recurrences, respectively (64). In our series, with a longer follow-up, the rate of recurrence was exactly the same (2.55% per year) and 3-fold greater than the one observed in the control group (0.79% per year). Of note, the rate of venous thrombosis in the control group was similar to the reported in previous studies including unselected patients with PV and ET (65,66).

Another remarkable aspect of the present study is the increased risk of major bleeding in patients with SVT. *De Stefano et al*. had reported an incidence of severe bleeding of 2.1% per year, very similar to the 2.4% rate observed in our series (64). The increased risk of bleeding could be explained by a wider use of anticoagulant agents, often associated with antiplatelet

agents and the coexistence of oesophageal varices with the latter being the main risk factor for major bleeding in a series including 518 MPN-SVT patients(60).

On the other hand, recently a higher incidence of second cancer has been reported in patients with MPN (67,68). The exposure to hydroxyurea, ruxolitinib or pipobroman, has been significantly associated with non-melanoma skin cancer (68). In our study and despite the frequent use of hydroxyurea, we did not observe a higher incidence of non-melanoma skin cancer in PV/ET cases presenting with SVT. This could be explained by the young age of these patients, because age and duration of sun exposure are essential risk factors for the development of non-melanoma skin cancer. Interestingly, when non-melanoma skin cancer was excluded from the analysis, a higher incidence of second cancer was observed in ET and PV patients presenting with SVT in comparison with their age- and sex-matched controls. To the best of our knowledge, this is the first study reporting this association, which calls for additional studies to confirm this observation.

The main limitation of our study arise from the data collected from registries that may be prone to some biases, and there is a non-trivial chance of potentially missing data, what might have influenced on the estimated frequency of second neoplasms. Additionally, it could be argued that including small centres may give rise to additional issues such as accurate diagnosis and managing of challenging situations, particularly SVT. Although it must be pointed out that most of the with SVT and controls included in the present study were rigorously registered from large reference hospitals. Finally, accurate diagnosis of MPN subtype may be particularly challenging in patients with SVT; for this reason, in all these centres isotopic red cell mass measurement is routinely performed in cases with SVT in whom MPN is suspected.

Finally, the third study evaluated the potential role for an immunological-mediated mechanism in the pathophysiology of porto-sinusoidal vascular disorder. The results of this study support that the finding of different autoantibodies is frequent in patients with PSVD and therefore its presence should not discard the diagnosis of PSVD or being a definite reason to diagnose an autoimmune liver disorder. Otherwise, our study shows that the presence of auto-Abs is significantly higher in patients with PSVD than in patients with cirrhosis, despite non-alcoholic steatosis hepatitis (NASH) patients were also included in the cirrhotic cohort and ANA have been shown to be positive in NASH cirrhosis in absence of autoimmune hepatitis (69).

When we focused in the specific auto-Abs found in PSVD patients, although there was a trend for increased reactivity for anti-SMA, ANA, and anti-tTG IgA, the only auto-Abs that were significantly increased were AECA. Although Anti-SMA Ab was also highly prevalent,

autoimmune hepatitis was disregarded because the biopsies did not have characteristic data of plasma cells, interphase hepatitis or hepatocyte rosetting, in addition to performing the simplified criteria for the diagnosis of AIH (70), the scale was <6 points in each patient.

It is worth highlighting that the presence of AECA might facilitate the diagnosis of patients with PSVD. Unfortunately, the diagnostic capacity of AECA was modest. However, AECA positivity, especially at a titer ≥1/16 together with low elastography (71) or low HVPG values, constitutes an additional parameter increasing the probability that the patient has a PSVD. By contrast, AECA negativity, although not discarding, will not reinforce PSVD suspicion.

In addition, the results of our study also suggest that AECA may have a pathophysiological role in PSVD. AECA have been identified as circulating auto-Abs targeting the EC (38). The pathogenic implication of AECA has been defined in other diseases such as systemic sclerosis(72). Targeted antigens of AECA may be different among various pathological conditions and even in the same disease (73).

Despite AECA have been previously described in patients with PSVD, its significance and targeted protein in PSVD is unknown. The definition of AECA is usually restricted to auto-Abs targeting the antigens expressed on the EC surface. Our indirect IFF analysis in HNL tissue clarified the binding capacity of serum AECA to vascular EC of the liver. Nonetheless, CytoELISA could detect some kinds of antibodies against cytoplasmic antigens and because of that we aimed to identify the antigens to which the serum of patients with positive AECA was reacting. We observed a band directed against a 68-72-kDa protein at WB. Several heat shock proteins (HSPs) with a molecular weight in the range of 68-72 kDa has been described in the literature(74).

Interestingly, HSP72 (within the HSP family) increases its endothelial expression in different stress situations (75). and it is an early and sensitive indicator of hepatotoxicity and hepatic injury (76,77). In addition, several studies show that ischaemia-reperfusion and hypoxia induce the expression of HSP72 in rat livers(78,79).

On the other hand, AECA have been suggested to contribute to the pathogenesis of systemic vasculitis and vasculitis-associated diseases(80), specifically through binding in the membrane of EC to phospholipids or HSP60, among others, activating and inducing EC apoptosis (78,79). EC apoptosis could further induce the expression of HSPs. Moreover, *Motoyama et al* (81) suggested that sinusoidal endothelial cells (SEC) are the more sensitive liver cells to HSP72 expression on injury, demonstrating indeed that all apoptotic SECs express HSP72. All these data suggest that AECA could play an active role in PSVD pathogenesis via an antibody-mediated

immune mechanism. Remarkably, apoptotic EC have an increased secretion of connective tissue growth factor (CTGF) that is able to promote fibrosis (82).

In that regard, levels of CTGF in sera and in liver tissue are known to be up-regulated in patients with PSVD(83), which is in keeping with a potential role of vascular endothelial cell apoptosis in the pathogenesis of PSVD(84). Sera from patients with SLE containing IgG AECA induce in cultured EC the expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1). Interestingly, soluble VCAM-1 serum levels are also elevated in PSVD patients, and VCAM-1 is expressed in vascular endothelial cells in liver of patients with PSVD (85).

All these findings together support the potential contribution of AECA in the pathogenesis of PSVD. Thus, we hypothesise that damage to EC, arising from different etiological insults, could be the first step in the pathophysiology of PSVD. Furthermore, the expression of the HLA-DR molecule on EC of portal venous radicles, secondary to portal venous damage, could facilitate the development of other autoimmune mechanisms, mainly cell mediated. All these immunological mechanisms (cell and antibody mediated) are not exclusive, and probably they can be present simultaneously. It is likely that an immune-mediated mechanism is only present in part of the patients with PSVD. Indeed, several etiological factors have been suggested for PSVD. It may be that some of these may cause a similar or very close clinical disease through non-immune-mediated mechanisms. This would be in relation to our findings that only around a quarter of patients with PSVD exhibit AECA. In conclusion, our findings suggest that AECA determination may be an additional tool to facilitate the diagnosis of PSVD. In addition, our study provides evidence that supports the contribution of humoral autoimmunity in a subgroup of patients with PSVD. AECA may activate EC inducing endothelial apoptosis contributing to PSVD lesions.

V.Conclusions

1) NGS allows the simultaneous evaluation of multiple genes associated with myeloproliferative neoplasms, even at low mutational levels.
2) NGS could identify *JAK2-exon12* mutations not previously detected by the conventional techniques.
3) NGS also identified high-molecular-risk HMR-variants associated with clonal haematopoiesis.
4) Patients with idiopathic/local factor SVT harbouring HRM-variants seem to be at a higher risk of recurrent splanchnic thrombosis.
5) Patients with PV and ET presenting with SVT seem to have a reduced survival compared to other patients with PV and ET of the same age and sex.
6) Patients with SVT died more frequently from complications related with to hepatic disease, major bleeding and second cancer (excluding non-melanoma skin cancer).
7) Indicating this excess of mortality seems more related by complications other than MPN progression.
8) The proportion of patients with at least one positive antibody seems higher in patients with PSVD compared with cirrhosis.
9) AECA determination could be an additional tool to ease the diagnosis of PSVD.
10) Humoral autoimmunity may play a role in a subgroup of patients with PSVD.
11) AECA may activate EC inducing endothelial apoptosis, what could contribute to PSVD lesions.

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