Etiology and Clinical Outcome of Budd-Chiari Syndrome and Portal Vein Thrombosis

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Etiology and Clinical Outcome of Budd-Chiari Syndrome and Portal Vein Thrombosis

Etiologie en klinisch beloop van Budd-Chiari syndroom en vena portae trombose

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INTRODUCTION

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VASCULAR LIVER DISORDERS

The liver receives approximately one-third of the resting cardiac output. Blood flow to the liver is supplied by both an arterial (hepatic artery) and a venous (portal vein) system and three hepatic veins provide drainage of blood from the liver to the inferior vena cava. The hepatic vascular system is quite dynamic and has the ability to function as a reservoir for blood within the general circulation. Different conditions can interfere with hepatic blood flow and cause disease. The most important clinical syndrome affected by obstruction within the liver vasculature is portal hypertension. Portal hypertension is defined by an increase in the pressure of the portal venous system which results from a disruption of normal blood flow at either a prehepatic, intrahepatic or posthepatic level. The most common cause of portal hypertension in the Western world is liver cirrhosis, leading to an elevated portal pressure due to an increased resistance to intrahepatic blood flow as a result of architectural distortion of the liver.

In the absence of liver cirrhosis, numerous less common disorders are known to cause, so-called, non-cirrhotic portal hypertension. Two rare diseases, characterized by thrombosis of the large hepatic vessels are Budd-Chiari syndrome (BCS) and portal vein thrombosis (PVT). Both these disorders share certain features, such as etiologic factors causing thrombosis and the development of portal hypertension, but are considered as separate disease entities based on the location of venous obstruction and their variable clinical presentation. BCS is defined as an obstruction of the hepatic venous outflow tract, ranging from the level of the small hepatic veins up to the junction of the inferior vena cava with the right atrium. Most cases of BCS in the Western world are caused by thrombosis of the hepatic veins, sometimes in combination with thrombosis of the inferior vena cava. The exact incidence of BCS is unknown but is estimated around 1 per million. Thrombotic occlusion of the portal vein is somewhat more common, especially as a complication in patients with liver cirrhosis. Non-cirrhotic PVT has a diverse etiology but a significantly better outcome than in patients with underlying liver cirrhosis or hepatobiliary malignancies.

BUDD-CHIARI SYNDROME

Budd-Chiari syndrome (BCS), first described in 1846 [1], is a rare clinical entity resulting from an occlusion of the hepatic venous outflow tract. Hepatic outflow obstruction related to right-sided cardiac failure or sinusoidal obstruction syndrome (SOS, also known as veno-occlusive disease [2]) is not included in the definition of BCS. In Europe and North America, the main cause of outflow obstruction is thrombosis of the hepatic veins. [3] Involvement or isolated obstruction of the inferior vena cava is encountered relatively more often in Asian countries, such as India and Japan. [4-5] Over the past years, improved imaging techniques and new

insights into potential causative factors have significantly contributed to the diagnosis and treatment of BCS. Nevertheless, due to the rarity of this disorder, most existent knowledge is based on data from (small) retrospective series.

Clinical manifestations of hepatic venous obstruction

Obstruction of the hepatic veins gives rise to several hemodynamic changes, such as a decreased sinusoidal blood flow and an increased sinusoidal blood pressure, which can lead to portal hypertension. Venous congestion also provokes ischemia and may subsequently cause necrosis of sinusoidal hepatocytes. Significant hypoxic damage can result in a deterioration of hepatic synthetic function. Over time, hepatocytes are replaced by fibrosis, predominantly localized in the centrilobular area. Nodular regeneration is also regularly seen in patients with BCS and ultimately, cirrhosis may develop. [6] Other potential consequences of hepatic venous obstruction are portal vein thrombosis and hypertrophy of the caudate lobe. In approximately 15-20 % of cases of BCS concomitant portal vein thrombosis is identified. [7-8] Because the caudate lobe is the only liver segment with direct venous drainage into the inferior vena cava, compensatory hypertrophy often occurs. Caudate hypertrophy itself can subsequently cause compression and stenosis of the inferior vena cava, further contributing to the already existent venous congestion. [9]

Clinical presentation of patients with BCS is heterogeneous and ranges from the absence of symptoms to severe liver failure. The classical triad consists of abdominal pain, ascites and hepatomegaly but other possible symptoms are nausea, fever and jaundice. [10] The severity of disease is influenced by the extent of thrombosis, the rapidity of onset and the ensuing effect of compensatory mechanisms such as the formation of collateral veins. In the past years, different classifications (i.e. acute, subacute and chronic) have been used to describe patients with BCS according to the duration and severity of symptoms. [5] However, the prognostic value of these descriptive categories has not been validated. Instead, more recent studies have attempted to determine distinct prognostic classes based on the outcome of clinical and laboratory assessments. [11-12]

Despite the major hemodynamic changes involving the liver, synthetic function is often relatively spared. However, this does not preclude a late decline in general condition and liver function. During the course of the disease, portal hypertension frequently develops and may be complicated by bleeding from gastroesophageal varices. In a significant number of patients signs of portal hypertension, such as splenomegaly or esophageal varices, are already present at diagnosis, implicating that an acute thrombotic event can be superimposed on a long-standing obstruction. Less common, an episode of gastrointestinal bleeding is the first presenting sign of BCS. [13-14] In contrast, ascites is an important complication of hepatic venous obstruction and a frequent cause of morbidity. Control of ascites is therefore important in the management of patients with BCS.

Etiology

BCS can be further classified as primary or secondary, depending on the underlying cause and the type of venous obstruction. If an endoluminal venous lesion is present, such as thrombosis or an inferior vena cava web, BCS is considered primary. The secondary form consists of venous obstruction caused by external invasion or compression of the venous lumen, as may be caused by malignant tumors or large cysts. [3] Whereas in the past many cases were designated as idiopathic [5, 15], it has nowadays been established that in most patients with BCS an underlying risk factor predisposing to thrombosis is present. Both inherited (e.g. Factor V Leiden mutation, deficiencies in protein C, protein S and antithrombin) and acquired (e.g. paroxysmal nocturnal hemoglobinuria, antiphospholipid syndrome) procoagulant disorders have been associated with BCS, of which myeloproliferative neoplasms are the most common. [16-17] (Table 1) When both overt and latent forms are taken into account, approximately 50% of patients with BCS is shown to have an underlying myeloproliferative neoplasm (MPN). [14, 18-19] Moreover, it has become clear that in a large proportion of patients more than one risk factor can be identified. [20] In studies of BCS-patients with a proven MPN, additional prothrombotic factors were found in more than 30% of the cases. [21-22]

Table 1. Risk factors for Budd-Chiari syndrome

Inherited
Factor V Leiden mutation
Prothrombin (Factor II) mutation
Protein C deficiency
Protein S deficiency
Antithrombin deficiency
Acquired
Myeloproliferative neoplasm
Paroxysmal nocturnal hemoglobinuria
Antiphospholipid syndrome
Behçet's disease
Oral contraceptives
Pregnancy and puerperium
Hyperhomocysteinemia

Diagnostic work-up

Presence of hepatic venous outflow obstruction should be suspected in patients with (acute onset of) ascites and painful hepatomegaly or when refractory ascites is present, typically in combination with relatively normal liver function tests. BCS should also be considered if liver disease is observed in patients with known thrombophilia. Physical examination and laboratory investigations are usually not very specific. In most cases diagnosis can be accurately assessed with non-invasive radiological imaging. Doppler ultrasonography is the

initial technique of choice and has high sensitivity and specificity. [23] Findings that support the diagnosis of BCS are absence of flow or retrograde flow in the hepatic veins and the presence of thrombosis within the hepatic veins or inferior vena cava. Other indicative features are intrahepatic or subcapsular collateral veins and failure to visualize the hepatic veins. [24-25] Computerized tomography (CT) and magnetic resonance imaging (MRI) are also frequently applied to demonstrate occlusion of the hepatic veins, inferior vena cava or both. With these latter techniques the liver parenchyma itself is usually better visualized to show perfusion details or necrotic areas. [26] Secondary causes of BCS, such as tumoral invasion or cysts causing venous compression, can also be identified with these different imaging modalities. Invasive hepatic venography is still regarded as the reference procedure but is nowadays only performed if venous pressure measurements are required.

A liver biopsy is not required to confirm the diagnosis of BCS but can be performed to rule out other causes. Due to the high risk of sampling error, a biopsy is insufficient to study the severity of BCS. [27] Typical histologic findings of hepatic venous outflow obstruction are congestion, loss of hepatocytes and fibrosis, most often in the centrilobular area. [28] Histologic abnormalities usually show an inhomogeneous distribution depending on the involved venous obstruction. (Figure 1) Other parenchymal changes that can be found in approximately 25% of patients along the course of the disease are regenerative nodules. These benign nodules are thought to develop as a result of an imbalance between arterial and portal blood flow. Usually, multiple regenerative lesions are present that can range in diameter from a few millimeters up to 7 cm. [6, 29] Although malignant hepatic lesions are uncommon in patients with BCS, it may be difficult to distinguish regenerative macronodules from hepatocellular carcinoma. [30] Moreover, a recent French study suggested that especially patients with a long-standing thrombosis involving the inferior vena cava may be at increased risk of developing hepatocellular carcinoma. [31]

An equally important part of the diagnostic work-up in patients with thrombosis of the hepatic veins is the identification of underlying thrombophilic factors. In a significant amount of patients multiple etiological factors can be identified. [20] Therefore, the presence of one thrombophilic factor should not preclude further investigations of other possible risk factors. Diagnosis of an MPN can prove to be difficult in patients with BCS because in many cases typical changes in peripheral blood (i.e. high levels of haemoglobin or platelets) are absent and conventional diagnostic criteria are often not met. [32] In the past, these so-called occult or latent forms could only be detected by bone marrow biopsy or the presence of endogenous erythroid colony formation. [18, 33] Recently however, the diagnosis of (occult) MPN's has been facilitated by the discovery of the Janus Kinase 2 (JAK2) mutation. This acquired gain-of-function mutation of the JAK2 tyrosine kinase can be demonstrated in the majority of patients with an MPN. [34-35] Furthermore, several studies have already pointed out that the JAK2 mutation is proving to be a reliable screening marker for MPN's in patients with BCS. [21-22, 36-37] Because not all cases of MPN are JAK2 positive and further characterization is often needed, a bone marrow biopsy will still be required in most patients.

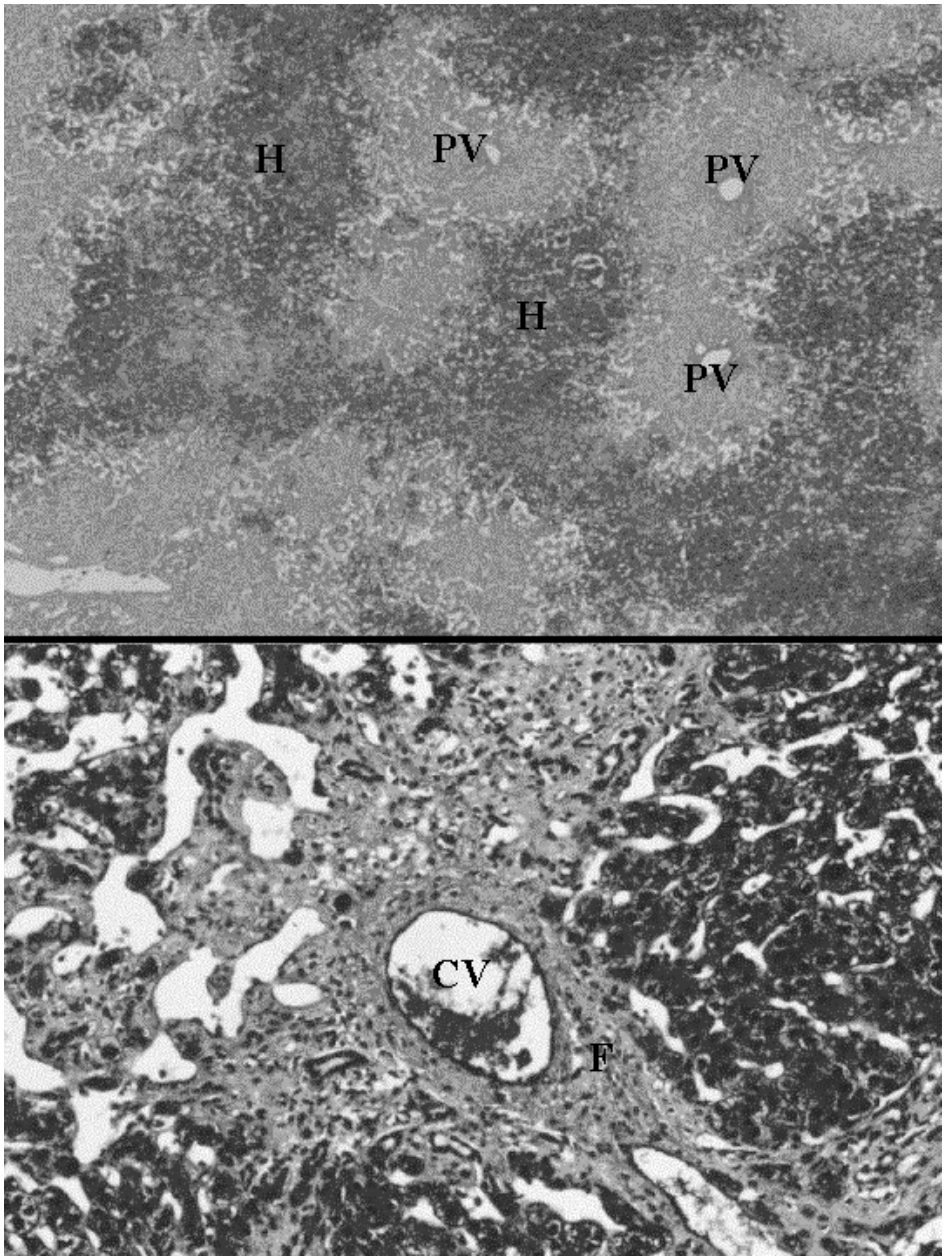

Figure 1. (Top panel) Liver biopsy specimen (Haematoxylin and Eosin (HE)-staining, enlarged 100x) depicting areas of hemorrhage (H) and congestion surrounding the central veins (zone 3). The periportal area (zone 1) around the portal vein (PV) branches is relatively spared. (Bottom panel) Further enlarged view of liver parenchyma (HE-staining, 200x enlarged) depicting a central vein (CV) of a liver lobulus surrounded by an area of fibrosis (F). This so called pericentral fibrosis is a typical finding in patients with BCS.

Treatment

Due to the rarity of the disorder, no controlled studies have been performed in patients with BCS. Therefore, most current knowledge and recommendations are based on case reports, retrospective studies and expert opinions. Furthermore, because experience with the treatment of this vascular liver disorder is often limited, all patients diagnosed with BCS should preferentially be referred to a specialized liver center. The first step in the treatment of patients with BCS is initiation of anticoagulant therapy to either achieve recanalization or prevent extension of the thrombosis. Although evidence remains circumstantial, lifelong anticoagulation is recommended in all patients with this life-threatening form of thrombosis, providing that there are no contraindications. [3] Underlying thrombophilic conditions should be identified and treated where possible. The next step in the management process concerns the manifestations and complications of liver disease. In the past, invasive treatment for patients with BCS was frequently applied and many patients were treated with surgical portosystemic shunts or liver transplantation. [38-41] Recently however, a more stepwise approach has been advocated where therapeutic procedures are performed in order of increasing invasiveness and based on the response to previous treatment. [42] (Figure 2) This is supported by the finding that some patients can be adequately treated in a non-invasive manner. [19] Nevertheless, if ascites and other complications cannot be controlled with anticoagulation and diuretics alone, further (invasive) treatment steps are required. Percutaneous transluminal angioplasty (PTA) has been successful in a number of patients but should only be performed if a short-length stenosis is present. [43-44] Systemic or local thrombolytic therapy has also been attempted as a recanalization procedure, with variable success. Recent evidence suggests that it should be executed with great caution due to the high risk of bleeding complications. [45] When these recanalization techniques are not eligible or unsuccessful at controlling symptoms of ascites and portal hypertension, a shunting procedure is indicated. Surgical

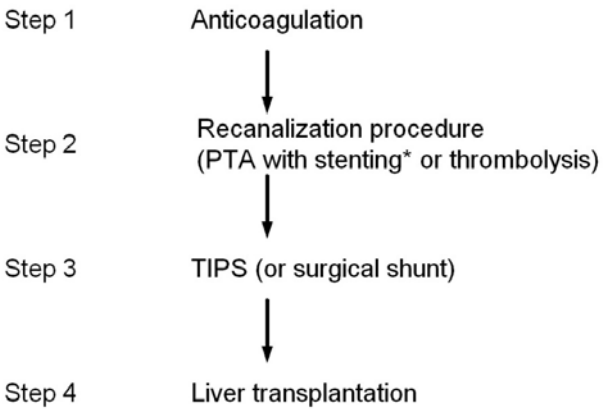

Figure 2. Treatment algorithm for patients with Budd-Chiari syndrome (BCS). *Only possible in case of short-length stenosis. PTA= percutaneous transluminal angioplasty, TIPS= transjugular intrahepatic portosystemic shunt.

portosystemic shunting has nowadays been almost completely abandoned as a treatment modality for patients with BCS. In a recent study it was performed in less than 2% of the patients. [19] Moreover, other studies have not been able to demonstrate a survival benefit of patients treated with surgical shunts. [12-13] This could be explained by a high perioperative mortality and a risk of shunt dysfunction or thrombosis. [46-47] Instead, more patients are currently being treated with a transjugular intrahepatic portosystemic shunt (TIPS) to lower portal venous pressure and decompress the hepatic sinusoids. Over the past years it has become increasingly clear that the outcome of TIPS in patients with BCS is good. The procedure is less invasive than surgical shunting, it can be successfully performed in most patients and there are fewer complications. [48-49] Furthermore, in high-risk patients, TIPS placement may even improve survival. [50] Nevertheless, when shunting procedures do fail and clinical deterioration occurs, orthotopic liver transplantation (OLT) is the last treatment option for patients with BCS. Patients presenting with fulminant liver failure should also be considered for liver transplantation. Survival rates and graft function after OLT in patients with BCS are comparable to patients transplanted for other causes. [51-52] Additionally, previous TIPS-insertion does not impair the outcome of transplantation [53] and in some cases TIPS-placement can therefore serve as a bridge to liver transplantation.

Prognosis

Prognosis of patients with BCS has dramatically improved in the past decades, which could be explained by a combination of earlier diagnosis, introduction of new treatment modalities and the routine use of anticoagulation. [54] Whereas before 1985 one-year survival rates of approximately 60% were reported [12, 14, 55], in more recent patient cohorts this number has increased to more than 80%. [12, 14, 42] Long-term survival in a large group of patients diagnosed with BCS from 1984 until 2001 was shown to be 69% and 62% at 5 and 10 years, respectively. [13] (Figure 3) From this same cohort a prognostic score was developed (Rotterdam BCS index) that identifies three distinct patient-groups with a good, intermediate and poor prognosis. The Rotterdam BCS index is based on four different clinical parameters (encephalopathy, ascites, prothrombin time and bilirubin) and can easily be calculated at diagnosis of BCS. [13] Whether specific underlying etiological factors influence the prognosis of patients with BCS is still unclear. Current evidence suggests that survival of patients with an MPN does not differ from patients without an underlying MPN. [21-22] Also, survival does not seem to be impaired by the recent shift in management leading to a less invasive treatment approach. [19, 42] In contrast, the presence of concurrent portal vein thrombosis has been associated with a poor prognosis in patients with BCS. [7-8] Further studies are warranted to investigate the effect of different prothrombotic factors on prognosis and to identify specific patients that require early invasive treatment.

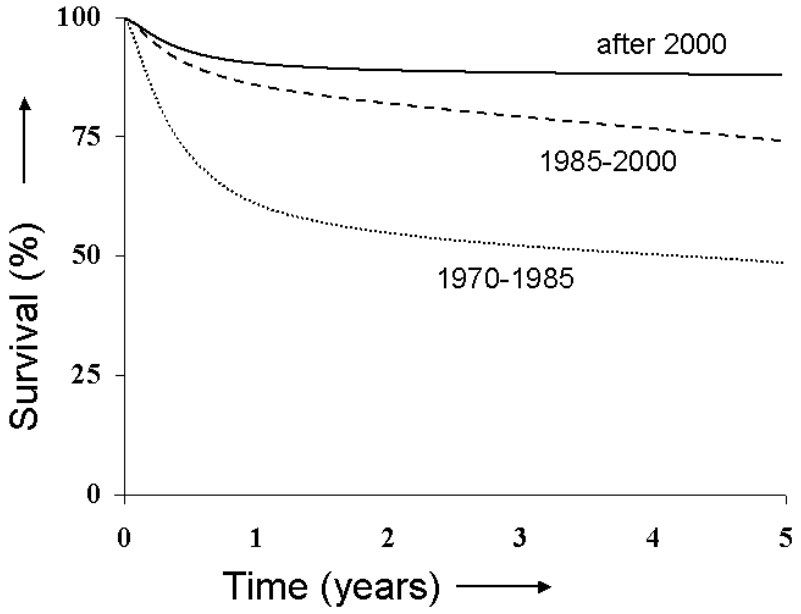

Figure 3. Survival curves of patients with Budd-Chiari syndrome (BCS) from different time periods. Adapted from Valla DC, reference [54]. Data on patient survival in different study periods is based on references [11-14, 19, 42 and 55].

PORTAL VEIN THROMBOSIS

The portal vein forms the backbone of the portal venous system that allows for blood from the digestive organs to flow towards the liver. Thrombosis of the portal vein can occur both in children and adults and results in significant hemodynamic changes. [56] Like in BCS and other forms of venous thrombosis, portal vein thrombosis (PVT) is associated with a number of different precipitating factors, both inherited and acquired. [16, 57-59] Though it is considered a rare disorder, a recent autopsy study showed the life-time risk of PVT in the general population to be 1%. [60] In adults, clinical presentation is highly variable but depending on the duration of symptoms and results of imaging, PVT can usually be classified as either acute or chronic. [61] In the past decade a number of, mainly retrospective, studies have been performed in patients with PVT. Results from these studies have significantly contributed to the current understanding of this vascular liver disorder. However, many questions remain unanswered and there is still much debate concerning the optimal treatment strategy for both acute and chronic PVT.

Clinical manifestations of acute and chronic PVT

An acute obstruction of the portal vein usually manifests itself as a sudden onset of abdominal pain, which may be very severe. Other symptoms that can occur are nausea, fever and diarrhea. [57] Whereas in the past, very few patients were diagnosed with acute PVT, due to increased awareness and improved imaging this disease entity is increasingly being recognized. [62] Although many patients will display some symptoms associated with PVT, a number of patients are completely asymptomatic. [57, 63] These patients are often only diagnosed by coincidence or when complications of chronic PVT occur. In response to thrombosis of the portal vein, portoportal and portosystemic collateral veins will develop to compensate for the decreased portal blood flow. [64-65] These collaterals may be present within several days after the venous occlusion and are eventually found in nearly all patients with a complete obstruction of the portal vein. [66] However, the amount, size and localization of collaterals differ strongly between patients. In addition to the development of collaterals, another compensatory mechanism that takes place is dilatation of the hepatic artery. [58] Nevertheless, despite the fact that hepatic blood flow is only minimally decreased as a result of these hemodynamic changes, portal venous pressure is inevitably increased. Therefore, complications related to portal hypertension, such as splenomegaly and gastroesophageal varices, are the main features of patients with long-standing, chronic PVT. At diagnosis of PVT, more than half of the patients will already have varices or signs of portal hypertensive gastropathy. [67-69] Furthermore, in 20-40% of cases, an episode of gastrointestinal bleeding will be the presenting symptom of an underlying chronic PVT. [57, 63]

Besides complications of portal hypertension, two other potential consequences of PVT are intestinal ischemia and portal biliopathy. If apart from the portal vein, the mesenteric veins are also obstructed, there is a substantial risk of intestinal ischemia and subsequent bowel infarction. [70] This is the most severe complication of acute PVT and often requires immediate surgical intervention. Fortunately, intestinal infarction occurs very infrequently, in a recent study less than 5% of patients with acute PVT suffered from this complication. [71] In patients with chronic PVT there is also a minor risk of intestinal ischemia and bowel infarction if there is secondary extension of thrombosis into the superior mesenteric vein. Another possible complication, portal biliopathy, denotes structural abnormalities of the intrahepatic or extrahepatic biliary tree that are related to the presence of a portal cavernoma. [72] These changes are most likely the result of either direct compression of bile ducts by the portal cavernoma or ischemic structuring. In the majority of patients with chronic PVT a certain degree of biliary tree involvement can be demonstrated [73-74], but most remain asymptomatic. Clinical manifestations such as jaundice, cholangitis or cholecystitis have been reported in approximately 10-30% of cases, especially in patients of older age and with longer disease duration. [73, 75-76]

Table 2. Risk factors for the development of portal vein thrombosis.

Local (hepato-biliary) factors	Systemic (thrombophilic) factors
Liver cirrhosis	***Inherited***
	Factor V Leiden mutation
(Hepato-biliary) malignancy	Factor II (prothrombin) mutation
	Protein C deficiency
Intra-abdominal infection/inflammation	Protein S deficiency
Pancreatitis	Antithrombin deficiency
Cholecystitis	
Diverticulitis	***Acquired***
Appendicitis	Myeloproliferative neoplasm
Inflammatory bowel disease	Antiphospholipid syndrome
Omphalitis	Paroxysmal nocturnal hemoglobinuria
	Oral contraceptives
Iatrogenous injury of the portal vein	Pregnancy or puerperium
Splenectomy	Hyperhomocysteinemia
Abdominal surgery	Malignancy
Umbilical vein catherization	

Etiology

Both local (hepatobiliary) and systemic (thrombophilic) risk factors have been associated with thrombosis of the portal vein. [16, 69, 77-78] (Table 2) In children, infectious causes of PVT, such as sepsis or omphalitis, are frequently present. Specifically in neonates, catheterization of the umbilical vein is a risk factor for development of PVT. [79-80] In the adult population, liver cirrhosis and hepatobiliary malignancies are the most common local precipitating factors that together account for a large proportion of cases of PVT. [60] In patients with liver cirrhosis, the reported incidence of PVT varies from 6% to 17%. [81-83] Patients with more advanced stages of cirrhosis have a higher risk of PVT than patients with compensated liver disease. [63] The incidence of PVT in patients with hepatocellular carcinoma (HCC) is 10-44% [84-86] and appears to increase even further when concurrent cirrhosis is present. [60] For this reason, diagnosis of PVT in a patient with liver cirrhosis should raise awareness for the presence of HCC. Other known local risk factors, such as pancreatitis, abdominal surgery and inflammatory bowel disease, are associated with a lower risk of PVT and are only encountered in a minority of patients. [57, 87-88] In contrast, it is now clear that in many patients with non-cirrhotic non-malignant PVT, a systemic, thrombophilic risk factor is present. [16, 77-78, 89] Of these factors, myeloproliferative neoplasms (i.e. polycythemia vera, essential thrombocythemia and myelofibrosis) are by far the most common. In a recent study, a myeloproliferative neoplasms (MPN) was found in 37% of patients with non-cirrhotic non-malignant PVT. [22] Less frequent systemic risk factors associated with PVT are Factor V Leiden mutation, prothrombin gene mutation and inherited deficiencies of protein C, protein S and antithrombin. [16, 77] Moreover, in concordance with venous thrombosis at other sites, the etiology of PVT is often multifactorial, as in many patients a combination of underlying risk factors can be identified. [20] This was not only demonstrated

in patients with non-cirrhotic non-malignant PVT [16, 22], but also in cirrhotic patients with PVT. [90] In a cohort of patients with liver cirrhosis and PVT, a concurrent systemic risk factor was present in 70% of patients. [91-92] Furthermore, patients with PVT also seem to be at an increased risk of developing other venous thromboembolic events. [93-94]

Diagnostic work-up

On physical examination the majority of patients with PVT will exhibit splenomegaly, but ascites is usually absent. Laboratory investigations provide little clues and unless an underlying liver disease is present liver function tests are mostly (near) normal. However, using non-invasive imaging techniques the diagnosis of PVT can easily be established. Doppler ultrasound, computerized tomography (CT) or magnetic resonance imaging (MRI) can all be applied to demonstrate either the absence of flow or the presence of a thrombus in the portal vein. [95-97] (Figure 4) Additionally, with these imaging modalities it is possible to visualize the extent of the thrombosis. The presence of a network of collateral vessels around the portal vein, a so-called portal cavernoma, is a typical feature of chronic PVT on imaging. [98] Moreover, in patients with long-standing thrombosis the portal vein itself often becomes a fibrotic cord and may be difficult to visualize.

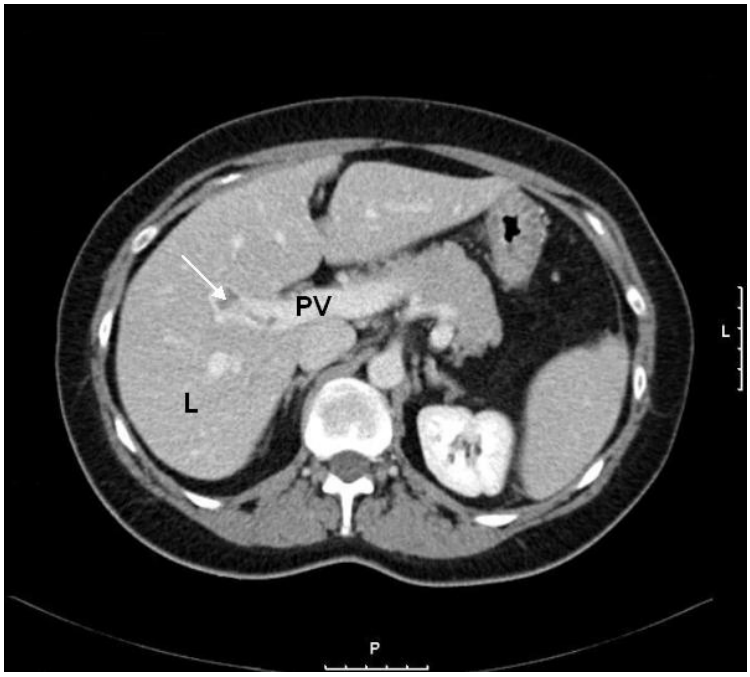

Figure 4. Computed tomography image of the liver (L) of a patient with portal vein thrombosis showing the presence of thrombotic material (arrow) in the lumen of the portal vein (PV).

Once PVT is diagnosed, patients should be screened for underlying etiologic factors. Of interest, in patients with PVT or underlying liver disease the diagnosis of certain thrombogenic factors may be impaired. Firstly, decreased hepatic synthetic function may result in lower plasma levels of protein C, protein S and antithrombin, thereby potentially masking a true deficiency or leading to an incorrect diagnosis of natural anticoagulant deficiency. [99] Secondly, characteristic features of a MPN may be absent due to splenomegaly or haemodilution. [32] The latter diagnostic problem can be solved by performing a bone marrow biopsy or by assessing the presence of endogenous erythoid colony formation. [100] Furthermore, discovery of the JAK2 mutation has further contributed to the diagnosis of MPNs. [35] In patients with polycythemia vera it has been shown that approximately 95% carry the JAK2 mutation, for essential thrombocythemia and myelofibrosis this mutation is present in 50-60% of patients. [34] Because the JAK2 mutation is not found in healthy controls, it has become a useful screening marker for MPN. In several studies of patients with non-cirrhotic non-malignant PVT 20-35% of the cases was JAK2 positive, underlining that MPN's are a major risk factor for the development of PVT. [101-103]

Treatment

Acute PVT. The management of patients with acute PVT is based on: (1) prevention of further thrombosis and therapy aimed at recanalization, (2) treatment of complications (e.g. bowel infarction) and concurrent disease and (3) identification and, if possible, treatment of underlying (thrombophilic) risk factors. Although no controlled studies have been performed, there is convincing evidence that rapid initiation of anticoagulation therapy results in either complete or partial recanalization in a significant amount of patients. Several retrospective series and a recent prospective study have all shown a beneficial effect of anticoagulation in patients with non-cirrhotic non-malignant PVT, with recanalization rates of approximately 45%. [62, 67, 71] Spontaneous improvement of portal vein patency was rarely seen in these studies. Therefore, the current consensus indicates that all patients with acute PVT should be treated with anticoagulation when there are no contra-indications. [104] A minimal treatment duration of 3 months is advised but, like venous thrombosis at other sites, this could be extended to 6 months. Moreover, in patients with proven systemic thrombophilia life-long anticoagulation therapy may be warranted due to the increased risk of new thrombotic events. [67, 93, 104]

Apart from anticoagulation, several other treatment modalities have also been employed to achieve recanalization of the obstructed portal vein. A number of case reports have successfully demonstrated the use of local thrombolysis in the early phase of PVT. [105-106] Recanalization has also been described after surgical thrombectomy or with percutaneous transhepatic angioplasty (PTA). [107-108] Nevertheless, experience with these techniques is limited and the risk of procedure-related complications and even mortality is substantial. [109-110] Consequently, their role in the treatment of acute PVT is still highly controversial.

In addition to its effect on recanalization, anticoagulation should also be initiated in the acute phase of PVT to prevent extension of the thrombosis. Extensive thrombosis of the mesenteric veins is mostly symptomatic and carries a high risk of intestinal ischemia. [63] Symptoms that may be present are severe abdominal pain and bloody diarrhoea. When intestinal infarction is suspected, immediate surgical intervention is required to resect necrotic parts of the bowel. If left untreated, bowel ischemia can lead to major complications such as intestinal perforation, shock, multi-organ failure and death. [70]

Chronic PVT. For patients with chronic PVT, therapy is mainly aimed at the treatment and prevention of complications of portal hypertension. Bleeding from gastroesophageal or ectopic (e.g. duodenal or rectal) varices is the most important complication of PVT-induced portal hypertension. Around 50% of patients will already have signs of varices at diagnosis and for that reason endoscopic screening for the presence of varices should be part of the diagnostic work-up in all patients with (chronic) PVT. In the case of non-cirrhotic non-malignant PVT, approximately 30% of patients will experience one or more episodes of gastrointestinal bleeding during follow-up. [57, 68] Despite the serious nature of complications, no controlled studies have been performed addressing the optimal management of variceal bleeding in patients with PVT. Therefore, current guidelines are mainly based on data from studies in patients with portal hypertension caused by liver cirrhosis, in the absence of PVT. [104] As has become clear from these studies, primary prevention of bleeding is recommended in patients with large (>5mm) varices. [111] Treatment with nonselective β-blockers and endoscopic band ligation are equally effective and both significantly reduce the risk of a first bleeding episode. [112] It has not been established which therapy should be preferred in patients with PVT, but pharmacologic treatment with β-blockers is probably more cost-effective. Endoscopic treatment as primary prevention could then be reserved for those patients with intolerance or contra-indications to β-blockers.

When prevention fails or when a patients presents with variceal hemorrhage, endoscopic therapy is the mainstay of treatment. Variceal band ligation is the preferred treatment modality for acute bleeding episodes but endoscopic sclerotherapy may also be applied. [104] For acute bleeding from gastric fundal varices, endoscopic variceal obturation with tissue adhesives seems to be most effective to control bleeding. [113]

After a first episode of variceal bleeding has been controlled, therapy is aimed at prevention of further events. In patients with PVT there have been a few studies addressing the prevention of rebleeding, specifically with endoscopic therapy. It was shown that endoscopic eradication of varices in patients with non-cirrhotic non-malignant PVT significantly reduced the risk of rebleeding. [114-116] The rate of rebleeding was reported to be 23% in the first year [116], which compares favourably to a rebleeding rate of approximately 31% in cirrhotic patients treated with endoscopic band ligation. [117] Studies investigating the effect of β-blockers on the prevention of rebleeding in patients with PVT have not been performed and their role in the secondary prophylaxis of variceal bleeding in these patients is therefore still unclear. [104]

Many patients with PVT-induced portal hypertension can be adequately managed with pharmacologic or endoscopic treatment. However, when these therapeutic options fail and in patients with recurrent variceal bleeding, a shunting procedure could be considered. Surgical shunts, preferably a distal splenorenal shunt, have proven to give durable decompression of the portal venous system. [118] Disadvantages that hamper the widespread application of these procedures are the considerable rates of morbidity and mortality and the high risk of shunt thrombosis. [81, 119] As a less invasive option, recent interest has gone out to the use of a TIPS. Several studies have reported the successful use of TIPS in the management of patients with PVT. [120-122] Nevertheless, a TIPS can only be performed in selected patients, as in many cases the procedure is technically not feasible due to extensive thrombosis (e.g. involving the splenic and mesenteric veins) or an inability to catheterize either the portal vein itself or collaterals forming the portal cavernoma. Future studies will have to determine the exact role of TIPS in the treatment of portal hypertension associated with PVT.

Treatment of portal biliopathy is only indicated in symptomatic patients. Endoscopic therapy with or without stent placement is effective in most cases of biliary obstruction or biliary stone formation. [123] When symptoms persist, a surgical intervention may be needed, aimed at the management of portal hypertension. A few studies performed in patients with portal biliopathy as a result of PVT, have illustrated that symptoms can be relieved with a portosystemic shunting procedure. [124-125] This diminishes the need for a secondary surgical bilioenteric anastomosis, which is associated with a high morbidity and mortality in these patients due to the extensive network of collaterals frequently surrounding the biliary structures. [126]

Whereas the role of anticoagulation has been quite well established in the treatment of patients with acute PVT, there is still much debate concerning its place, if any, in the management of chronic PVT. The significant risk of bleeding complications from gastroesophageal varices is often seen as a contraindication. Nevertheless, the high prevalence of systemic thrombophilia would support treatment with anticoagulation, as it has been reported that patients with PVT and an underlying thrombogenic risk factor have an increased risk of developing further thrombotic events. [67-68, 94] Moreover, it was shown that anticoagulation therapy decreased the incidence of new thrombotic episodes in these patients whilst the risk and severity of variceal bleeding was not altered. [68] This would support the use of anticoagulation in patients with chronic PVT and proven thrombophilia. Whether anticoagulation should be considered in patients with PVT and underlying liver cirrhosis is even less clear. One study has suggested that anticoagulation therapy may prove useful in a subgroup of patients with cirrhosis and PVT that are candidates for liver transplantation. [127] The presence of PVT in patients undergoing liver transplantation is associated with more complex surgical procedures and an increased rate of complications. [128-129] Treatment with anticoagulation in cirrhotic patients with PVT awaiting transplantation resulted in recanalization in 42% of cases and succesfully prevented extension of thrombosis. [127] Still, despite these favourable results of anticoagula-

tion, evidence is minimal and more studies are needed to define whether treatment with anticoagulation truly has a beneficial effect in patients with chronic PVT. Current consensus, solely based on expert opinion, indicates that life-long anticoagulation therapy should be considered in patients with PVT in whom an underlying thrombophilic risk factor has been identified. [104]

Prognosis

The prognosis of patients with PVT is mainly determined by the underlying cause of thrombosis and not by the complications of portal hypertension. [57, 69] Whereas in earlier studies many patients died as a result of variceal bleeding [130], recent data suggests that mortality related to gastrointestinal hemorrhage is uncommon. [67] In a large cohort of 172 patients with PVT, death due to variceal bleeding occurred in 2% of the patients. [69] Furthermore, in a recent short-term prospective study in patients with non-cirrhotic non-malignant PVT, no deaths due to variceal bleeding were reported. [71] The prognosis of PVT-patients without underlying cirrhosis or malignancy can therefore be considered as good, with 5- and 10-year survival rates of 90% and 80%, respectively. [69] Outcome is worse in patients with liver cirrhosis because in this group liver function is already impaired and there is a higher risk of (liver-associated) complications. Survival after liver transplantation was shown to be significantly lower in cirrhotic patients with concomitant PVT as compared to cirrhotic patients without PVT. [127] Clearly, the presence of an underlying malignancy also substantially affects survival. It has been reported that patients with HCC that develop PVT during the course of the disease have a very poor prognosis. [93] In one study, 5-year survival of PVT-patients with malignancy was only 8%. [69] Another factor that has a negative impact on survival is intestinal ischemia complicated by bowel infarction. In patients with mesenteric vein thrombosis mortality rates may vary between 20 and 50%. [70] Conversely, underlying systemic risk factors do not seem to influence prognosis, although long-term follow-up data of patients with PVT and known thrombophilia is lacking. A recent study demonstrated that the presence of a MPN does not affect 5-year survival rates. [22]

CONCLUSIONS

Vascular liver disorders, such as BCS or PVT, constitute a rare group of liver diseases. Over the past decades, increased awareness and advances in noninvasive imaging techniques have facilitated the diagnosis of these vascular diseases. For patients with BCS, long-term survival has dramatically improved with the increased use of anticoagulation and the introduction of TIPS. PVT generally has a more benign disease course and better clinical outcome, although complications of portal hypertension may sometimes be difficult to manage. The role of an-

ticoagulation treatment is less clear in PVT, in part based on the high risk of gastrointestinal bleeding and the finding that many patients present with a more chronic form of thrombosis. Regarding the etiology of thrombosis, several studies have underlined the intriguing relationship between myeloproliferative neoplasms and both BCS and PVT. Still, there remains a significant amount of patients in whom no known risk factors for thrombosis are found, suggesting that certain prothrombotic factors contributing to the pathogenesis of these disorders have not yet been identified. Despite recent advances, many aspects concerning the (multifactorial) etiology, optimal treatment and outcome of BCS and PVT have not yet been fully elucidated.

AIMS AND OUTLINE OF THE THESIS

This thesis focuses on the etiology and clinical outcome of patients with BCS and non-cirrhotic PVT. Several studies are based on data from a large prospective study initiated by the European Network for Vascular Disorders of the Liver (EN-Vie). The EN-Vie study has provided a unique database of clinical information on two cohorts of patients with BCS or PVT from nine European countries.

Although there is a clear overlap in the spectrum of prothrombotic factors that can cause BCS or PVT, there may also be factors that are specifically related to thrombosis at a certain location. **Chapter 2** investigates the site-specificity of thrombosis by comparing the risk factor profile of patients with BCS to that of patients with PVT. Additionally, the prevalence of inherited thrombophilia is studied in a cohort of matched, healthy control subjects.

In **Chapter 3 and 4** potentially unknown risk factors for thrombosis are explored. The role of hypofibrinolysis as a possible cause of thrombosis is investigated in the first study by comparing plasma levels of several factors involved in fibrinolysis between patients with BCS from the EN-Vie study and a cohort of healthy, sex- and age-matched controls. As a measure of overall fibrinolytic potential, the results of a clot-lysis assay are also studied. In the second study, differences in clot-binding proteins between patients with BCS and healthy controls are evaluated using a proteomic approach.

Chapter 5 describes a cohort of patients with BCS and underlying paroxysmal nocturnal hemoglobinuria (PNH) from the EN-Vie study. PNH is a rare disorder of hematopoietic stem cells whereby patients have a high risk of developing venous thrombosis, especially cerebral and hepatic vein thrombosis. The study compares the disease presentation and prognosis of BCS-patients with concomitant PNH to patients without PNH, with a specific focus on the therapeutic options, such as TIPS-placement.

The role of anticoagulation therapy in patients with non-cirrhotic PVT is evaluated in **Chapter 6**. Using data from a large single-center cohort, the effects of anticoagulation are studied, with special emphasis on bleeding and thrombosis risk. From the same cohort, the study

presented in **Chapter 7** focuses on a subgroup of patients with PVT, namely those with an underlying myeloproliferative neoplasm (MPN). This long-term follow-up study investigates the complications and treatment strategies that are relevant to this specific patient group.

Finally, **Chapter 8** describes the outcome of pregnancy in a multicentric cohort of patients with non-cirrhotic PVT. A significant amount of females that are affected by PVT are women of child-bearing age. There is little data on the risks associated with pregnancy in these patients and the potential effects on fetal health.

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Chapter 2

ETIOLOGIC FACTORS UNDERLYING BUDD-CHIARI SYNDROME AND PORTAL VEIN THROMBOSIS: CLUES FOR SITE-SPECIFIC THROMBOSIS

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ABSTRACT

Various risk factors for thrombosis have been associated with both Budd-Chiari syndrome (BCS) and non-cirrhotic, non-malignant portal vein thrombosis (PVT). To date, it has not been fully established whether there are also differences in hypercoagulable states underlying these two forms of abdominal thrombosis. The aim of our study was to identify factors that may explain the site specificity of both rare forms of venous thrombosis. Underlying risk factors and multifactorial etiology of thrombosis were studied in 160 patients with BCS and 102 patients with acute PVT from the EN-Vie study, a prospective European collaboration, and compared with 116 controls. The presence of the FVL mutation was associated with an increased risk of BCS (OR 3.9, CI 1.3-11.9) but not of PVT (OR 1.0, CI 0.2-4.7) compared to healthy controls. In contrast, the FII mutation was significantly related to PVT (OR 10.1, CI 2.2-47.8) but not to BCS (OR 2.1, CI 0.4-11.1), as compared to the controls. Homozygous MTHFR mutation was not associated with either BCS or PVT. Comparing both patient groups, a myeloproliferative neoplasm (MPN) was more common in BCS-patients compared to patients with PVT (39% vs. 22%, respectively, p=0.009). Polycythemia vera appeared to be the predominant subtype of MPN in BCS (56% of MPN-cases) whereas in patients with PVT essential thrombocythemia was most frequently diagnosed (52% of MPN-cases, p=0.002). The type, number and specific combinations of etiologic factors did not affect clinical presentation in both BCS-patients and PVT-patients.

*Conclusion*s. There are significant differences in the risk profile leading to thrombosis in BCS or PVT. Inherited thrombophilia and MPN subtypes seem to be related to thrombosis at a specific site.

INTRODUCTION

Budd-Chiari syndrome (BCS) is a rare clinical entity resulting from an obstruction of the hepatic venous outflow tract. [1] In Europe and North America, the main cause of outflow obstruction is thrombosis of the hepatic veins, either with or without extension of thrombosis into the inferior vena cava. [2] A second, more common, disorder involving the liver vasculature is thrombosis of the portal vein. Portal vein thrombosis (PVT) is regularly encountered as a complication of liver cirrhosis or local malignancy [3-4], but is infrequent in the absence of cirrhosis or hepatobiliary tumors. Whereas in the past many cases of BCS and non-cirrhotic, non-malignant PVT remained idiopathic, it is now clear that these forms of venous thrombosis are associated with a number of hereditary and acquired thrombophilic conditions. [5-7] Many of these hypercoagulable states, such as those induced by Factor V Leiden (FVL) mutation, factor II (FII, prothrombin) gene mutation, protein C deficiency and oral contraceptive use, are also well established risk factors for the development of deep venous thrombosis of the lower limbs. [8-11] In addition to these individual risk factors, it is also increasingly recognized that in many patients with BCS or PVT there is clustering of thrombophilic states. [12-13] This is in line with the designation of venous thrombosis as a multicausal disease. [14]

Myeloproliferative neoplasms appear to be specifically associated with an increased risk of abdominal vein thrombosis. [15-16] Recent discovery of a mutation in the Janus kinase 2 gene (JAK2 V617F), as a specific marker for the presence of a myeloproliferative neoplasm (MPN) [17], has provided further evidence that either an occult or overt MPN is present in a large proportion of patients with BCS or PVT. [18-19] Another disorder with an apparent predisposition for thrombosis at specific locations is paroxysmal nocturnal hemoglobinuria. Patients with this acquired disorder of hematopoietic stem cells are at high risk of developing thrombosis in cerebral or abdominal veins. [20-21]

Whether there are also differences in etiologic factors underlying BCS and PVT has not been extensively investigated. Risk factors for thrombosis have mostly been described in studies focusing on either one disorder separately or in reports considering cases of BCS and PVT together, as a combined entity of splanchnic vein thrombosis. Apart from factors inducing hypercoagulability, both changes in blood flow and vessel wall abnormalities have also been considered as part of Virchow's triad leading to venous thrombosis. In this light, in addition to certain shared prothrombotic factors there may also be differences between risk factors involved in triggering thrombosis at distinct sites. [22] Many previous studies have been retrospective or included only a limited number of patients, which makes it difficult to draw accurate conclusions. In the current study we aimed to identify specific risk factor profiles underlying thrombosis in two large, identically collected prospective cohorts of patients with BCS and acute PVT. Furthermore, as it is yet unknown whether the presence of multiple prothrombotic factors or specific combinations of risk factors influence disease presentation in these patients, we have also studied the relationship between underlying etiology and clinical characteristics.

PATIENTS AND METHODS

Study cohort

Data for this study was obtained from the EN-Vie study, a prospective European collaboration including newly diagnosed patients with BCS and PVT. Patients with PVT and underlying liver cirrhosis or abdominal malignancies were excluded from the study. As previously described [13, 23], consecutive patients with BCS or PVT were recruited during a study period of two years from, respectively, nine and seven different European countries. At the end of the study period a total of 163 patients with BCS and 138 patients with non-cirrhotic non-malignant PVT were included. At baseline (date of diagnosis) and at different time-points during follow-up, extensive data was collected on clinical symptoms, etiology, laboratory parameters, radiologic findings, interventions and outcome. Risk factors for thrombosis were investigated as reported previously. [12-13] Furthermore, the study protocol included the collection of blood samples from all patients for storage of plasma and DNA.

For the most accurate assessment of underlying risk factors and to ensure the most homogenous patient cohort, only cases presenting with acute PVT (n=102) were considered for this study. Diagnostic criteria for acute PVT were imaging evidence of solid material within the portal vein lumen or its left or right branch, and the absence of porto-portal collaterals. Because information on underlying etiology was not available for three patients with BCS, these were excluded from the current study, leaving 160 eligible cases. Thus, the final cohort consisted of 160 patients with BCS and 102 patients with acute PVT. For each of these patients the number and type of underlying risk factors for thrombosis were evaluated. When more than one prothrombotic factor had been identified, etiology was considered multifactorial.

The EN-Vie study was conducted with approval from all national and, if necessary, local ethical committees, in accordance with the nation-specific rules. All patients agreed to participate in the study by means of a written informed consent.

Case-control study

At inclusion in the EN-Vie study, all patients with BCS were asked to provide a control person. Controls were healthy non-relatives and had to be of the same gender, ethnic background and age (with a range of 5 years) as the patient. Controls were only considered eligible if they did not have a previous history of thrombosis and were not using anticoagulation. If patients were not able to provide a control person, the study coordinating centres attempted to find equally matched controls from their own resources. A written informed consent was obtained from all control subjects participating in the study.

As for the patients, blood samples were collected from the controls by means of venapuncture. From these samples, plasma was obtained through centrifugation and DNA was

extracted according to local standard methods. All plasma and DNA samples were stored centrally at -70°C until analysis.

Mutation analysis in cases and controls

DNA-samples from patients with BCS, patients with acute PVT and healthy controls were used to assess the prevalence of three different mutations implicated in venous thrombosis: FVL (G1691A) mutation, FII (G20210A) mutation and the C677T mutation of methylenetetrahydrofolate reductase (MTHFR). Furthermore, the Janus Kinase 2 (JAK2) V617F mutation was determined as a marker for a myeloproliferative neoplasm. All mutation analyses were performed using a polymerase chain reaction (PCR) according to previously described methods. [8, 24-26] All analyses were performed at the Service d'Hématologie Biologique at the Hôpital Beaujon, Clichy, France. In patients for whom no DNA sample was available for central analysis, the results of locally performed mutation analyses were included when present.

Statistical analysis

Comparison of parameters was done using a Student's T-test for continuous variables and a Chi-square or Fisher's Exact test for categorical variables. In the case-control study, the relative risk of either BCS or PVT associated with the presence of the tested mutations as compared to healthy controls was estimated as an odds ratio (OR) and corresponding 95% confidence interval (CI) using logistic regression. Results were corrected for age and sex. A p-value of <0.05 was considered to be statistically significant. All statistical analyses were performed with the Predictive Analytic Software (PASW) for Windows, version 17.0.2 (SPSS Inc., Chicago, IL).

RESULTS

Characteristics at time of diagnosis of the patients with BCS (n=160) and the patients with acute PVT (n=102) are shown in Table 1. Median age of the controls was 37 years (range 16-75 years) and 42% were males. Sex distribution did not differ significantly between the two patient groups although the percentage of females was higher among BCS-cases. Patients diagnosed with acute PVT were significantly older than those diagnosed with BCS (median age 48 vs. 37 years, $p<0.001$). Ascites and hepatomegaly were present in more than two-thirds of the cases with BCS whereas abdominal pain and fever were the most common clinical manifestations in patients with acute PVT.

Table 1. Characteristics at diagnosis of patients with BCS or acute PVT

	BCS (n=160)	PVT (n=102)	p
Males, n (%)	67 (42)	52 (51)	0.149
Age at diagnosis (yrs)§	37 (26-48)	48 (37-59)	<0.001
History of thrombosis, n (%)	34 (21)	20 (20)	0.778
Abdominal distension	110 (69)	17 (18)	<0.001
Abdominal pain, n (%)	98 (63)	93 (91)	<0.001
Fever, n (%)	24 (15)	48 (47)	<0.001
Ascites, n (%)	132 (83)	39 (38)	<0.001
Hepatomegaly, n (%)	108 (68)	25 (25)	<0.001
Splenomegaly, n (%)	82 (51)	39 (40)	0.086
ALT, xULN§	1.4 (0.3-3.5)	1.1 (0.4-1.8)	0.002
Bilirubin, µmol/l§	31 (17-45)	15 (7-22)	0.002
Creatinine, µmol/l§	80 (66-93)	76 (63-89)	0.001

ALT, alanine aminotransferase; ULN, upper limit of normal. § Values represent median (interquartile range).

Table 2 displays the prevalence of the different tested mutations in BCS-patients, PVT-patients and controls. Mutation frequencies of the patient groups were compared to the controls and corrected for age and sex. As shown, presence of the FVL mutation was associated with a significantly increased risk of BCS with an OR of 3.9 (CI 1.3-11.9). This was not seen for acute PVT, in these patients prevalence of the FVL mutation was similar to that of the controls. In contrast, the FII mutation was clearly related to development of PVT (OR 10.1, CI 2.2-47.8) whereas the risk of BCS was not significantly increased (OR 2.1, CI 0.4-11.1) with this mutation when compared to the controls. Interestingly, presence of the MTHFR mutation was not associated with an increased risk of thrombosis in both patients with BCS and those with PVT. These results did not change when only cases with a homozygous mutation were considered. The JAK2 mutation was not found in any of the controls whereas it was detected in 29% and 17% of the patients with BCS or PVT, respectively (both p<0.001).

The differences in the role of FVL or FII mutation as a causal factor of either BCS or PVT were further underlined when the prevalence of etiologic factors was compared between the two patient groups. (Table 3) FVL mutation, as well as a myeloproliferative neoplasm (MPN) and paroxysmal nocturnal hemoglobinuria, were more frequently encountered in patients with BCS as compared to cases with PVT. There was also a trend towards a higher frequency of systemic disorders (e.g. connective tissue disease) in patients with BCS (11% vs. 5% in PVT-patients) but this difference failed to reach statistical significance (p=0.103). On the contrary, FII mutation was present in 14% of the PVT-patients but in only 4% of the BCS-patients (p=0.002). Local inflammatory factors were also more common as an underlying risk factor in cases with PVT.

Table 2. Analysis of prothrombotic mutations in patients with BCS or acute PVT and healthy controls

	control (n=116)	BCS (n=160)	p	OR§	95% CI	acute PVT (n=102)	p	OR§	95% CI
FVL mutation, n (%)	4 (4)	18 (12)	0.017	3.894	1.275-11.891	3 (3)	1.000	1.000	0.211-4.730
FII mutation, n (%)	2 (2)	5 (4)	0.390	2.085	0.391-11.115	14 (14)	0.003	10.121	2.151-47.615
MTHFR mutation, n (%)	61 (55)	59 (51)	0.594	0.866	0.509-1.472	41 (53)	0.638	0.862	0.464-1.602
MTHFR homozygotes, n (%)	13 (12)	17 (15)	0.440	1.361	0.622-2.978	9 (12)	0.959	0.976	0.379-2.514
JAK2 mutation, n (%)	0 (0)	35 (29)	<0.001	-	-	14 (17)	<0.001	-	-

OR, odds ratio; CI, confidence interval; FVL, factor V Leiden; FII, factor II; MTHFR, methylenetetrahydrofolate reductase; JAK2, janus kinase 2. § Versus control group, corrected for age and sex.

Table 3. Frequency of underlying risk factors for thrombosis in patients with BCS or acute PVT

	BCS (n=160)		PVT (n=102)		p
Myeloproliferative neoplasm§	55/143	39%	21/94	22%	0.009
JAK2 mutation	35/121	29%	14/82	17%	0.053
PNH	15/77	19%	0/39	0%	0.003
FVL mutation	18/147	12%	3/95	3%	0.014
FII mutation	5/144	4%	14/98	14%	0.002
PC/PS/AT deficiency	11/147	8%	6/93	7%	0.762
Antiphospholipid antibodies	37/150	25%	28/98	29%	0.494
Systemic disorder†	17/160	11%	5/102	5%	0.103
Hormonal risk factor‡	35/93	38%	17/50	34%	0.667
Local factor$	21/160	13%	29/102	28%	0.002

JAK2, janus kinase 2; PNH, paroxysmal nocturnal hemoglobinuria; FVL, factor V Leiden; FII, factor II; PC, protein C; PS, protein S; AT, antithrombin. § As detected by either bone marrow examination or positive JAK2 status. When these tests were not available, evidence of MPN was based on the presence of spontaneous erythoid colony formation, but only when other criteria suggestive of MPN were also present (n=3).† Connective tissue disease, sarcoidosis, vasculitis or Behçet's disease. ‡ Pregnancy, puerperium or hormonal contraception, considered in female patients only. $ Inflammatory bowel disease, intra-abdominal or systemic infection.

Considered as one of the most important risk factors for BCS and non-cirrhotic, non-malignant PVT, a MPN was diagnosed in 39% and 22% of the cases, respectively (p<0.009). The JAK2 mutation was identified in 29% of tested patients with BCS as compared to 17% of PVT-patients (p=0.053). For both patient groups, approximately one-quarter of the cases had previously been diagnosed as having a MPN whereas in the majority of cases thrombosis of the hepatic or portal veins was the first manifestation of an underlying MPN. (Table 4) Interestingly, there appeared to be a difference regarding the distinct subtypes of MPN identified in both forms of thrombosis. In patients with BCS, polycythemia vera was the predominant type of MPN, present in 56% of the cases. Essential thrombocythemia was the most frequently encountered type of MPN in patients with PVT. Idiopathic myelofibrosis was also more common in PVT-patients as compared to BCS-patients (14% versus 4%, respectively). The frequency of JAK2-positive MPN's was comparable for both categories of patients. In cases with BCS, 21 of 24 tested patients (88%) with polycythemia vera were JAK2 positive. In the subgroup of patients with either essential thrombocythemia or myelofibrosis, the proportion of cases that were JAK2 positive was similar between BCS-patients and PVT-patients (67% vs 69%, respectively).

In the majority of patients with BCS or PVT, at least one risk factor for the development of thrombosis could be found. Still, in 25 cases with BCS (16%) and 19 patients with acute PVT (19%) no etiologic factors were identified. On the other hand, a multifactorial etiology of thrombosis, considered as the presence of more than one prothrombotic factor, was found in 46% and 48% of the patients with BCS or PVT, respectively. To evaluate the potential impact of multiple underlying risk factors on clinical presentation, we compared the characteristics at diagnosis of patients with no risk factors, patients with one etiologic factor and patients with more than one risk factor. For both BCS and PVT, there were no significant differences in

Table 4. Details of myeloproliferative neoplasms diagnosed in patients with BCS or acute PVT

	BCS (n=55)		PVT (n=21)		p
Previous history of MPN	13	24%	6	29%	0.657
Type of MPN[§]					0.002
Polycythemia vera	30	56%	3	14%	
Essential thrombocythemia	10	19%	11	52%	
Idiopathic myelofibrosis	2	4%	3	14%	
Unclassified	9	17%	3	14%	
Occult	3	6%	1	5%	
JAK2 positive MPN[†]	35/43	81%	14/19	74%	0.513

MPN, myeloproliferative neoplasm; JAK2, janus kinase 2. § In one case of BCS the type of MPN could not be determined because the only evidence was presence of the JAK2 mutation and patient died before further examinations (e.g. bone marrow or laboratory testing) could be performed. † Considered in cases with MPN that were tested for the JAK2 mutation.

patient characteristics, clinical symptoms and extent of thrombosis based on the number of identified prothrombotic factors. (data not shown)

Focussing on the different types of risk factors identified in patients with BCS, there was no apparent clustering of etiologic factors. Myeloproliferative neoplasms were the most frequently diagnosed underlying cause, present in 39% of the cases. Compared to BCS-patients without a MPN, cases with a MPN were older (median age 46 vs. 34 years, p=0.001) and more often had ascites and splenomegaly at diagnosis (96% vs. 73% and 66% vs. 46%, respectively, both p<0.05). Other individual risk factors did not clearly influence clinical characteristics at diagnosis of BCS. In the cohort of patients with PVT, there were also no particular combinations of etiologic factors that frequently occurred together. However, an extension of thrombosis into the superior mesenteric or splenic vein was found significantly more often in PVT-patients with a MPN as compared to those without (86% vs. 53%, p=0.019). Contrarily, in patients with PVT based on a local risk factor, involvement of the superior mesenteric or splenic vein was uncommon, present in 38% of the cases as compared to 67% of the cases without local risk factors (p=0.020). Moreover, in 59% of the PVT-patients with a local risk factor thrombosis of the main portal vein was incomplete or thrombosis involved only a single portal vein branch.

DISCUSSION

This study involves the two largest cohorts of prospectively included patients with BCS and acute PVT so far. To identify factors that determine the site-specificity of these two forms of abdominal vein thrombosis, we have evaluated the distribution of etiologic factors in both patient groups and performed a case-control study to estimate the risk of thrombosis associated with four known thrombophilic mutations. Moreover, considering the current understanding that a multifactorial etiology of thrombosis is common in both BCS and PVT, we have also investigated the influence of the presence of multiple prothrombotic factors on disease presentation.

We confirmed that the Factor V Leiden mutation is significantly associated with BCS, as indicated by an almost fourfold increased relative risk. The FII mutation, in contrast, does not appear to be related to the presence of BCS. Remarkably, the opposite is true for PVT, where the G20210A mutation in the prothrombin gene but not the FVL mutation poses a greatly increased risk of thrombosis. These apparent differences were also observed when the frequency of both mutations was compared between the two patient groups. An association between PVT and the FII mutation has previously been reported by others [3, 27], but results are conflicting as in some studies no significant relationship was demonstrated. [5, 28] In a study by Denninger *et al.*, comparing two relatively small cohorts of patients with BCS and patients with PVT, no significant difference was found in the frequency of the FII mutation. Regarding the FVL mutation, an increased incidence was described among BCS-patients, but this study lacked a control group. [12] Overall, most earlier studies provided conclusions based on a small number of cases that, in part, resulted in inconsistent findings. Our work overcomes these previous limitations, with two large patient series combined with a group of healthy controls, and yields strong evidence that there is a differential role for FVL and FII mutation in the etiology of BCS and PVT.

Concerning the distinct association of FVL and FII mutation with these two forms of thrombosis, little is known about possible differences in thrombotic tendency. Both mutations convey an increased risk of venous thrombosis, albeit through a disruption at different points in the coagulation cascade. Some studies have suggested that the prothrombin gene mutation, in contrast to the FVL mutation, is associated with an increased risk of ischemic stroke, coronary heart disease or peripheral arterial disease. [29-31] However, these findings were not confirmed in a large meta-analysis studying the role of prothrombotic mutations in arterial thrombosis. [32] Given that both mutations induce hypercoagulability in a similar way, it could be hypothesized that other, vascular bed-specific factors are responsible for the susceptibility to thrombosis in either the hepatic or portal veins, but this remains to be elucidated.

As opposed to the increased risk associated with the FVL and FII mutation, no relationship was found between presence of the MTHFR mutation and BCS or PVT. Although a few studies have suggested an association between this mutation and either BCS or PVT [33-34], the causal role of the MTHFR mutation is not well established. Even in patients with deep venous thrombosis of the lower extremities, doubts have arisen regarding the contribution of this mutation to the development of thrombosis. [35-36] Given these findings, we believe that the role of the MTHFR mutation in thrombosis in patients with BCS or PVT, if any, is at most only minor.

As expected, the JAK2 mutation was not detected in any of the controls but was present in a significant number of cases with BCS or PVT. Over the past years it has become increasingly clear that detection of this mutation is a useful screening tool for the diagnosis of an underlying MPN. [37-38] An interesting finding in this study was that the type of MPN seemed to differ between the two patient groups, with polycythemia vera being the major underlying form in BCS-cases where essential thrombocythemia was more common in PVT. This discrepancy

has not been described before, although this may in part be due to the limited size of most reported patient cohorts. Nevertheless, in a large retrospective series including a total of 237 BCS-patients, 83% of the patients with a MPN had polycythemia vera. [39] In another study, including PVT-patients, that also provided information on MPN type, essential thrombocythemia was the most frequently detected form, present in 14 of 23 MPN-cases (61%). [27]

Thrombosis, both arterial and venous, is a well-recognized complication in patients with MPN. [40] However, despite extensive investigations, the precise pathogenetic mechanism leading to thrombosis in MPN-patients has not yet been elucidated. Abnormal platelet function, leukocyte activation and changes in platelet-leukocyte interaction have all been implicated as causative factors. [41-43] Reviewing various large cohort studies of patients with MPN, the spectrum of thrombosis differs somewhat between cases with polycythemia vera and those with essential thrombocythemia. Venous thrombosis is more common in polycythemia vera, accounting for approximately one-third of all thrombotic events in these patients. [44-45] In essential thrombocythemia, arterial thrombosis represents the majority of thrombotic complications. [46-47] Involvement of the microcirculatory system is also common in these patients and may manifest as erythromelalgia, transient ischemic attacks, headache or visual and hearing defects. These symptoms arise as a result of platelet thrombi that form in small vessels of the end-arterial circulation and typically occur in patients with thrombocytosis. [48-49] Considering the unique anatomy of the portal vein, one could speculate that essential thrombocythemia may predispose to PVT through platelet-mediated microvascular thrombosis of small portal venules, ultimately leading to overt thrombosis of the portal vein. This would be supported by the finding that in 18 of 21 PVT-patients with a MPN, thrombosis extended into either one or both portal vein branches. This may influence the treatment of PVT in the presence of an MPN, since these patients may benefit especially from platelet aggregation inhibitors, like aspirin. Still, this is the first study to demonstrate differences in the type of MPN underlying either BCS or PVT and further studies will be required to confirm these findings.

Regarding the role of multifactorial etiology, our results show that the presence of multiple risk factors for venous thrombosis does not influence disease presentation or the extent of thrombosis, both in patients with BCS and those with PVT. However, because the follow-up of this study was limited, we did not investigate the effect of multifactorial etiology on disease outcome. Potential effects of a multifactorial etiology on clinical outcome may only become evident after a longer time period. In patients with deep venous thrombosis of the lower extremity, the presence of more than one systemic risk factor for thrombosis is associated with an increased rate of future thrombotic events. [50-51] Whether this also applies to patients with BCS or PVT, remains to be investigated. Further studies, with a longer follow-up period, are required to evaluate if the long-term outcome of these patients is influenced by the presence of multiple prothrombotic factors. The same holds true for specific types of underlying risk factors, such as the presence of a MPN. Although there was no obvious clustering of risk factors in both patient groups, there was some effect of specific etiologic factors on the

extent of thrombosis in PVT-patients. A thrombosis confined to the portal vein or one of its branches was frequently encountered in patients with a local risk factors whereas an extended thrombosis involving the superior mesenteric vein or splenic vein was present in the majority of cases with an underlying MPN. These findings may reflect a differential impact of local inflammatory factors as compared to a more generalized hypercoagulable state that is found in MPN-patients. Nevertheless, other studies are needed to determine whether disease outcome and prognosis differs between patients with PVT resulting from local inflammatory factors and patients with PVT that have an underlying MPN.

Despite the extensive thrombophilia screening that was performed in the majority of patients, not all cases were systematically tested for all currently known risk factors. Moreover, in 15% of the patients with BCS and 19% of the patients with PVT no risk factors for thrombosis could be identified. Even though these numbers are lower than reported in other studies, it is possible that some etiological factors may have been missed. Another aspect to consider is the fact that, given the complex etiology of these disorders, certain factors that contribute to the development of thrombosis at these sites have possibly not yet been identified. Both these issues would potentially result in an underestimation of the number of risk factors and therefore, it is possible that the proportion of patients with a multifactorial etiology of thrombosis is, in fact, even higher than what we found in this study.

Concluding, this study demonstrates that presence of the FVL mutation is clearly associated with BCS, whereas FII mutation is a strong risk factor for PVT. The MTHFR mutation, even in homozygous form, is not a significant risk factor for either of these vascular liver disorders. Polycythemia vera appears to be more common in BCS and essential thrombocythemia seems more prevalent in PVT. Although there remains a clear overlap in the spectrum of etiologic factors associated with thrombosis in BCS and PVT, the finding that some factors appear related to thrombosis at a certain location, supports the idea of vascular bed-specific homeostasis.

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Chapter 3

IMPAIRED FIBRINOLYSIS AS A RISK FACTOR FOR BUDD-CHIARI SYNDROME

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ABSTRACT

In Budd-Chiari syndrome (BCS) thrombosis develops in the hepatic veins or inferior vena cava. To study the relationship between impaired fibrinolysis and BCS, we measured plasma levels of fibrinolysis proteins in 101 BCS-patients and 101 healthy controls and performed a plasma-based clot lysis assay. In BCS-patients, plasminogen activator inhibitor 1 (PAI-1) levels were significantly higher than in controls (median 6.3 vs. 1.4 IU/ml, $p<0.001$). Thrombin-activatable fibrinolysis inhibitor and plasmin inhibitor levels were lower than in controls (13.8 vs. 16.9 µg/ml and 0.91 vs. 1.02 U/l, both $p<0.001$). Median plasma clot lysis time (CLT) was 73.9 min in BCS-patients and 73.0 min in controls ($p=0.329$). A subgroup of cases displayed clearly elevated CLTs. A CLT above the 90th (93.1 min) or 95th (98.1 min) percentile of the controls was associated with an increased risk of BCS, with odds ratios of 2.4 (95%CI 1.1-5.5) and 3.4 (95%CI 1.2-9.7), respectively. In controls, only PAI-1 activity was significantly associated with CLT. Analysis of single nucleotide polymorphisms of fibrinolysis proteins revealed no significant differences between cases and controls.

Conclusions. This case-control study provides the first evidence that an impaired fibrinolytic potential, at least partially caused by elevated PAI-1 levels, is related to the presence of BCS.

INTRODUCTION

Budd-Chiari Syndrome (BCS) is a rare but life-threatening liver disease that is characterized by an obstruction of venous blood flow from the liver, irrespective of the underlying cause. [1] In the Western world, most cases of BCS are caused by thrombosis of one or more hepatic veins, with or without concomitant thrombosis of the inferior vena cava. The etiology of thrombosis in these specific veins is complex but in the past decades it has become clear that both inherited and acquired factors leading to an increased thrombotic tendency are involved. [2-4] To date, an underlying cause can be identified in the majority of patients with BCS and in a significant number of cases a combination of risk factors is present. [5-6]

Myeloproliferative neoplasms are the most common cause of thrombosis in BCS and either overt or latent forms are found in approximately 50% of all patients. [7-8] Another focus of extensive etiological study has been on disorders of coagulation. The mechanism of clot formation is dependent on the interaction of a large number of pro- and anticoagulant factors. The balance between these factors is crucial for normal hemostasis and disturbances of this balance may lead to a thrombotic tendency. Several disorders of the clotting system which lead to a hypercoagulant state and which are known to be involved in venous thromboembolism, have also been associated with BCS, e.g. Factor V Leiden mutation, prothrombin gene mutation and protein C deficiency. [2-3] So far, little attention has been addressed to disorders of the fibrinolytic system as a risk factor for BCS.

Fibrinolysis is the process through which fibrin clots are dissolved by the action of plasmin. Plasmin, an active enzyme that is formed from its inactive precursor plasminogen degrades fibrin into fibrin degradation products. Two main proteins that are responsible for the activation of plasminogen are tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). [9] The profibrinolytic actions of plasmin, t-PA and u-PA are all regulated by a number of specific inhibitors. Plasminogen activator inhibitor-1 (PAI-1) is the main inhibitor of t-PA and u-PA whilst both plasmin inhibitor (also known as α_2-antiplasmin) and thrombin-activatable fibrinolysis inhibitor (TAFI) are important in the inhibition and down regulation of plasmin. [10] The overall fibrinolytic activity is based on the balance between these profibrinolytic enzymes and their inhibitors, together with their affinity for fibrin and the regulatory effects of fibrin itself.

Disturbance of the fibrinolytic balance causing hypofibrinolysis can lead to decreased clot degradation and is a potential risk factor for venous thrombosis. Despite some conflicting results in previous studies [11-15], recent evidence suggests that there is a relationship between reduced fibrinolytic activity and the risk of venous thrombosis. [16-17] In the current study we have investigated the role of hypofibrinolysis as a risk factor for BCS.

PATIENTS AND METHODS

Study population

Patients with BCS were recruited from the EN-Vie study cohort. The EN-Vie Study, a prospective, multicenter cohort study of patients with BCS from 9 European countries, has been described previously. [6] In short, consecutive patients newly diagnosed with BCS were enrolled in different countries. At time of diagnosis and during follow-up, data concerning clinical condition and etiology and results of radiology, pathology and laboratory assessments were collected. BCS was defined as an hepatic venous outflow obstruction and its manifestations, regardless of the cause and regardless of the level of obstruction, ranging from the small hepatic veins to the entrance of the inferior vena cava into the right atrium. This definition did not include hepatic outflow obstruction caused by congestive heart failure or sinusoidal obstruction syndrome (previously known as veno-occlusive-disease). From October 2003 until October 2005, a total of 163 patients were included in the study.

After patients were enrolled in the study, they were asked to provide their own healthy control. Controls were healthy non-relatives and had to be of the same gender, ethnic background and age (with a range of 5 years) as the patient. Furthermore, controls were only included if they did not have a history of previous thrombosis. When patients were unable to provide controls themselves, the study coordinating centres attempted to find equally matched controls from their own resources.

Blood samples were collected from both patients (at time of diagnosis) and controls by means of venapuncture in tubes containing 0.11M trisodium citrate. Plasma was prepared by centrifugation at 2000g for 10 minutes and DNA was extracted from whole blood according to local standard methods. Both plasma and DNA samples were transported to the Erasmus University Medical Center in Rotterdam and stored at –70 °C until analysis.

The EN-Vie Study was conducted with approval from all national and, if necessary, local ethical committees, in accordance with the nation-specific rules. All patients and controls agreed to participate in the study by means of a written informed consent.

Only patients for whom stored plasma samples and a matched control person were available, were considered eligible for the current study. For each patient, Rotterdam prognostic scores were calculated as defined previously. [18] All laboratory assessments were performed at the Laboratory of Hematology of the Erasmus University Medical Center Rotterdam.

Measurement of fibrinolytic markers in plasma

In plasma samples of both patients and controls, we determined levels of four different fibrinolysis proteins, fibrinogen and factor XIII (FXIII). Plasma levels of fibrinogen were measured as a function of thrombin clotting time by using the Clauss method. [19] Levels of t-PA antigen

in plasma were assayed by a slightly modified commercially available enzyme-linked immunosorbent assay (t-PA Antigen Elisa Reagent Kit, Technoclone, Vienna, Austria). The activity levels of PAI-1 and TAFI were measured using a chromogenic assay (Chromolize PAI-1, Trinity Biotech, Berkeley Heights, USA and Actichrome TAFI activity kit, American Diagnostica, Stamford, USA, respectively). Plasmin inhibitor activity levels were determined by a chromogenic assay using the Coamatic Plasmin Inhibitor kit of Chromogenix (Instrumentation Laboratory, Milan, Italy). FXIII levels in plasma were also measured, using a spectrometric assay (Berichrom FXIII kit, Dade Behring, Newark, USA) adapted to a microtiter plate format, with pooled control plasma (Factor Assay ConTrol plasma (FACT), lot 222e1, George King Bio-Medical Inc, Overland Park, USA) as a calibrator.

Plasma fibrinolytic potential

Lysis of a tissue factor-induced plasma clot by exogenous t-PA was studied essentially as described previously with some small modifications. [16] Plasma (60µl) was diluted 1.7 fold in Hepes buffer (25 mM Hepes, 137 mM NaCl, 3.5 mM KCl, 3 mM $CaCl_2$, 0.1% bovine serum albumin (BSA), pH 7.4). The diluted plasma samples (85µl) were added to the wells of a microtiter plate containing 15µl of a reaction mixture. The reaction mixture was composed of tissue factor (TF- Innovin, Dade Behring, Marburg, Germany), $CaCl_2$, t-PA (Actilyse, Boehringer Ingelheim, Ingelheim, Germany) and phospholipid vesicles (consisting of 40% L-α-dioleoylphosphatidylcholine (PC), 20% L-α-dioleoylphosphatidylserine (PS), and 40% L-α-dioleoylphosphatidylethanolamine (PE), all from Sigma, St Louis, USA, prepared according to [20-21]). Innovin was prepared according to the manufacturer's instructions, by reconstituting the supplied lyophilized powder with 10 ml of deionized water and it went through a freeze/thawing cycle before it was used in the assay.

The final concentrations in the clotted plasma were as follows: tissue factor 1000 times diluted, 17 mM $CaCl_2$, 25 ng/ml t-PA and 10 µM phospholipid vesicles. After mixing the diluted plasma and the reaction mixture, each clot was covered with paraffin oil (50µl, Merck – No.107162) and the plate was placed immediately in a prewarmed (37°C) incubation chamber of a microplate reader (Perkin Elmer, Turku, Finland). The optical density at 405 nm was monitored every minute for 400 min. The clot lysis time (CLT) was defined as the time from the midpoint of clear to maximum turbid transition, which characterizes clot formation, to the midpoint of the maximum turbid to clear transition, which represents clot lysis. A prolonged CLT in comparison to the control group CLT is indicative of hypofibrinolysis while a short CLT can indicate hyperfibrinolysis.

Analysis of common polymorphisms

All individuals were genotyped for several previously identified single nucleotide polymorphisms (SNPs) of FXIII [100G/T (Val34Leu)], PAI-1 [-675 4G/5G], TAFI [1040C/T (Thr325Ile) and 1538A/T], t-PA [-7351C/T and 27739G/A] and fibrinogen [-148C/T]. [22-27] The genotype for these seven SNPs was determined by a duplex polymerase chain reaction (PCR) followed by a restriction fragment length analysis. Different PCR-mixtures and PCR-conditions were used for all polymorphisms.

Statistical analysis

Plasma levels of TAFI, plasmin inhibitor, fibrinogen and FXIII as well as the CLTs displayed a normal distribution. Because t-PA antigen and PAI-1 activity levels had a skewed distribution, a log-transformation was performed before these variables were analyzed. The results of all parameters were compared between the cases and controls using the Student's T-test. The association between fibrinolytic parameters in cases and different measures of liver function was assessed using Spearman's Rank Correlation test. The relative risk of BCS associated with an elevated CLT was estimated as an odds ratio (OR) and corresponding 95% confidence interval (CI) using logistic regression, adjusted for sex and age. Percentiles (70^{th}, 80^{th}, 90^{th} and 95^{th} percentile) of the CLT measured in the control group were used as cut-off levels. Determinants of the CLT were evaluated in controls with multiple linear regression, including the log-transformed values for PAI-1 activity and t-PA antigen. Differences between cases with and without a CLT above the 90^{th} percentile were assessed using the Mann-Whitney U test. The relationship between the different polymorphisms and BCS was investigated with ORs (and 95% CI) calculated by logistic regression. A p-value of <0.05 was considered to be statistically significant. All statistical analyses were performed with the Statistical Package for Social Sciences for Windows, version 14.0 (SPSS, Chicago, USA).

RESULTS

From the total EN-Vie cohort of 163 patients with BCS, plasma samples for this study were available for 107 patients (66%). Six patients were subsequently excluded because a control person was missing, leaving 101 eligible sex- and age-matched case-control pairs.

Patient characteristics are shown in Table 1. The patient and control group were comparable with respect to age, sex and race. Baseline characteristics of the 101 patients in the study group were similar to those of the total EN-Vie cohort (n=163), indicating that the current study population was a representative patient sample. Median age at diagnosis was 37 years (range: 16-84) and 42% of the patients were males. Concurrent portal vein thrombosis

was present in 13% of the patients. An underlying myeloproliferative disorder (MPN) was identified in 35 patients (35%).

Table 1. Baseline characteristics of patients with BCS

Patient characteristics	n=101
Age, median (range)	37 (16-84)
Sex; males	42
Race; Caucasian	74
Location of venous occlusion	
HV occlusion	47
IVC occlusion	2
Combined HV and IVC occlusion	52
Concurrent portal vein thrombosis	13
Rotterdam BCS prognostic index§	
Class I	29
Class II	51
Class III	20

Unless stated otherwise, values are expressed as number of cases and corresponding percentage, n (%). § As a result of missing values, the Rotterdam BCS prognostic index could not be calculated in 1 patient; HV= hepatic vein, IVC= inferior vena cava.

Plasma fibrinolytic parameters

As shown in Figure 1, levels of t-PA antigen (Figure 1A, median 6.8 vs. 2.9 ng/ml) and PAI-1 activity (Figure 1B, median 6.3 vs. 1.4 IU/ml) were significantly increased in cases versus controls (both $p<0.001$). In contrast, TAFI activity-levels were lower in patients as compared to the controls (Figure 1C, median 13.8 vs. 16.9 µg/ml, $p<0.001$). Plasmin inhibitor activity was also decreased, with a median level of 0.91 U/l in cases and 1.02 U/l in controls (Figure 1D, $p<0.001$).

Because the liver synthesizes most coagulation factors and fibrinolytic proteins and patients with BCS may exhibit various degrees of liver dysfunction, we evaluated the association between the fibrinolytic parameters and different liver function tests. PAI-1 activity showed no significant correlation with any liver function test. For t-PA antigen there was a minor correlation with albumin levels and TAFI-activity was associated with both albumin and bilirubin levels. Plasmin inhibitor activity correlated with factor V and bilirubin levels. However, all correlation coefficients were less than 0.4, indicating at most only a moderate relationship. (Table 2)

Plasma levels of fibrinogen and FXIII were also measured, given their important role in fibrin formation and in the cross-linking of fibrin fibers, respectively. Levels of fibrinogen were higher in cases as compared to controls (Figure 1E, median 2.9 vs. 2.8 g/l), but this difference failed to reach statistical significance ($p=0.072$). FXIII concentration however, was significantly lower

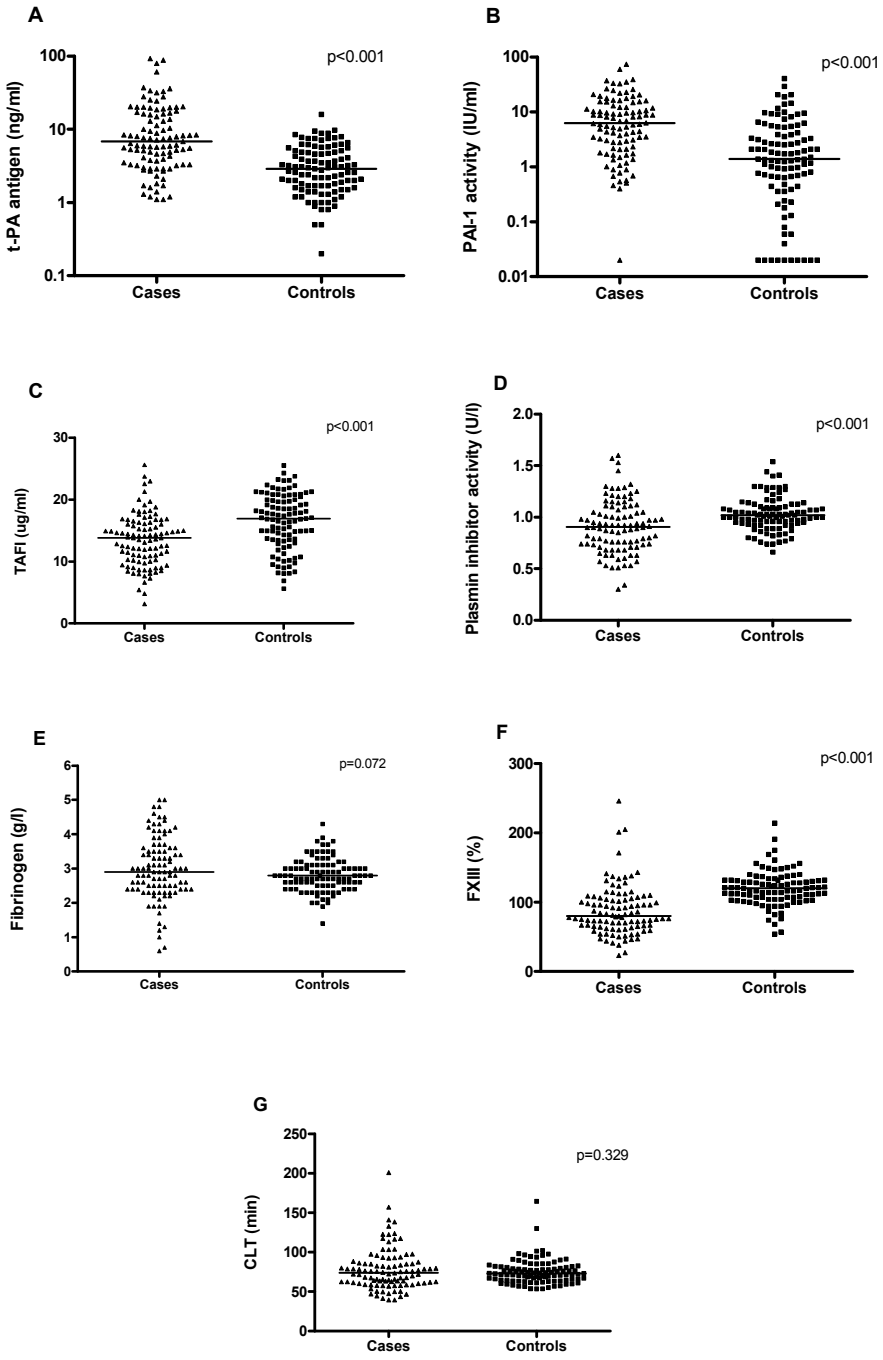

Figure 1. Comparison of plasma levels of t-PA antigen (A), PAI-1 activity (B), TAFI-activity (C), plasmin inhibitor activity (D), fibrinogen (E) and factor XIII (F) between patients with Budd-Chiari syndrome (cases, n=101) and healthy controls (n=101). For t-PA antigen and PAI-1 the log-transformed values are depicted. (G) Clot lysis times (in minutes) of patients with Budd-Chiari syndrome (cases, n=100) and healthy controls (n=101) as measured using a plasma-based in vitro clot lysis assay. Individual data-points and median values are given.

in the BCS-patients (Figure 1F, median 80% vs.120%; p<0.001). Association with liver function tests revealed only a minor inverse correlation between fibrinogen and bilirubin levels. (Table 2)

Table 2. Associations of fibrinolytic parameters in BCS-patients with different measures of liver function

	Correlation coefficient§					
	PAI-1	t-PA	TAFI	PI	FXIII	Fibrinogen
Albumin	-0.124	-0.388*	-0.273*	0.011	0.166	-0.085
Bilirubin	-0.044	0.185	-0.377*	-0.356*	-0.069	-0.315*
ALT	0.166	0.085	0.037	0.146	0.054	-0.076
Factor V‡	-0.007	-0.125	0.329	0.384*	0.052	0.244

*§ Spearman's rho correlation coefficient. * p<0.05. ‡ Factor V levels were only determined in 29 patients.*
CI= confidence interval, PAI-1= plasminogen activator inhibitor-1, t-PA= tissue-type plasminogen activator, TAFI= thrombin-activatable fibrinolysis inhibitor, PI= plasmin inhibitor, FXIII= factor XIII, ALT= alanine aminotransferase.

To evaluate whether the presence of an underlying MPN had an effect on any of the fibrinolytic parameters, we compared the plasma levels of all fibrinolysis proteins between cases with and without evidence of a MPN. In BCS-patients with a MPN (n=35) median TAFI level was 11.9 µg/ml as compared to 14.6 µg/ml in patients without MPN (n=66, p<0.001). Plasmin inhibitor levels were also lower in MPN-cases than in non-MPN cases, 0.79 vs. 0.97 U/l, respectively (p=0.009). Levels of PAI-1 activity, t-PA antigen, fibrinogen and FXIII did not differ significantly between both patient groups.

Plasma fibrinolytic potential

As a measure of overall fibrinolytic potential we performed an *in vitro* clot lysis assay. The clot lysis time (CLT) results for both cases and controls are depicted in Figure 1G. For the total study population, CLTs did not differ between the two groups (median 73.9 min in cases vs. 73.0 min in controls, p=0.329). Also, when cases were subdivided into prognostic classes according to the Rotterdam BCS index, there were no significant differences between patient classes and when these classes were compared to their controls (data not shown). Nevertheless, we identified a subgroup of BCS-patients with an increased CLT when compared with the controls. To investigate the potential contribution of an increased CLT to the presence of BCS, different cut-off levels were set, according to the 70th, 80th, 90th and 95th percentile of CLTs in the control population. Using values below the cut-off as a reference, the relative risk of BCS for CLTs above the cut-off points augmented with increasing values of the cut-off. (Table 3) With the 90th percentile as a cut-off value (CLT 93.1 min), 21 cases had CLTs above this level as compared with 10 controls, corresponding with a more than two-fold increased relative risk of BCS (OR 2.4; 95% CI:1.1-5.5). This risk was even higher when the 95th percentile (CLT of 98.1 min) was used as a cut-off value (OR 3.4; 95% CI:1.2-9.7).

Table 3. Risk of BCS according to CLT

Cut-off percentile	CLT (min)	No. of controls	No. of cases	OR (95% CI)§
70	78.5	30	41	1.7 (0.9-3.0)
80	84.9	20	30	1.8 (0.9-3.4)
90	93.1	10	21	2.4 (1.1-5.5)
95	98.0	5	15	3.4 (1.2-9.7)

§ ORs adjusted for age and sex; CLT= clot lysis time, OR= odds ratio, CI= confidence interval.

To determine which factors were associated with the CLT in the control population, we performed a multiple linear regression analysis including PAI-1, t-PA antigen, TAFI, plasmin inhibitor, fibrinogen and FXIII. PAI-1 activity levels appeared to be the most important determinant of CLT, with a corresponding regression coefficient of 3.36 (CI 1.46-5.26) and a p-value of 0.001. Regression coefficients for t-PA antigen, TAFI, plasmin inhibitor, fibrinogen and FXIII were not statistically significant. Indeed, when we compared BCS-patients with CLTs above the 90th percentile to patients with CLTs below the 90th percentile, it became apparent that cases with an increased CLT had significantly higher levels of PAI-1 activity. (Table 4) There was no significant difference between CLTs in patients with an underlying MPN as compared to cases without MPN (p=0.573).

Table 4. Comparison of BCS-patients with normal and prolonged CLT (>90th percentile)

Parameter	Prolonged CLT§	Normal CLT§	P‡
PAI activity, IU/ml	15.5 (6.5-24.6)	5.1 (1.0-13.3)	<0.001
t-PA antigen, ng/ml	14.9 (2.5-27.4)	5.9 (1.1-10.8)	0.003
TAFI, µg/ml	12.6 (9.4-15.8)	14.0 (10.9-17.0)	0.713
Plasmin inhibitor, U/l	0.97 (0.75-1.19)	0.90 (0.74-1.07)	0.472
Fibrinogen, g/l	3.1 (2.3-3.9)	2.8 (2.2-3.4)	0.058
Factor XIII, %	83 (58-109)	79 (59-100)	0.949

Median values (interquartile range) are given in each group. § Prolonged CLT was defined as a CLT above the 90th percentile of the control population (93.1 min), patients with a CLT below the 90th percentile were defined as having a normal CLT. ‡ Mann-Whitney U test; CLT= clot lysis time, PAI-1= plasminogen activator inhibitor-1, t-PA= tissue-type plasminogen activator, TAFI= thrombin-activatable fibrinolysis inhibitor, FXIII= factor XIII.

As the majority of patients with BCS are given anticoagulation immediately after diagnosis, we wanted to estimate the potential effect of anticoagulant treatment on clot lysis times and the fibrinolytic parameters. Therefore, we evaluated the clotting times of control samples as measured in the clot lysis assay. Median clotting time of control samples was 5.28 minutes (interquartile range 4.14). Considering a clotting time longer than 10 minutes as abnormal, we identified 10 patients in the study population with a clotting time above this cut-off level.

Of these patients, 6 cases also displayed a prolonged CLT (above 90th percentile). Exclusion of these patients and their matched controls from the analysis did not affect the outcome of the plasma measurements. (data not shown) Furthermore, although the size of the risk was somewhat attenuated, there remained a clear trend towards an increased risk of BCS in cases with a CLT above the 90th or 95th percentile, with ORs of 1.8 (CI 0.8-4.4) and 2.7 (CI 0.8-9.0), respectively.

Analysis of common polymorphisms

Over the past years, different polymorphisms have been described in the genes for PAI-1, t-PA, TAFI, fibrinogen and FXIII. To investigate the genotype distribution in patients with BCS, the genotypes for seven common polymorphisms were analyzed and compared between cases and controls. Table 5 displays the results of the genotype analysis. There were no significant differences between cases and controls.

Table 5. Distribution of polymorphisms between BCS-patients and controls

	Cases	Controls	OR (95% CI)
FXIII (Val34Leu)			
GG, n (%)	44 (46)	40 (40)	1§
GT, n (%)	48 (50)	53 (53)	0.8 (0.5-1.5)
TT, n (%)	4 (4)	7 (7)	0.5 (0.1-1.9)
T-allele (frequency)	29%	34%	
PAI-1 (4G/5G)			
4G4G, n (%)	19 (20)	26 (27)	1§
4G5G, n (%)	70 (74)	62 (65)	1.5 (0.8-3.1)
5G5G, n (%)	6 (6)	7 (7)	1.2 (0.3-4.1)
5G-allele (frequency)	43%	40%	
TAFI (1040)			
CC, n (%)	43 (44)	46 (46)	1§
CT, n (%)	43 (44)	41 (41)	1.1 (0.6-2.0)
TT, n (%)	11 (11)	12 (12)	1.0 (0.4-2.5)
T-allele (frequency)	34%	33%	
TAFI (1583)			
AA, n (%)	51 (51)	45 (45)	1§
AT, n (%)	34 (34)	40 (40)	0.8 (0.4-1.4)
TT, n (%)	14 (14)	16 (16)	0.8 (0.4-1.8)
T-allele (frequency)	31%	36%	
t-PA (-7351)			
CC, n (%)	48 (49)	42 (42)	1§
CT, n (%)	41 (42)	45 (45)	0.8 (0.4-1.4)
TT, n (%)	9 (9)	13 (13)	0.6 (0.2-1.6)
T-allele (frequency)	30%	36%	
t-PA (27739)			
AA, n (%)	77 (84)	81 (84)	1§
AG or GG, n (%)	15 (16)	16 (16)	1.0 (0.5-2.1)
G-allele (frequency)	9%	8%	
Fibrinogen (-148)			
CC, n (%)	49 (57)	52 (54)	1§
CT, n (%)	29 (34)	36 (38)	0.9 (0.5-1.6)
TT, n (%)	8 (9)	8 (8)	1.1 (0.4-3.0)
T-allele (frequency)	26%	27%	

§ Reference category; OR= odds ratio, CI= confidence interval, PAI-1= plasminogen activator inhibitor-1, t-PA= tissue-type plasminogen activator, TAFI= thrombin-activatable fibrinolysis inhibitor, FXIII= factor XIII.

With respect to the FXIII Val34Leu gene variant (a valine to leucine substitution at codon 34), there is evidence suggesting an interaction with plasma fibrinogen levels. Therefore, we determined the genotype distribution of this polymorphism in cases and controls depending on the plasma levels of fibrinogen. High fibrinogen concentration was defined as plasma levels above the 75th percentile in controls (3.1 g/l). Using this definition, 43 cases and 27 controls had high fibrinogen levels, compared to 58 cases and 74 controls with normal fibrinogen levels (levels <75th percentile). With the FXIII Val/Val genotype as a reference, we calculated ORs for BCS associated with carriership of the Leu-allele. In the group with high fibrinogen levels, heterozygous and homozygous carriership of the Leu-allele was associated with an OR of 0.5 (CI: 0.2-1.5) and 0.1 (CI: 0.0-1.6), respectively. The presence of either the Val/Leu or the Leu/Leu genotype corresponded with an OR of 0.5 (CI: 0.2-1.2). For the group with normal fibrinogen levels, the ORs for Leu-allele heterozygotes, homozygotes or both heterozygotes and homozygotes were 1.1 (CI: 0.5-2.2), 1.0 (CI: 0.2-5.0) and 1.1 (CI: 0.5-2.2), respectively. Similar results were obtained when we used the 90th percentile of fibrinogen levels in controls (3.5 g/l) as a cut-off level. (data not shown) These results suggest that at high fibrinogen levels, the relative risk of BCS is lower in carriers of the FXIII Leu-allele as compared to individuals with the Val/Val genotype.

DISCUSSION

This case-control study is the first study to show that impaired fibrinolysis is a potential risk factor for BCS. In patients with deep venous thrombosis it has recently been shown that an impaired plasma fibrinolytic potential is a risk factor for thrombosis. [16-17] Whether alterations in the fibrinolytic system can also predispose to thrombosis in patients with BCS has not yet been investigated. In the current study group of BCS-patients and healthy controls we measured the plasma concentration of several factors involved in fibrinolysis (t-PA, PAI-1 TAFI and plasmin inhibitor) or the final step of fibrin clot formation (fibrinogen and FXIII). As an overall measure of plasma fibrinolytic potential we performed a plasma-based *in vitro* clot lysis assay.

Levels of individual components of the fibrinolytic pathway were clearly altered in patients with BCS as compared to the group of healthy controls. In patients, plasma concentrations of t-PA antigen and PAI-1 activity were significantly higher. PAI-1 is an important inhibitor of fibrinolysis and it has been hypothesized that elevated plasma levels are a risk factor for venous thrombosis. Several studies have found a relationship between elevated PAI-1 levels and venous thrombosis. [14, 28-30] In PAI-1 transgenic mice, it was shown that PAI-1 expression is related to the development of venous thrombosis. [12] Still, a few prospective studies have failed to show a relationship between PAI-1 levels and venous thrombosis, suggesting that the predictive power of elevated PAI-1 may be low. [13, 31-32] In contrast to PAI-1, t-PA promotes

fibrin degradation and the increased concentration of t-PA antigen present in patients with BCS may thus seem to counteract the inhibitory effects of PAI-1 on fibrinolysis. However, t-PA measured in plasma is largely inactive because it rapidly forms a complex with PAI-1. [33] The high levels of t-PA antigen we found in this study probably reflect the presence of these t-PA/PAI-1 complexes and are likely the result of an elevated PAI-1 concentration. [34] An alternative explanation for increased circulating t-PA/PAI-1 complexes could be that there is a decreased hepatic clearance of these complexes. In addition to its synthetic function, the liver is also involved in the clearance of many fibrinolytic proteins. Indeed, there was a relationship between t-PA levels and the severity of liver disease, as expressed by the Child-Pugh classification. (data not shown) Although this classification is developed for patients with liver cirrhosis, it could indicate that the levels of t-PA antigen were affected by an impaired clearance of complexed t-PA. In contrast, we did not find a correlation between Child-Pugh class and PAI-1 activity, suggesting that an impaired hepatic clearance was not a major causative factor responsible for the increased PAI-1 levels. (data not shown) Moreover, the levels of PAI-1 activity we measured do not include the complexed form of PAI-1 and should therefore be interpreted differently. Our findings are in line with data from a study of fibrinolytic parameters in patients with liver cirrhosis, showing increased levels of t-PA antigen with increasing severity of liver disease but no significant change of PAI-1 activity levels as compared to healthy controls. [35]

Only one relatively small study has investigated the status of components of the fibrinolytic system in patients with BCS. Dayal *et al.* [36] studied t-PA and PAI-1 levels in 27 BCS-patients and compared them to subsets of patients with other liver diseases and to a group of 20 healthy controls. In contrast to our findings, they found no significant differences between the groups. Only 3 of their patients with BCS displayed elevated levels of t-PA and PAI-1. Our study consisted of a considerably larger cohort of patients. Furthermore, cases were included in a prospective manner which may partially account for these differences in results. The substantially increased PAI-1 activity we found in patients with BCS provides the first evidence that fibrinolysis may be impaired in these patients. As yet, it is unclear what factors could account for these elevated PAI-1 levels. There is evidence that fibrinolysis may be impaired in patients with an underlying MPN, such as polycythemia vera and essential thrombocytosis. [37-38] In this study, CLTs and PAI-1 activity levels did not differ significantly between cases with and without an underlying MPN. However, this does not exclude an effect of an MPN on fibrinolysis as there may be an interplay of different mechanisms leading to elevated PAI-1 levels. Further studies will have to be performed to establish the causal role of PAI-1 levels in the pathogenesis of thrombosis in BCS-patients.

To further characterize the status of the fibrinolytic system in BCS, the plasma fibrinolytic potential was determined with an *in vitro* plasma clot lysis assay. We found that median CLTs were not significantly different between BCS-patients and controls. However, a subset of patients had a clearly prolonged CLT. Calculation of cut-off levels in the control group revealed

that a CLT above the 90th or 95th percentile was associated with an increased risk of BCS (OR of 2.4 and 3.4, respectively). Further characterization of patients who had signs of hypofibrinolysis showed that they had significantly higher plasma levels of PAI-1. The concentration of TAFI, fibrinogen and factor XIII did not differ between patients with and without hypofibrinolysis, suggesting that PAI-1 might be the most important determinant of hypofibrinolysis in these patients. This was also supported by the finding that PAI-1 was significantly associated with CLTs in the control population.

TAFI did not seem to contribute to the observed hypofibrinolysis as TAFI levels in plasma of patients with BCS were actually lower than in controls. Activity levels of plasmin inhibitor were also decreased in the cases with BCS. Interestingly, we found that both TAFI and plasmin inhibitor levels were significantly lower in BCS-patients with an underlying MPN, as compared to cases without signs of a MPN. It is well known that in MPN-patients both hemorrhagic and thrombotic complications can occur. [39] Whether changes in the fibrinolytic system contribute to bleeding or thrombosis in these patients has not yet been elucidated. Although evidence is scarce, one other study has also shown that levels of plasmin inhibitor were significantly decreased in patients with chronic MPN. [40] Our findings suggest that the levels of TAFI and plasmin inhibitor were, in part, influenced by the presence of an underlying MPN.

FXIII levels were significantly lower in cases as compared to controls. FXIII is responsible for the cross-linking of fibrin fibers during clot formation. A severe deficiency of FXIII is associated with a bleeding tendency but slightly decreased levels of FXIII do not seem to impair its function significantly. [41] Moreover, it has been suggested that increased levels of FXIII have a protective effect on the development of venous thrombosis. [42] Our findings are in line with that report and may even indicate a pathogenetic role of decreased FXIII levels in BCS. However, this needs to be further investigated.

An important confounder when studying the haemostatic system in patients with liver disease is the potential effect of impaired liver function itself. Most clotting factors are exclusively synthesized by the liver and it is well known that in patients with acute or chronic liver failure major changes in both pro- and anticoagulant pathways can occur. [43] Nevertheless, deterioration of synthetic function predominantly occurs along the course of parenchymal liver diseases whereas in vascular liver disorders protein synthesis is mostly well preserved. In the patient group we studied, we found no major correlations between several measures of liver function and the levels of PAI-1, t-PA antigen, TAFI, plasmin inhibitor, fibrinogen and FXIII. Therefore, we believe that the observed differences in plasma fibrinolytic parameters between BCS-patients and controls cannot be explained by disturbances in liver synthetic function alone. Moreover, PAI-1 is not only secreted by hepatocytes but can also be synthesized by endothelial cells, smooth muscle cells and adipocytes. [44] These alternative sites of PAI-1 synthesis may partly compensate for a potential decrease of PAI-1 synthesis resulting from liver dysfunction.

Increased levels of PAI-1 activity can be difficult to interpret, considering that PAI-1 is found to be an acute phase reactant. [45] To evaluate the possible influence of an acute phase response on PAI-1 activity in BCS-patients, we studied the association between PAI-1 levels and plasma concentration of fibrinogen, which is also considered an acute phase protein. There was no correlation between PAI-1 activity and fibrinogen levels in the cases (Spearman's rho correlation coefficient -0.037, p=0.713). These findings illustrate that PAI-1 activity levels were probably not elevated as a result of an acute phase response.

Another factor that could have influenced our findings is the fact that many patients were already treated with anticoagulants when plasma samples were taken. Anticoagulation affects not only the clotting cascade but may also alter the fibrinolytic system. In our study population we identified 10 patients with a clearly prolonged clotting time, for whom a significant effect of anticoagulant treatment on the CLTs could not be excluded. However, subsequent exclusion of these patients did not substantially change the results of our analyses, suggesting that the effect of anticoagulation on fibrinolytic parameters in our study was probably negligible.

Despite previous studies, showing that certain single nucleotide polymorphisms of PAI-1, t-PA, FXIII and TAFI influence the risk of venous thrombosis [42, 46-50], our results did not show an association between any of the studied polymorphisms and thrombosis in BCS-patients. With respect to the two TAFI-gene polymorphisms, this is in accordance with a recent study from our group. [49] In patients with splanchnic vein thrombosis (i.e. BCS or portal vein thrombosis) there was no association between TAFI C1040T and TAFI T1583A polymorphisms and the risk of splanchnic vein thrombosis. For the FXIII Val34Leu polymorphism, it has been suggested that there is an interaction with fibrinogen levels. [51] At high fibrinogen concentrations the 34Leu variant has been associated with a protective effect against venous thrombosis and coronary artery disease. [52-53] In line with these findings, we also observed a trend towards a decreased risk of hepatic vein thrombosis in FXIII Leu-allele carriers with high fibrinogen levels. Carriership of the Leu-allele was associated with an OR for BCS of 0.5 (CI: 0.2-1.2) in individuals with high fibrinogen as compared to an OR of 1.1 (0.5-2.2) in individuals with normal fibrinogen. However, our results failed to reach statistical significance, which may be due to the relatively small number of patients and controls.

In conclusion, this study provides the first evidence that an impaired fibrinolysis may play a role in the pathogenesis of BCS. Although there was no significant overall difference in plasma fibrinolytic potential between patients and controls, a subgroup of patients with a prolonged CLT could be identified in whom the risk of BCS was clearly increased. These patients were characterized by increased plasma levels of PAI-1 but not by high TAFI-levels. Hypofibrinolysis may thus prove to be a previously unknown risk factor for the development of BCS.

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Chapter 4

PROTEOMIC ANALYSIS REVEALS THAT APOLIPOPROTEIN A1 LEVELS ARE DECREASED IN PATIENTS WITH BUDD-CHIARI SYNDROME

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ABSTRACT

Budd-Chiari syndrome (BCS) is a rare vascular liver disorder caused by thrombosis of the hepatic veins. In some patients no known thrombophilic factor can be identified. This study aims to identify novel factors that might play a role in thrombosis in BCS-patients by using a proteomic approach. The abundance of plasma clot-bound proteins was compared between 9 BCS-patients and 9 controls by two-dimensional difference gel electrophoresis. The protein with the most significant decrease in patients was identified with mass spectrometry. Plasma levels of this protein were measured and the results were validated in a large cohort of BCS-patients. A total of 26 protein spots significantly differed ($p<0.001$). The spot with the most significant decrease in patients was identified by mass spectrometry as apolipoprotein A1 (apo A1). The mean level of apo A1 in plasma of these BCS-patients (0.74 g/l) was also significantly lower than in controls (1.45 g/l, $p=0.002$). This finding was validated in a large cohort of 101 BCS-patients and 101 controls (0.97 g/l vs. 1.32 g/l, $p<0.001$). There was no major correlation between plasma levels of apo A1 and various liver function tests.

Conclusions. BCS-patients show decreased clot-bound protein abundance and plasma levels of Apo A1. Decreased levels of apo A1 may play a role in the etiology of thrombosis in BCS-patients and possibly in other patients with venous thrombosis.

INTRODUCTION

Venous thrombosis is a frequent cause of morbidity and mortality in the Western world, with an incidence rate of approximately 1 per 1000 patient-years. [1-2] The most common manifestations of venous thrombosis are deep venous thrombosis of the lower extremities and pulmonary embolism. Other localizations, such as thrombosis of the cerebral or abdominal veins, are seen infrequently. When thrombosis involves the hepatic veins or the inferior vena cava, blocking the outflow of blood from the liver, it is referred to as Budd-Chiari syndrome (BCS). [3] BCS is a rare vascular liver disorder but if left untreated, liver failure or death may ensue. [4] As is the case with thrombosis at other locations, various inherited and acquired factors have been identified that are associated with BCS. [5-8] Moreover, current evidence suggests that BCS is a multifactorial disease that often develops in the presence of more than one risk factor. [9] Although one or more underlying causes can be found in the majority of patients, there are still cases in which none of the known risk factors are present.

Fibrin clot formation is the final step of a complex cascade of reactions that represents the coagulation system. Dysregulation of any of the numerous components of the clotting cascade and the fibrinolytic system can potentially disrupt the hemostatic balance, leading to an increased tendency of either bleeding or thrombosis. Alterations in the plasma concentration of certain proteins influencing blood coagulation (e.g. elevated levels of factor VIII or a protein C deficiency) can therefore be involved in the onset of venous thrombosis. [10-11] Several proteins bind to fibrin as an essential step in their mechanism of action (e.g. thrombin, factor XIII, plasminogen and tissue-type plasminogen activator). [12] Consequently, some prothrombotic abnormalities may also be reflected by changes in the concentration of proteins that bind to a plasma clot.

In this study we aimed to identify novel factors that may play a role in venous thrombosis observed in patients with BCS by using a proteomic approach. To this end, we prepared plasma clots in vitro by addition of thrombin to freshly frozen plasma samples and compared the plasma clot composition of BCS-patients and healthy controls using two-dimensional fluorescence-based difference gel electrophoresis (2D-DIGE). [13] Furthermore, the specific protein found to have the most significant decrease in abundance in cases versus controls was identified with mass-spectrometry. Subsequently this finding was validated in a large case-control study using plasma samples of BCS-patients and controls.

PATIENTS AND METHODS

Materials

Urea, thiourea, CHAPS, DTT and iodoacetamide were obtained from Fluka (St. Louis, MO, USA). Aprotinin (Trasylol) was obtained from Bayer (Leverkusen, Germany). Tris (PlusOne), CyDyes, DeStreak, IPG buffer pH 3-10 and immobiline strips were obtained from GE Healthcare (Uppsala, Sweden). The anchorchip plate, α-cyano-4-hydroxycinnamic acid matrix and the Ultraflex-II apparatus were from Bruker Daltonics (Bremen, Germany). Thrombin was obtained from Sigma-Aldrich (St. Louis, MO, USA). Colloidal blue staining kit was obtained from Invitrogen (Paisley, UK) and Trypsin Gold was obtained from Promega Corporation (Madison, WI, USA).

Plasma samples from cases and controls used for in vitro clot formation

To examine the plasma clot composition of patients with BCS and healthy controls, blood samples were collected from 9 consecutive patients with BCS admitted to the Department of Gastroenterology and Hepatology of the Erasmus Medical Center in Rotterdam, the Netherlands. For each patient a healthy, non-related control person was recruited from department personnel. Controls were of the same sex, ethnicity and age (with a range of five years) as the patient. Furthermore, controls did not have a previous history of thrombosis or malignancy and were not using oral contraceptives. Peripheral blood samples were obtained from both patients and controls by means of venapuncture and collected in tubes containing 0.11 M trisodium citrate. Platelet-free plasma was acquired by a two-step centrifugation method (10 minutes at 2,000g and 10 minutes at 20,000g) at 4°C and subsequently stored at –70 °C until further analysis.

This study was conducted with approval from the ethics committee of the Erasmus University Medical Center. All patients and controls agreed to participate by means of a written informed consent.

Sample preparation for 2D-DIGE

Clots of 500 µl citrated plasma were prepared by adding calcium chloride (20 mM) and thrombin (1 NIH U/ml) for the initiation of coagulation and aprotinin (100 KIU/ml) to prevent proteolytic degradation. [14] After an incubation period of 2 hours at room temperature, the clots were extensively washed by permeating them with 10 ml Tris-buffered saline (50 mM Tris-HCl, 100 mM NaCl, pH 7.4) containing aprotinin (100 KIU/ml) overnight at 4°C. The clots were compacted by centrifugation, washed with deionized water and noncovalently clot-bound proteins were extracted with 150 µl rehydration buffer (7M urea, 2M thiourea, 4%

(w/v) CHAPS, 30 mM Tris-HCl, pH 8.5) for 1 hour at room temperature. Samples of 50 μl were labeled with the N-hydroxysuccinimide esters of Cy3 or Cy5 minimal fluorescent cyanine dyes. Five patient samples were labeled with Cy3 minimal dye and the other 4 patients with Cy5 minimal dye. The matched controls were labeled with Cy5 and Cy3 minimal dye, respectively. The samples were randomized to Cy3 and Cy5 labeling to minimize dye-based artifacts. Labeling was performed using 400 pmol dye per 50 μl sample, containing about 1.5 μg protein. Samples were labeled on ice for 30 minutes and quenched with 0.2 mM lysine. A pool of all 9 patient and 9 control samples was prepared and 50 μl was labeled with Cy2 minimal dye. This sample was used as an internal standard. For 2D-gel electrophoresis, each labeled patient sample was pooled with the labeled matched control sample and the labeled internal standard and analyzed simultaneously to reduce gel-to-gel variations. The total volume of sample was adjusted to 185 μl with rehydration buffer, 0.9% IPG-buffer 3-10 pH range and 1.2% (v/v) DeStreak.

2D-DIGE

The 9 pools containing the labeled samples of the BCS-patients, their matched controls and the internal standard were run on 9 different gels. The presence of the internal standard in each pool facilitated gel-to-gel matching. The proteins were separated in the first dimension with a 17 cm immobiline drystrip with a 3-10NL pH range. The isoelectric focusing was carried out at 20°C on the IPGphore II with the following running protocol: 12 h at 30 V (rehydration), 1 h at 500 V, 1 h at 1000 V, and 60000 Vh at 8000 V, with a 50 μA limit per gel.

The strips were equilibrated in equilibration buffer (6 M urea, 50 mM Tris-HCl pH 8.8, 20% (v/v) glycerol, 2% (w/v) SDS) with 1% (w/v) DTT for 15 minutes followed by a second equilibration step with equilibration buffer with 1% (w/v) iodoacetamide for 15 minutes. For the second dimension the strips were run on 10.5% polyacrylamide gels (20 x 22.4cm) at 12 mA/2 gels constant current for 18.5 h.

Image acquisition and analysis

After 2D-DIGE separation, gels were scanned using Typhoon 9410 (Amersham Pharmacia Biotech) at 100-micron resolution. 2-D images from the Cy2-, Cy3-, and Cy5-labeled protein fractions were scanned using a 488, 532 and 633 nm laser, respectively. Gel images were cropped using ImageQuant TL software and image analysis was performed with Decyder V6.5 software (Amersham Pharmacia Biotech). Spot detection was performed using the differential in-gel analysis (DIA) module by setting 7500 as estimated number of spots. The Cy2, Cy3, and Cy5 images of each gel were merged and spot boundaries were detected. Spots resulting from non-protein source, like dust particles, were filtered out by removing spots with a slope greater than 1.3. The gel with the highest spot-count was assigned as the master gel, which was used as

a template. Gel-to-gel matching of the standard spot maps of each gel was performed using the biological variation analysis (BVA) software module to ensure that the same protein spots were compared between gels. Normalized Cy3 and Cy5 spot volumes were compared to the corresponding Cy2 standard spot volume within each gel, which gave a standardized abundance.

Mass spectrometry

Preparative gels were run with 185 μl unlabeled internal standard following the same procedure as described above. The gels were stained with Colloidal blue staining kit for 3 hours and destained with deionized water overnight, as recommended by the manufacturer.

For mass spectrometry, protein spots were manually excised from the preparative gel, washed in deionized water and destained in 30% (v/v) acetonitrile (ACN)/50 mM NH_4HCO_3. Destained gel pieces were washed briefly with deionized water, vacuum dried and rehydrated in 4 μl trypsin digest solution (75 μg/ml trypsin gold in 20 mM NH_4HCO_3, pH 8.0) for digestion overnight at room temperature. Peptide extraction was performed with 5 μl of 50% ACN/0.1% trifluoroacetic acid. The extracted sample was spotted on an anchorchip plate with saturated α-cyano-4-hydroxycinnamic acid matrix solution in 100% ACN (1:1). Digested peptide fragments were analyzed in a Matrix Assisted Laser Desorption/Ionization – Time of Flight (MALDI-ToF) mass spectrometer using an Ultraflex-II apparatus. Flexanalysis 2.4 and Biotools 3.1 software were used for data processing. The obtained mass spectra were analyzed using peptide mass finger print spectra with the online Matrix Science Database with MASCOT software (www.matrixscience.com). The MSDB database 20060831 (3239079 sequences; 1079594700 residues) was searched with the Mascot parameters set as follows: Taxonomy, homo sapiens; mass tolerance, 100 ppm; maximum one missed cleavage per peptide; fixed modification of carboxymethylation of cysteine residues; variable modification of partial oxidation of methionine residues. Scores above MSDB database threshold of 64 were considered significant ($p<0.05$).

Plasma samples from large case-control study

To validate findings from the proteomic study in a larger case-control population, we used plasma samples from the EN-Vie Study. The EN-Vie Study, as described previously, is a prospective multicenter observational study of patients with BCS. [8, 15] During the study period of two years a total of 163 newly diagnosed patients with BCS were included from nine European countries. Apart from recorded data on clinical parameters, underlying etiology and treatment outcome, blood samples were also collected. Furthermore, each enrolled patient was asked to provide a sex- and age-matched (with a range of five years) control person. Controls had to be of the same race as their matched cases and had to have no previous history of thrombosis. If patients were unable to provide a control person themselves, the

national study coordinating centers attempted to find equally matched controls from their own resources. From all control subjects blood samples were obtained. As for patients, blood samples were collected through venapuncture in tubes containing 0.11 M trisodium citrate. From all blood samples, plasma was acquired by centrifugation at 2000g for 10 minutes. Plasma samples were stored at –70 °C at one central facility until analysis.

The EN-Vie study was conducted with approval from all national and, if necessary, local ethical committees, in accordance with the nation-specific rules. All patients and controls agreed to participate in the study by means of a written informed consent.

For this case-control study, only patients for whom stored plasma samples and a matched control person were available were considered eligible.

Measurement of apolipoprotein A1 and HDL cholesterol levels in plasma

In all plasma samples, those from the cases and controls used for in vitro clot formation and 2D-DIGE and those from the EN-Vie study, the concentration of apolipoprotein A1 (apo A1) was determined with a Beckman Coulter nephelometer using commercially available monoclonal antibodies. Levels of HDL cholesterol were measured in the samples from the EN-Vie study with an enzymatic colorimetric test (HDL-C plus 3rd generation, Roche Diagnostics, Mannheim, Germany) and expressed as percentage of the mean level of the control group. This level amounted to 1.0 mM, but might be somewhat underestimated because of the use of citrate in the samples.

Miscellaneous methods

Data on plasma levels of albumin, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and factor V were obtained from the EN-Vie Study database. All these assays were performed locally. To correct for possible variation between different laboratory assessments, values of AST and ALT were analyzed as a fraction of the upper limit of normal, which was calculated using the local cut-off values.

Statistical analyses

The protein abundance in plasma clots of patients with BCS and controls was compared using the Student's T-Test within the Decyder software. Differences in apo A1 levels in plasma were tested using the non-parametric Mann-Whitney U Test for the 9 BCS patients or the Student's T-Test for the larger cohort of BCS-patients from the EN-Vie study. Spearman's Rho correlation coefficients were calculated as a non-parametric measure of correlation between apo A1 levels and liver function tests. A p-value of <0.05 was considered statistically significant. All statistical analyses were performed with the Statistical Package for Social Sciences for Windows, version 15.0 (SPSS, Chicago, IL).

RESULTS

Plasma samples used for in vitro clot formation were collected from nine patients with BCS, 7 females and 2 males, with a median age at diagnosis of 35 years (range 16-54 years). Underlying etiologic factors were myeloproliferative neoplasms (n=4), homozygous Factor V Leiden mutation (n=1), antiphospholipid antibodies (n=3) and oral contraceptives (n=2). In two patients no risk factors could be identified and in three patients two prothrombotic factors were present. The median age of the controls (7 females and 2 males) was 31 years (range 22-49 years).

Differences in plasma clot composition between BCS-patients and controls

After in vitro plasma clot formation, the protein composition of clots from patients with BCS was compared to that of plasma clots from controls. On the master gel, 1369 different protein spots were detected after 2D-DIGE analysis. All other gels of case-control pairs were matched

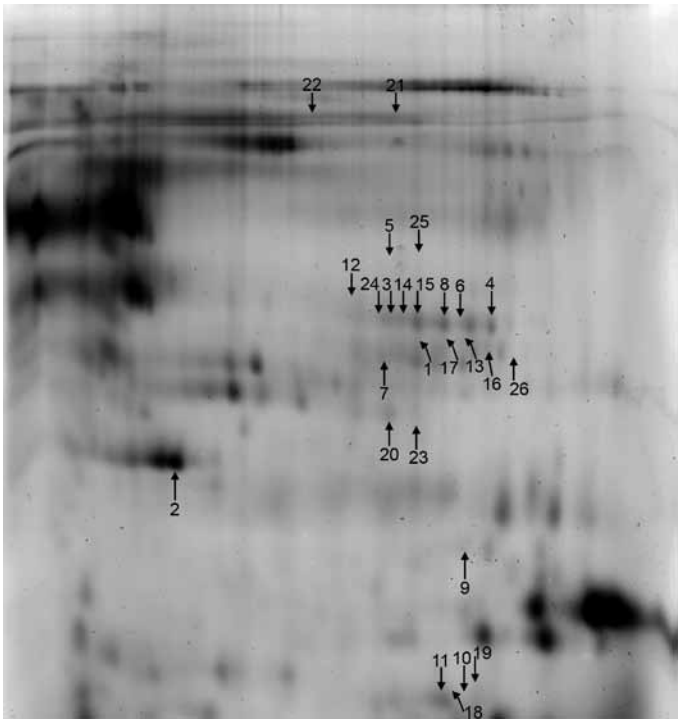

Figure 1. Image of the master gel of 2D-DIGE, displaying proteins that significantly differed in abundance between BCS-patients and controls, analyzed with Decyder software. The arrows on the 10.5% polyacrylamide gel indicate the 26 protein spots that are statistically significantly ($p<0.001$) different between patients with Budd-Chiari syndrome and healthy controls. Some arrows point to a protein spot that is not visible on this scan of the master gel, however with Decyder software a protein spot was detected and shown to be different between patients and controls. Spot number 2 was identified with mass spectrometry as apolipoprotein A1 with a mass of 28 kDa. The other 25 protein spots are not yet identified.

and compared with the master gel and this resulted in the detection of 26 protein spots that statistically significantly ($p<0.001$) differed in abundance between patients and controls. (Figure 1) The protein spot with the most significant decrease ($p=6.6 \times 10^{-5}$) was spot number 2. (Table 1) The mean value for the standardized abundance of patient samples for this spot was 0.70 as compared to 1.65 for the controls, corresponding to a 2.4-fold lower abundance in patients samples. (Figure 2) This protein spot was excised from preparative gels and analyzed using MALDI-TOF-MS. The protein was identified as apolipoprotein A1 (apo A1) with the following identification details; NCBI gi | 90108664, mass 28061Da, Mascot score 76-237, number of peptides matched 18-24, sequence coverage (%) 54-81, with a p-value between 3e-19 and 0.0036 (n=4). The other 25 spots with a statistically significant difference in protein abundance between patients and controls have not yet been identified.

Table 1. Protein spots with a statistically significant difference in abundance between controls and patients with Budd-Chiari syndrome as analyzed with Decyder software

Spot number	Average ratio (controls/patients)	P-value
1	-4.31	2.70E-05
2	2.35	6.60E-05
3	-6.94	0.00013
4	-13.19	0.00013
5	-4.34	0.00014
6	-12.49	0.00016
7	-8.15	0.00016
8	-7.27	0.00018
9	-8.1	0.00019
10	-3.64	0.00024
11	-2.63	0.0003
12	-5.1	0.00032
13	-4.22	0.00034
14	-9.67	0.00037
15	-5.01	0.00037
16	-4.96	0.00047
17	-4.34	0.0005
18	-3.7	0.00055
19	-2.45	0.00061
20	-2.73	0.00073
21	2.35	0.00076
22	3.09	0.00077
23	-4.08	0.00079
24	-5.51	0.00081
25	-3.21	0.0009
26	-3.03	0.00097

The average ratio is the ratio of the standardized abundance of controls and patients. Positive ratios indicate that the standardized abundance in controls is higher than in patients. Negative ratios indicate that the protein abundance in patients is higher than in controls.

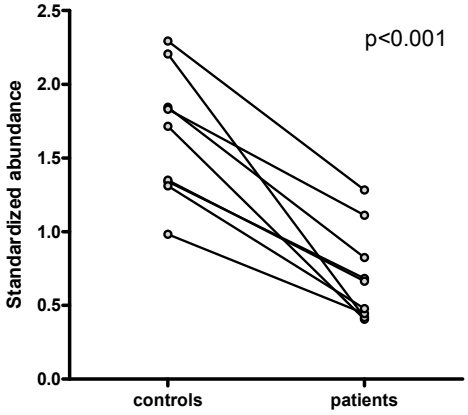

Figure 2. Protein abundance of apo A1 of BCS-patients and their matched controls. Statistical analysis was performed using the BVA module of DeCyder software and shows on average a 2.4 fold lower abundance for apolipoprotein A1 in the patient plasma clots compared to control plasma clots. Individual data points are indicated and lines connect the standardized abundance data of patients with their matched controls that were analyzed on a single gel.

Plasma levels of apo A1

The low abundance of apo A1 in plasma clots of BCS-patients was also seen in plasma levels. Mean (± SD) plasma level of apo A1 in these 9 cases was significantly lower than in the controls, 0.74 (± 0.21) g/l vs. 1.45 (± 0.31) g/l, respectively (p=0.002), corresponding to a 2.0 fold difference in plasma levels.

To validate these findings we used plasma samples from the EN-Vie Study cohort. From this study, plasma samples for measurement of apo A1 levels were available for 107 patients. However, for six patients there was no control person and these patients were excluded from the analysis, leaving 101 eligible case-control pairs. Mean age of the patient population was 38 years (range 16-83) and 42% were males. Results from the apo A1 assay are shown in Figure 3A.

Patients with BCS had significantly lower apo A1 levels in plasma as compared to the controls (mean ± SD 0.97 ± 0.36 g/l vs. 1.32 ± 0.24 g/l, p<0.001). The pattern of the patient values might suggest the existence of a subgroup with low levels of apo A1. When the group of patients with apo A1 levels below 0.85 g/l were compared to those with higher apo A1 levels, it became clear that the subgroup of cases with lower apo A1 levels included relatively more patients with a myeloproliferative neoplasm (20 of 36 (56%) vs. 15 of 63 (24%), respectively, p=0.001). No other differences in underlying etiology of thrombosis or characteristics at diagnosis were found between these two apparent subgroups.

The plasma levels of apo A1 correlated with plasma levels of HDL cholesterol (HDL-C) in BCS-patients from the EN-Vie study cohort (Pearsons correlation coefficient of 0.863,

p<0.001). Mean (±SD) HDL-C levels were 71% (± 32) in patients as compared to 100% (± 26) in controls (p<0.001). (Figure 3B)

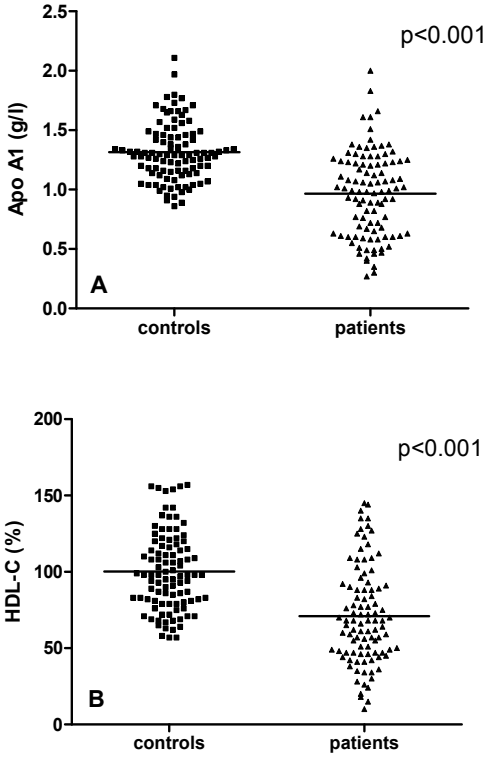

Figure 3. Apolipoprotein A1 levels (A) and HDL cholesterol levels (B) in plasma of patients with Budd-Chiari syndrome and healthy controls. Individual data points and mean values are given.

Apo A1 levels and liver function tests

Because apo A1 is synthesized by the liver, an impaired liver function may influence the plasma levels. To estimate the effect of decreased hepatic synthetic function on apo A1 levels in patients with BCS, we determined the correlation between apo A1 and different parameters of liver function in the EN-Vie study cohort.

As shown in Table 2, there was an association between apo A1 levels and several parameters of liver function. However, the correlations were only weak, with correlation coefficients lower than 0.35. Furthermore, when we compared the apo A1 levels of cases with normal albumin levels (>35 g/l, n=34) to their matched controls, BCS-patients still had significantly lower apo A1 levels than their healthy controls (1.08 ± 0.36 g/l vs. 1.31 ± 0.23 g/l, p=0.002) (Figure 4). To assure that albumin levels were not artificially elevated, we assessed whether

patients had received prior albumin infusion. None of the cases with albumin levels in the normal range had been treated with albumin.

Table 2. Correlation between apo A1 levels and parameters of liver function in patients with BCS

		Apo A1	
	N	Spearman's Rho	P
Albumin	99	0.319	0.005
Bilirubin	97	-0.211	0.038
ALT	87	-0.197	0.067
AST	86	-0.217	0.044
Factor V	28	0.327	0.089

ALT, alanine aminotransferase; AST, aspartate aminotransferase.

DISCUSSION

In this study we have used a proteomic approach to investigate differences in proteins bound to a plasma clot in order to detect novel players in the regulation of hemostasis that may be associated with the development of venous thrombosis in BCS. To our knowledge this is the first report on analysis of plasma clot proteins in patient samples using proteomics as a detection method. Previously we have shown that this technique could be used to identify new fibrin-binding proteins in plasma. [14] In 2D-DIGE an internal standard is run on each gel together with a patient sample and a control sample. This allows a direct comparison of protein abundance between series of samples without interference by gel-to-gel variation. Using 2D-DIGE, 9 samples of patients with BCS were compared with samples of their matched controls. A total of 26 protein spots significantly differed ($p<0.001$) in abundance between cases and controls and were either increased or decreased in the patient samples. The protein spot displaying one of the most significant differences between both groups was identified with mass spectrometry as apo A1. The other 25 protein spots with a different abundance in cases and controls still need to be identified. The standardized protein abundance of apo A1 was reduced by a factor 2.4 in BCS-patient samples. A similar reduction was found in the plasma concentration of apo A1 in these 9 patients. The findings of the first part of this study were confirmed in a validation study by measuring the concentration of apo A1 in plasma in a large cohort of 101 BCS-patients. Apo A1 was found to be significantly decreased compared to the healthy, matched controls. HDL cholesterol levels were also found to be decreased in patients compared to controls, and correlated well with Apo A1 plasma levels as seen before. [16]

Information on specific lifestyle factors was not recorded, however patients and controls were of the same age and race and were recruited from the same geographical area. In some patients with BCS, hepatic synthetic function may be impaired as a result of venous thrombosis which can result in venous congestion and hepatocyte necrosis. Because apo A1, like albumin,

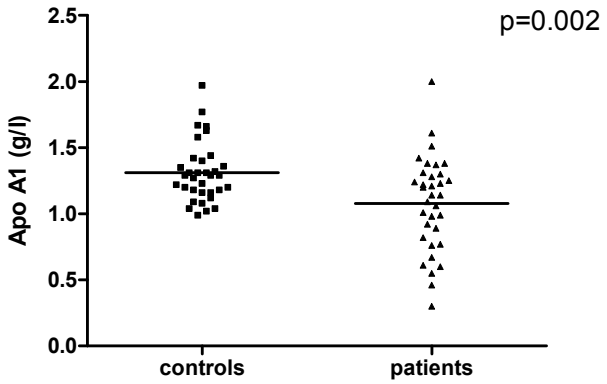

Figure 4. Apolipoprotein A1 levels in plasma of cases with normal albumin levels (>35g/l; n=34) compared to their healthy controls. Individual data points and mean values are given.

is one of the main proteins synthesized by the liver, the observed differences in plasma levels of apo A1 between BCS-patients and healthy controls could have been caused by the liver disease in the former group. However, in our study group there was only a weak correlation between apo A1 levels in plasma and different liver function tests. Moreover, in a subgroup of BCS-patients with normal albumin levels, the concentration of apo A1 was still significantly lower as compared to their healthy matched controls. Due to the marked clinical heterogeneity of BCS and the finding that many patients have a more or less acute-on-chronic form of disease presentation [15, 17], it is difficult to clearly distinguish between acute and chronic forms of BCS. Still, when we compared patients with an acute onset of symptoms to those with a more chronic development of symptoms, apo A1 levels were comparable between both groups (data not shown). Overall, the decreased plasma concentration of apo A1 and also the decrease in standardized protein abundance of apo A1 found in plasma clots of BCS-patients cannot entirely be explained by an impaired liver synthetic function in these patients.

A low apo A1 level has previously been reported as a marker of liver fibrosis. [18-19] Therefore, we cannot exclude that fibrosis might partially explain the low levels of apo A1 found in BCS-patients. Nevertheless, we found no association between apo A1 levels and the presence of fibrosis and/or cirrhosis in a subgroup of 26 BCS-patients of whom a liver biopsy sample was available (data not shown). Hence, we believe that low levels of apo A1, next to other risk factors, might play a causal role in the development of venous thrombosis in BCS, in addition to being a consequence of venous thrombosis. It is of interest that apo A1 levels appeared to be particularly low in BCS-patients with an underlying myeloproliverative neoplasm. It has been shown that in myeloproliferative neoplasms, which represent a major risk factor for BCS, HDL levels can be markedly decreased due to an increased catabolism of apo A1. [20] Further studies are needed to clarify this association and its potential role in the development of venous thrombosis.

The mechanism through which decreased levels of apo A1 could result in venous thrombosis in patients with BCS is not yet clear. Apo A1 is the main protein component of the reverse cholesterol transporter HDL. For arterial thrombosis plasma levels of HDL are known to be inversely related with risk. [21] For venous thrombosis the role of HDL and apo A1 is less clear. However, there are strong indications that the same inverse relation between plasma levels and thrombosis risk in arterial thrombosis is seen with venous thrombosis. [22-24] Furthermore, it is thought that arterial thrombosis and venous thrombosis share common risk factors. [24-25] In a recent study by Eichinger et al., low levels of apo A1 in plasma were associated with a significantly higher risk of recurrent venous thrombosis. [26] These data are in conflict with the results of a population-based prospective study that did not find an association between HDL cholesterol and venous thromboembolism. [27] HDL can be involved in hemostasis in several ways. [28] One way, shown by Griffin et al., is that HDL can enhance the activated protein C pathway. [29] This pathway is part of the natural anticoagulant system and activation results in a prolongation of the prothrombin time, which is correlated with the plasma levels of apo A1. [29] Consequently, when apo A1 concentration in plasma is decreased, this may potentially result in an impaired haemostatic balance and thereby an increased tendency for thrombosis. Previous studies have shown that defects in the protein C pathway may indeed result in BCS, both in an experimental animal model [30] and in humans [5]. Another potential mode of action, recently published by Dahlbäck and colleagues, is that anionic phospholipids lose their procoagulant properties when incorporated into HDL. [31] Still, further studies are required to elucidate the exact mechanism through which apo A1 interacts with the hemostatic system, including the possible role of the clot binding of apo A1.

In conclusion, using 2D-DIGE as a detection method, we have shown that the protein composition of in vitro formed plasma clots differs between patients with BCS and healthy controls. Apo A1 is significantly less abundant in plasma clots of BCS-patients and this difference is caused by lower plasma levels of apo A1 in this group. Although the precise causative mechanism has not yet been elucidated, this is the first evidence that decreased apo A1 levels may play a role in the development of venous thrombosis in patients with BCS. Decreased apo A1 levels may also contribute to other manifestations of venous thrombosis.

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Chapter 5

PAROXYSMAL NOCTURNAL HEMOGLOBINURIA IN BUDD-CHIARI SYNDROME: FINDINGS FROM A COHORT STUDY

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ABSTRACT

A well recognized cause of Budd-Chiari syndrome (BCS) is paroxysmal nocturnal hemoglobinuria (PNH). PNH is an acquired disorder of hematopoietic stem cells, characterized by intravascular hemolysis and venous thrombosis. Testing for this hematological disorder should be considered in all BCS-patients. Using data from the EN-Vie Study, a multi-center study of 163 patients with BCS, we investigated the relationship between BCS and PNH in 15 patients with combined disease and compared the results to 62 BCS-patients in whom PNH was excluded. Median follow-up for the study group (n=77) was 20 months (range 0-44 months). BCS-patients with PNH presented with a significantly higher percentage of additional splanchnic vein thrombosis (SVT) as compared to BCS-patients without PNH (47% vs. 10%, p=0.002). During follow-up, type and frequency of interventions for BCS was similar between both groups. Six patients with BCS and PNH were successfully treated with a transjugular intrahepatic portosystemic shunt (TIPS). Of 15 patients with PNH, six underwent allogenic stem cell transplantation after diagnosis of BCS. PNH was successfully cured in five cases. There was no significant difference in survival between BCS-patients with and without PNH.

Conclusions. This study shows that despite a higher frequency of additional SVT, short-term prognosis of BCS-patients with PNH does not differ from BCS-patients without PNH. Treatment with TIPS can be safely performed in patients with PNH. Stem cell transplantation appears to be a feasible treatment option for PNH in BCS-patients.

INTRODUCTION

Obstruction of the hepatic venous outflow tract, either at the level of the hepatic veins or the inferior vena cava, is referred to as the Budd-Chiari Syndrome (BCS). [1] This vascular liver disorder is characterized by the presence of hepatomegaly, abdominal pain and ascites and carries a significant risk of complications and death. Young adults in the third and fourth decades of life are most frequently affected, with a female predominance. Most cases of BCS are the result of thrombosis. [2] In recent years, many different thrombophilic factors (e.g. Factor V Leiden mutation, antiphospholipid syndrome, protein C deficiency) have been identified that are associated with the development of hepatic vein thrombosis. [3-5] Myeloproliferative neoplasms are the most common underlying cause and can be identified in approximately half of all patients with BCS. [6-7]

Another haematological disorder that has been related to BCS, is paroxysmal nocturnal hemoglobinuria (PNH). [8-9] PNH is a rare acquired disorder of a pluripotent hematopoietic stem cell caused by a somatic mutation in the phosphatidylinositol glycan class A (PIG-A) gene. [10-11] The product of this gene is involved in the synthesis of glycosylphophatidylinositol (GPI), a glycolipid structure that attaches specific proteins to the cell membrane. [12-13] As a result of the mutation, a clone of affected PNH-cells arises that are deficient in all GPI-anchored surface proteins. Two clinically important proteins absent on PNH-cells are the complement regulatory proteins CD55 and CD59. [14] The most common manifestations of the disease are (complement-mediated) intravascular hemolysis, bone marrow failure and venous thrombosis. The latter complication occurs in up to 40% of all patients with PNH and is the main cause of morbidity and mortality. [8-9, 15-16] Remarkably, there is a predisposition for thrombosis of the intra-abdominal and cerebral veins, with more than one third of thrombotic episodes located in the hepatic veins or the inferior vena cava. [8-9, 17] The presence of PNH thus infers a high risk for the development of BCS.

Due to the rarity of both disorders, few studies are available that specifically address the relationship between PNH and BCS. Most current data stems from small retrospective series and case reports. Recently a large prospective study of patients with BCS from 9 European countries was concluded (EN-Vie study). [18] Results from this study were used to investigate the clinical presentation, treatment outcome and prognosis of patients with BCS and PNH.

PATIENTS AND METHODS

Patients

From the EN-Vie study cohort we obtained data on baseline characteristics, treatment and survival of BCS-patients with and without underlying PNH. The EN-Vie study, as described

previously [18], is an observational study in which newly diagnosed patients with BCS were consecutively enrolled in 9 different European countries. The presence of BCS was defined as an hepatic venous outflow obstruction and its manifestations, regardless of the cause and the level of obstruction, ranging from the small hepatic veins to the entrance of the inferior vena cava into the right atrium. Venous obstruction due to right-sided heart failure and sinusoidal obstruction syndrome (SOS; previously known as veno-occlusive-disease) were not included in this definition. The EN-Vie Study was conducted with approval from all national and, if necessary, local ethical committees, in accordance with the nation-specific rules. All patients agreed to participate in the study by means of a written informed consent.

From October 2003 until October 2005, a total of 163 patients with BCS were included. During the study period, data concerning clinical condition, etiology and results of radiology, pathology and laboratory assessments were collected. Study guidelines were available for all participating centers and recommended testing for PNH as part of the etiology work-up.

For patients identified as having PNH, a questionnaire was sent out to their respective physicians 20 months after study closure to obtain additional information on survival, further thrombotic events, stem cell transplantation and other specific treatment for PNH.

Statistical analyses

Baseline characteristics and applied treatment modalities were compared between BCS-patients with and without underlying PNH. For continuous variables the Mann-Whitney U test was applied. Categorical variables were assessed with either the Chi-square test or the Fisher's Exact test. Differences in survival during follow-up were calculated using the log-rank test. A p-value of <0.05 was considered to be statistically significant. All statistical analyses were performed with the Statistical Package for Social Sciences for Windows, version 14.0 (SPSS, Chicago, IL).

RESULTS

Baseline characteristics

From the total study cohort of 163 patients, 10 patients were already known to have PNH before diagnosis of BCS (6.1%). Additionally, 67 patients were tested for the presence of PNH by either flow cytometry (84%), Ham's test (7%) or both (9%). Five of these tests (7.5%) were positive for PNH. Study findings of all patients with PNH (n=15) were subsequently compared to the patients in whom the presence of PNH was excluded (n=62). Clinical characteristics of both groups are presented in Table 1. To ensure that the group of BCS-patients without PNH was a representative sample of the total EN-Vie cohort, baseline characteristics of these

patients were compared to the EN-Vie patients that were not tested for PNH (n=86). Results were not significantly different. (data not shown)

As shown in Table 1, sex distribution, age at diagnosis of BCS and the type of thrombosis did not differ between BCS-patients with and without PNH. However, evaluation of radiological findings revealed that the frequency of splanchnic vein thrombosis (SVT; i.e. portal vein, mesenteric vein or splenic vein thrombosis) at presentation was significantly higher in patients with PNH as compared to the non-PNH-patients (47% vs. 10%, p=0.002). Concurrent portal vein thrombosis was most common and occurred in one-third of the patients with BCS and PNH. In contrast, this was the case in less than 10% of the patients without PNH (p=0.022).

Table 1. Baseline characteristics of BCS-patients with and without PNH

	PNH-patients (n=15)	Non-PNH-patients (n=62)	P
Males, n (%)	6 (40)	26 (42)	0.891
Age at diagnosis, median (range)	29 (18-62)	37 (16-74)	0.280
Caucasian race, n (%)	12 (80)	36 (58)	0.116
Type of occlusion, n (%)			1.000
Isolated HV occlusion	7 (47)	31 (51)	
Isolated IVC occlusion	0 (0)	1 (2)	
Combined HV and IVC occlusion	8 (53)	30 (49)	
Ascites, n (%)	12 (80)	49 (79)	1.000
Hepatomegaly, n (%)	8 (53)	42 (68)	0.294
Splenomegaly, n (%)	6 (40)	37 (60)	0.168
Splanchnic vein involvement, n (%)	7 (47)	6 (10)	0.002
Portal vein occlusion, n (%)	5 (33)	5 (8)	0.022
Laboratory at baseline§, median (range)			
Bilirubin, µmol/l	32 (8-135)	32 (5-325)	0.675
ALT, x ULN	1.2 (0-36)	1.4 (0-294)	0.696
LDH, x ULN	2.7 (1-5)	1.0 (0-25)	0.001
Creatinine, µmol/l	71 (36-251)	80 (53-330)	0.069
Haemoglobin, mmol/l	5.8 (4.8-9.9)	9.1 (4.2-12.3)	<0.001
Leukocyte count, $x10^9$/l	6.6 (3.1-9.3)	11.1 (1.4-30.2)	0.003
Platelet count, $x10^{12}$/l	67 (15-274)	250 (20-773)	<0.001
Rotterdam BCS index‡, n (%)			0.452
Class I	4 (27)	20 (33)	
Class II	6 (40)	30 (49)	
Class III	5 (33)	11 (18)	

HV, hepatic vein; IVC, inferior vena cava; ALT, alanine aminotransferase; ULN, upper limit of normal; LDH, lactate dehydrogenase; MELD, model for end-stage liver disease; § Laboratory values at baseline were not available for all patients; ‡ Prognostic classification of patients with BCS, calculated as previously defined [ref. 21].

Specific disease characteristics of the BCS-patients with PNH are presented in Table 2. Results of flow cytometry, available for 10 patients, showed that in all but one patient more than half of the circulating granulocytes were affected by PNH (PNH-clone size >50%). Because there is a close relationship between PNH and both aplastic anemia and myelodysplastic syndrome, we assessed whether there was concurrence of these disorders in patients with BCS and PNH. Of the 15 patients with PNH, a second bone marrow disorder was present in 8 (53%) of them. Five patients had signs of aplastic anemia and three were diagnosed with a myelodysplastic syndrome. Furthermore, in two of these patients there was a concurrent myeloproliferative neoplasm. Like PNH, myeloproliferative neoplasms are a major etiologic factor for the development of BCS. Other risk factors for thrombosis, either inherited or acquired, could also be identified in patients with BCS and PNH. In total, additional thrombotic risk factors were present in 10 patients with PNH (67%). (Table 2)

Table 2. Disease characteristics of patients with PNH and BCS

	Patients with BCS and PNH (n=15)
PNH-clone size in %, median (range)[§]	
Erythrocytes	31 (5-65)
Granulocytes	70 (3-99)
Additional etiologic factors, n (%)[‡]	
Myeloproliferative neoplasm	2 (13)
Inherited coagulation abnormality	1 (7)[¥]
Acquired thrombogenic risk factor	6 (40)[£]
Hormonal risk factor[$]	3 (33)[#]
Additional bone marrow disorder, n (%)	
Aplastic anemia	5 (33)
Myelodysplastic syndrome	3 (20)
Specific treatment for PNH, n (%)[€]	
Corticosteroids	5 (33)[a]
Cyclosporin	5 (33)[a]
Anti-thymocyte globulin (ATG)	1 (7)[β]
Eculizumab	2 (13)[&]
Other (supportive) therapy[%]	11 (73)

§ Data on the PNH-clone size in erytrocytes and granulocytes (as measured by flow cytometry) was available for 9 and 10 patients, respectively; ‡ Categories are not mutually exclusive, some patients have more than one additional risk factor; ¥ One case with antithrombin deficiency; £ Four cases with hyperhomocysteinemia and two cases with antiphospholipid antibodies; $ Only considered in female patients; # Two cases with a recent pregnancy and one case with oral contraceptive use; € In most patients more than one treatment modality was applied; a In 4 patients treatment was initiated before diagnosis of BCS; β Treatment was initiated before diagnosis of BCS; & In both cases, eculizumab therapy was started after diagnosis of BCS; % Blood transfusion, iron replacement therapy, folate, splenectomy or androgens.

Of the 5 patients in whom BCS was the first presentation of PNH, we evaluated if, apart from the flow cytometry results, there were any other signs or symptoms suggestive of PNH. Two patients had been previously diagnosed with a different bone marrow disorder, one with aplastic anemia and one with a myelodysplastic syndrome. Of the other three patients,

all displayed signs of anemia, which was combined with thrombocytopenia in one patient. A markedly elevated plasma level of lactate dehydrogenase, as a possible clue for intravascular hemolysis, was only present in one patient.

Treatment of BCS

Median follow-up for the total group of BCS-patients with and without PNH (n=77) was 20 months (range 0-44 months). In the period of study one patient with PNH was lost to follow-up (at 30 months) and two PNH-patients died, at 14 and 26 months after diagnosis respectively. Causes of death were progressive liver failure and multi-organ failure. Table 3 displays the different therapeutic interventions that BCS-patients with or without PNH underwent during follow-up. There were no significant differences in the frequencies that these treatment modalities were applied in patients with and without underlying PNH. Almost all patients were treated with anticoagulation, which was initiated within the first week after BCS-diagnosis in 12 of 14 (87%) PNH-patients and in 37 of 56 (66%) non-PNH-patients (p=0.202). Of patients with PNH, 12 cases were anticoagulated with oral coumarine derivatives, 1 patient was treated with low molecular weight heparin and 1 patient was given danaparoid after an episode of heparin-induced thrombocytopenia. Despite the fact that 10 patients were previously known to have PNH, only 2 were on anticoagulant treatment before BCS developed, both because of another thrombotic episode in the past (pulmonary embolism and sagittal sinus thrombosis, respectively). In these two patients adequate treatment with anticoagulation did not prevent development of hepatic vein thrombosis. Furthermore, during follow-up there was either an extension of thrombosis or occurrence of a new thrombotic event in four patients with PNH (27%) despite anticoagulant treatment.

Table 3. Treatment during follow-up of BCS-patients with and without PNH

	PNH-patients (n=15)	Non-PNH-patients (n=62)	P
Anticoagulation	14 (93)	56 (90)	1.000
Diuretics	8 (53)	35 (57)	0.827
Thrombolysis or PTA	2 (13)	12 (19)	0.725
TIPS§	6 (40)	18 (29)	0.535
OLT	0 (0)	11 (18)	0.109

Values expressed as number of cases and percentage, n (%). PTA, percutaneous transluminal angioplasty; TIPS, transjugular intrahepatic portosystemic shunt; OLT, orthotopic liver transplantation; § Numbers represent cases with a successful TIPS procedure; TIPS-placement failed in 1 PNH-patient and 4 non-PNH patients.

In seven patients with PNH a TIPS-procedure (transjugular intrahepatic portosystemic shunt) was performed. The other eight patients with PNH were not treated with TIPS, either because there was no or only minimal ascites (n=4), because ascites was under control with diuretics or paracenthesis (n=3) or because TIPS-placement was considered not feasible due to an extensive thrombosis of the portal vein, superior mesenteric vein and splenic vein (n=1). TIPS-

placement was successful in 6 out of 7 PNH-patients (86%), as compared to 18 of 22 (82%) successful TIPS-procedures in BCS-patients without PNH. Polytetrafluoroethylene (e-PTFE) covered stents were used in 4 of 6 PNH-patients (67%) and 12 of 18 non-PNH patients (67%). Indications for TIPS in the patients with underlying PNH were refractory ascites in five patients, uncontrollable variceal bleeding in one patient and progressive liver failure in another patient. In one patient with PNH and concurrent portal vein thrombosis, TIPS-placement was attempted but proved to be technically infeasible. However, in two other PNH-patients with a thrombosis of the portal vein, the TIPS-procedure was successful. In total, 8 patients with PNH had additional portal vein thrombosis, either present at diagnosis of BCS (n=5) or appearing during follow-up (n=3). Apart from the three cases that underwent TIPS, portal vein thrombosis developed after TIPS-placement in one patient, TIPS was not indicated in three cases and TIPS was considered infeasible in another patient. During follow-up TIPS-dysfunction occurred in two BCS-patients with PNH. For one patient a single revision with dilation of the TIPS was sufficient to restore adequate blood flow. The second patient required multiple interventions due to recurrent TIPS-occlusion, which were eventually unsuccessful. At the last follow-up, a complete thrombosis of the TIPS was seen on imaging, with the presence of collaterals from the portal system to the vena cava inferior. Of note, an orthotopic liver transplantation (OLT) was not carried out in any of the patients with BCS and PNH whereas in the group without underlying PNH it was performed in 18% of the patients (p=0.109).

Treatment of PNH

All but two patients with PNH received some form of treatment, mostly consisting of blood transfusions combined with more specific therapy, such as corticosteroids or cyclosporine. (Table 2) Because allogenic stem cell transplantation is the only potential curative therapy for PNH, we evaluated the frequency and outcome of this treatment modality. Out of 15 patients with BCS and PNH, stem cell transplantation was considered as a therapeutic option in 9 patients. Due to the absence of a suitable donor, two patients could not be transplanted. For a third patient the extent and severity of the thrombosis (BCS with concurrent portal vein, mesenteric vein and splenic vein thrombosis) was the reason for not performing a stem cell transplantation. Eventually, six BCS-patients with underlying PNH were treated with allogenic stem cell transplantation. Characteristics of these patients and further details of their transplantation are given in Table 4. One patient died shortly after stem cell transplantation as a result of multi-organ failure but in the remaining five patients PNH was successfully treated. Of these five patients, two patients also underwent a TIPS-procedure either before or after stem cell transplantation to relieve symptoms of hepatic congestion.

Table 4. Characteristics of BCS-patients with PNH that underwent allogenic stem cell transplantation (SCT)

	Sex	Age§	Other bone marrow disorder(s)	Details SCT	Outcome SCT	TIPS
1.	F	37	Aplastic anemia	SCT with reduced intensity conditioning	Alive, no recurrence	Unsuccessful attempt
2.	F	27	Myelodysplastic syndrome, myelofibrosis	1st SCT with myeloablative conditioning 2nd SCT (reduced intensity conditioning) performed because of high risk of myelodysplastic syndrome 3rd SCT with myeloablative conditioning	Acute myeloblastic leukaemia after 2nd SCT. Successful treatment of bone marrow disorders after 3rd SCT. Alive.	-
3.	M	39	-	Patient underwent 2 SCT's before diagnosis of BCS, due to disease recurrence a 3rd and 4th SCT were undertaken (both with reduced intensity conditioning).	Alive, with successful treatment of PNH after last SCT	TIPS performed 1 month after last SCT
4.	M	18	Aplastic anemia	SCT with myeloablative conditioning	Alive, no recurrence	-
5.	M	51	Myelodysplastic syndrome	SCT performed with reduced intensity conditioning when the patient's clinical condition started to deteriorate	Died 3 months after SCT as a result of multi-organ failure	-
6.	F	28	Aplastic anemia	SCT performed with reduced intensity conditioning	Alive, no recurrence	TIPS procedure 3 months before SCT

All stem cell transplantations were performed within 3 years after onset of BCS. BCS, Budd-Chiari Syndrome; PNH, paroxysmal nocturnal hemoglobinuria; TIPS, transjugular intrahepatic portosystemic shunt. § Age at diagnosis of Budd-Chiari syndrome.

Survival

To investigate whether the survival of BCS-patients with PNH differed from those without PNH, we compared the follow-up of the two groups of patients. There was no significant difference in short-term survival between BCS-patients with and without underlying PNH. (Figure 1) In the additional year of follow-up of patients with PNH none of the patients died, resulting in an estimated 2-year survival of 93%.

DISCUSSION

This study reports the largest series of consecutive patients with BCS and underlying PNH. Previous studies addressing this rare combination of disorders have mostly consisted of case reports and therefore, little is still known about the clinical presentation, outcome of treatment and prognosis of these patients. In the current study we have compared the disease course of fifteen patients with BCS and PNH to a cohort of BCS-patients in whom PNH was excluded as an underlying cause.

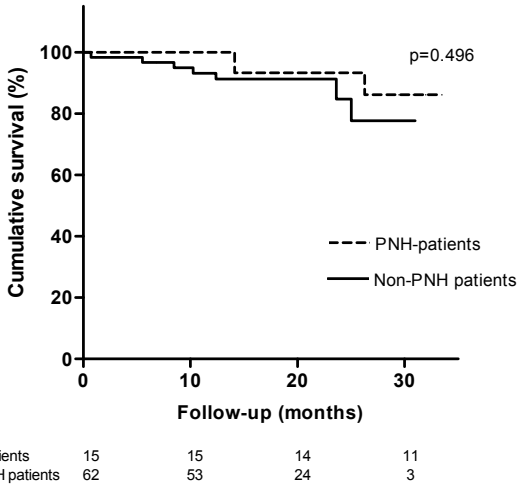

Figure 1. Kaplan-Meier survival curve of BCS-patients with and without PNH. Cumulative survival after diagnosis of BCS. P-value calculated using the log-rank test.

Previous studies have shown that PNH may be responsible for up to 10% of the cases of BCS. [19-22] In the current study we found that in 6.1% of the BCS-patients PNH had already been established before BCS occurred. Furthermore, in the group of patients tested for PNH, we found a prevalence of this disorder of 7.6%. From these data it is reasonable to assume that PNH is responsible for approximately 6-8% of cases of BCS. Because 86 patients with BCS were not tested for PNH, it is possible that some patients may have been missed. Even though this number is likely to be small, it should be emphasized that testing for PNH in patients with BCS needs to be part of the standard etiology-screening, as diagnostic signs may not be very specific, e.g. only slight anemia.

Clinical manifestations at diagnosis of BCS did not differ between patients with and without PNH with respect to the type of thrombosis, liver function tests and the presence of ascites, hepatomegaly and splenomegaly. The most striking difference in presentation was the more frequent concurrence of SVT in patients with PNH. Because PNH is associated with a major risk of thrombosis [8, 17], this may explain the increased rate of additional thrombosis in these patients. The increased tendency for thrombosis in PNH-patients is further illustrated by the finding that after diagnosis of BCS either extension of the thrombosis or a new thrombotic event occurred in four of fifteen cases (27%), despite treatment with anticoagulation.

Interestingly, none of the patients known with PNH before the occurrence of BCS received anticoagulation as primary prophylaxis. Two patients were on anticoagulant therapy for secondary prophylaxis after a previous venous thrombotic event. Still, in these patients, treatment with anticoagulation could not prevent the development of BCS. There is currently no consensus on whether PNH-patients without a history of thrombosis should be anticoagulated. Nevertheless, a few studies have shown that the risk of thrombosis and other

disease manifestations is correlated to the size of the PNH-clone, specifically the amount of mutated granulocytes. [15, 23] Therefore, it has recently been suggested that patients with a PNH-clone larger than 50%, without significant thrombocytopenia or other contraindications, may benefit from primary prophylaxis with anticoagulation. [24] Of the ten patients in this study previously known with PNH, data on the granulocyte clone-size was available for eight. In all of these eight patients more than 50% of the granulocytes was derived from the PNH-clone (range 55-99%), indicating that primary thromboprophylaxis would have been warranted in these individuals and stresses the importance of determining the size of the PNH-clone.

Because anticoagulation clearly does not prevent the development of venous thrombosis in all patients, other therapeutic options for PNH should also be considered. The only potential curative treatment for PNH is allogenic hematopoietic stem cell transplantation, but this therapy carries a high risk of morbidity and mortality. One study of 57 PNH-patients that underwent bone marrow transplantation reported that normal bone marrow function was restored in approximately 50% of patients, with a 2-year survival rate of 56%. [25] Given the presence of a major thrombotic complication in patients with PNH and established BCS, it is unclear whether stem cell transplantation is a feasible treatment option in these patients. In our patient-cohort, a stem cell transplantation was performed in six patients with BCS and PNH. In five patients PNH was successfully treated, with a median follow-up after transplantation of 17 months (range 4-35 months). These results indicate that BCS should not be considered as a contraindication for stem cell transplantation in patients with PNH. However, the number of PNH-patients in this study is small and follow-up after transplantation is not very long. Furthermore, the therapeutic options for PNH have recently been extended with the discovery and development of eculizumab, a humanized monoclonal antibody directed against complement component C5. Treatment with this complement inhibitor has greatly reduced hemolysis and the severity of anemia in patients with PNH. [26-27] Moreover, the rate of thromboembolic events was significantly lower in patients treated with eculizumab as compared to non-treated patients. [28] In the current study group, treatment with eculizumab was initiated in two patients, but in both cases BCS had already been established. Whether this drug will become the mainstay of treatment for PNH, thereby also reducing the need for stem cell transplantation, remains to be resolved. Nevertheless, the availability of specific treatment modalities for PNH further emphasizes the importance of screening for PNH in patients that present with BCS.

During the follow-up period, different treatment modalities for BCS were applied to the same extent in PNH-patients and non-PNH-patients. Besides anticoagulation and diuretics, TIPS was the most frequently performed intervention. Approximately half of the patients with PNH underwent a TIPS-procedure, which was successful in 86% of cases, as compared to a 82% success-rate in patients without PNH. Furthermore, patients with underlying PNH did not require interventions in an earlier phase after diagnosis and the rate of complications

Table 5. Overview of treatment and outcome in published cases of BCS and underlying PNH*

					Treatment of BCS				Treatment of PNH§						
Case	Year	Sex	Age$	PNH prior to BCS	anticoagulation	thrombolysis / PTA	TIPS / PSS	OLT	transfusions	medical therapy¥	SCT	NA	Follow-up (years)	Outcome	Remarks
1 [35]	1991	f	29	no	+	+						x	<1	alive	-
2 [36]	1992	f	47	yes		+	+					x	<1	deceased	PSS due to clinical deterioration
3 [34]	1992	f	28	yes	+	+				+			15	alive	stenting of IVC 14 years after diagnosis
4 [33]	1993	m	54	yes	+			+		+			3	alive	second OLT due to thrombotic complications
5 [38]	1994	f	30	yes	+				+				2	alive	-
6 [47]	1994	f	33	yes	+	+						x	6	alive	-
7 [47]	1994	f	22	yes	+	+						x	2	alive	-
8 [43]	1996	m	11	no							+		3	alive	successful SCT
9 [44]	1998	m	31	no	+		+					x	<1	alive	bleeding complications after TIPS
10 [42]	1998	f	34	yes	+	+	+		+	+			<1	deceased	TIPS due to worsening ascites
11 [32]	1999	f	45	no				+				x	7	deceased	recurrence of BCS 7 years after OLT
12 [41]	2002	f	12	yes	+		+			+	+		4	alive	successful SCT
13 [45]	2003	f	38	yes	+	+						x	<1	deceased	-
14 [31]	2003	m	28	no	+			+				x	15	alive	recurrence of BCS after OLT
15 [39]	2003	f	35	yes	+				+				1	alive	-
16 [51]	2005	m	24	no	+		+			+	+		2	alive	successful SCT (2 attempts)
17 [46]	2006	m	27	yes		+					+		2	alive	successful SCT
18 [46]	2006	m	34	yes	+	+						x	2	alive	-
19 [46]	2006	f	14	yes		+					+		<1	deceased	died from complications after SCT
20 [49]	2007	m	15	yes	+		+					x	3	alive	bleeding complications after TIPS
21 [48]	2007	f	39	yes		+				+			<1	deceased	-
22 [40]	2007	f	32	yes	+			+			+		6	alive	successful SCT (2 attempts)
23 [37]	2008	f	19	no	+					+			<1	alive	treatment with eculizumab
24 [52]	2008	m	19	yes	+	+		+	+	+			7	alive	long-term anticoagulation with aspirin
25 [50]	2009	f	29	no	+							x	<1	deceased	-

** Only cases published after 1990 and providing sufficient data on treatment and outcome were considered; $ Age at diagnosis of BCS; § Treatment of PNH was considered both before and after diagnosis of BCS; ¥ Medical therapy for PNH consisted of steroids (n=7), cyclosporine (n=2), granulocyte colony-stimulating factor (G-CSF, n=1), antithymocyte globulin (ATG, n=1) or eculizumab (n=1).PTA, percutaneous transluminal angioplasty; TIPS, transjugular intrahepatic portosystemic shunt; PSS, (surgical) portosystemic shunt; OLT, orthotopic liver transplantation; SCT, stem cell transplantation; NA, information on treatment of PNH not available; IVC, inferior vena cava.*

and need for re-interventions was not different from BCS-patients without PNH. (data not shown) In one PNH-patient with concurrent portal vein thrombosis TIPS-placement failed. However, in two other cases a TIPS was successfully inserted despite additional portal thrombosis. Although evidence is scarce, the presence of portal vein thrombosis should probably not be considered a definite contraindication for TIPS. In two small series of patients with liver cirrhosis and portal vein thrombosis, TIPS-placement was successfully performed. [29-30] Orthotopic liver transplantation (OLT) was not carried out in any of the PNH-patients. This may indicate that the manifestations of BCS could be adequately managed with other treatment modalities in these patients. However, it is also possible that PNH was considered to be an important contra-indication for liver transplantation based on the high frequency of thrombotic complications and the risk of recurrence of BCS after OLT. [31-33]

To correlate the findings from our study to other reports of patients with BCS and underlying PNH, we reviewed the literature from 1990 for case-reports describing the treatment and outcome of this specific patient group. The outcome of BCS-patients with PNH described in case-reports published before 1986 has already been summarized in a previous study. [19] The findings from 22 case-reports describing a total of 25 patients with BCS and PNH are presented in Table 5. [31-52] In this series of cases, PNH was diagnosed before presentation of BCS in the majority of the patients. Treatment of BCS mainly consisted of anticoagulation, with or without additional interventions such as thrombolysis or TIPS-placement. Six patients with BCS and PNH were treated with allogenic stem cell transplantation, which was successful in five cases. These findings underline the feasibility of TIPS and stem cell transplantation in the treatment of BCS-patients with PNH.

Based on the results of our study, data from the series of case reports and current treatment recommendations, we have summarized the therapeutic options for BCS-patients with PNH. (Figure 2) However, it must be emphasized that controlled studies have not been performed and that the place of eculizumab in the treatment of PNH has not yet been fully established. The management of this specific patient group obviously requires a multidisciplinary approach and therapy should be guided by the severity of clinical symptoms associated with both disorders.

Mortality in patients with PNH is for a large part due to thrombotic complications and approximately 20% of all deaths can be attributed to BCS. [8-9, 17, 23] Especially for patients with BCS it has been suggested that the presence of PNH is associated with a poor prognosis, with mortality rates as high as 67%. [19] Still, in the overview of case reports presented, survival appeared to be improved in comparison with earlier studies. Less than one-third of the cases died, with most deaths occurring within the first year after diagnosis of BCS. In line with this observation, a recent study on incidence-case data of patients with PNH and abdominal vein thrombosis, published between 1953 and 2006, showed that mortality rates of PNH-patients

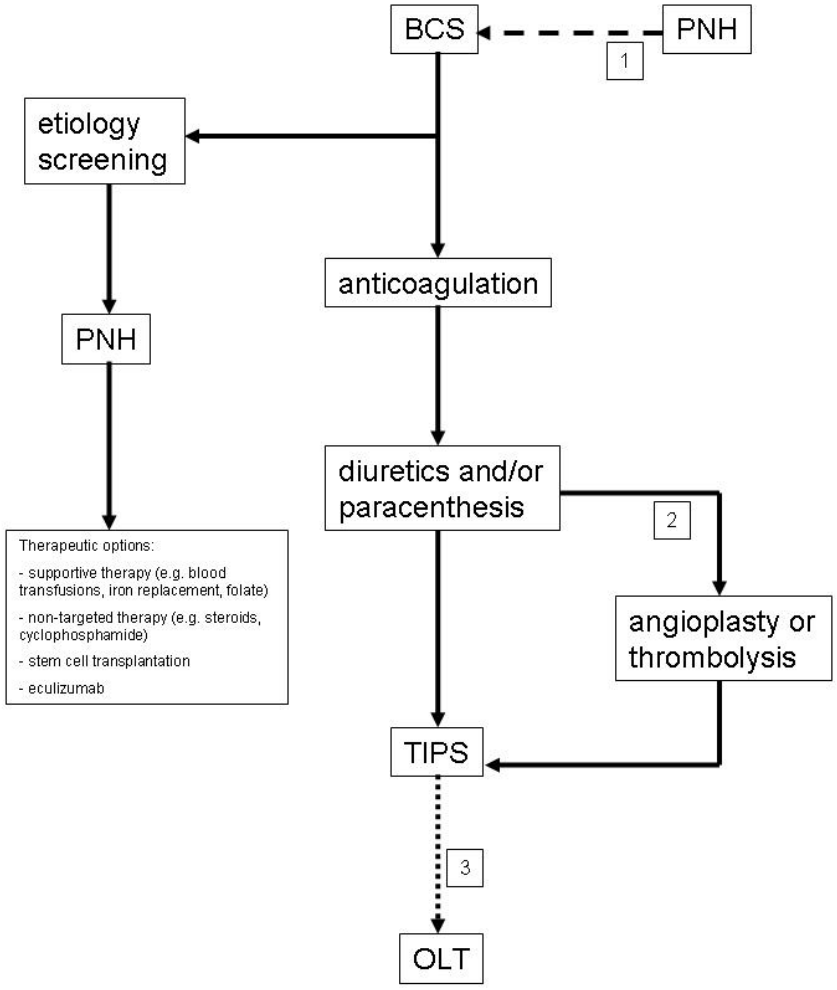

Figure 2. Overview of therapeutic options in patients with BCS and underlying PNH. [1] In some patients, the diagnosis of PNH has already been established before BCS develops. Therapeutic options are essentially similar as compared to patients in whom BCS is the first presentation of PNH. An important additional consideration in patients with previously diagnosed PNH is whether prophylactic anticoagulation is warranted (e.g. in cases with a PNH-clone >50%). [2] Angioplasty, with or without stenting, can be considered in patients with a focal or segmental obstruction of the hepatic venous outflow tract. Systemic or local thrombolysis may be performed in patients with recent thrombosis, mostly in combination with angioplasty and/or stenting. [3] Given the high rate of thrombotic complications that has been observed in BCS-patients with PNH undergoing liver transplantation, the presence of PNH can be considered a relative contraindication for OLT in these cases.

suffering from BCS have decreased in the past decades. [53] Furthermore, in accordance with the gross similarities in clinical presentation and therapeutic interventions, we did not find a significant difference in survival between BCS-patients with and without PNH. However, the follow-up period of this study is limited and future studies are required to establish if survival rates of these two groups remain comparable over time.

In summary, these results from the EN-Vie Study show that in patients with BCS and underlying PNH, the frequency of additional SVT is significantly higher than in BCS-patients without PNH. However, treatment during follow-up and short-term prognosis are relatively better than previously reported and are not affected by the presence of PNH. Our data also indicate that allogenic stem cell transplantation can be successfully applied to cure PNH in patients with BCS.

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Chapter 6

ANTICOAGULATION IN PATIENTS WITH NON-CIRRHOTIC EXTRAHEPATIC PORTAL VEIN THROMBOSIS

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Submitted

ABSTRACT

In patients with non-cirrhotic extrahepatic portal vein thrombosis (PVT) anticoagulation therapy should be considered when thrombosis is of recent onset or when a prothrombotic state is present. The aim of this study was to assess the effect of anticoagulation therapy on gastrointestinal bleeding and recurrent thrombotic events in patient with non-cirrhotic PVT. All consecutive patients with non-cirrhotic PVT, seen at our hospital from 1985 to 2009 were enrolled in this study. Data were collected by systematic chart review. One hundred-twenty patients (36% male; median age 44 years, range 16-87) were followed after the diagnosis PVT was made (median follow up 5.5 years, range 0.1-32.5). Forty patients had recent PVT, 71 patients had chronic PVT and in nine cases the onset was unknown. Sixty-six patients were treated with anticoagulation therapy. In thirty-seven patients 83 bleeding events occurred (variceal bleeding n=52, gastro-intestinal non-variceal bleeding n=31). Re-bleeding risk was 19% at one year, 46% at five years and 49% at ten years. Gastrointestinal bleeding at presentation (HR 2.1, p=<0.01), ascites (HR 2.0, p=0.01) and use of anticoagulation therapy (HR 2.0, p=<0.01) were significant predictors of (re)bleeding. Anticoagulation therapy had no effect on the severity of gastro-intestinal bleeding. Two out of four patients with fatal gastrointestinal bleeding were using anticoagulants. Twenty-two new thrombotic events occurred in nineteen patients (venous n=15, arterial n=7). Overall thrombotic risk was 4% at one year, 8% at five years and 27% at ten years. Of all new thrombotic events, 74% occurred in patients with a prothrombotic disorder. Anticoagulation therapy tended to lower the risk of a new thrombosis (HR0.2, p= 0.1), yet the only significant predictor of a new thrombotic event was presence of a prothrombotic disorder (HR 3.1, p= 0.03). New thrombotic events were significantly associated with poor survival (HR 3.1, p=0.02). Bleeding (HR 1.6, p=0.2) and anticoagulant treatment (HR 0.5, p=0.2) had no significant effect on survival.

Conclusions. In PVT patients new thrombotic events were mainly observed in patients with prothrombotic disorders. Anticoagulation therapy tended to prevent recurrent thrombosis but also significantly increased the risk of gastrointestinal (re)bleeding. This finding suggest that anticoagulation therapy should be used selectively and with caution in PVT patients.

INTRODUCTION

Extrahepatic portal vein thrombosis (PVT) is an important cause of non-cirrhotic portal hypertension. Variceal bleeding is one of the major complications of PVT. [1-5] Several local risk factors for developing PVT, including cirrhosis, hepatobiliary malignancies and pancreatitis, and systemic risk factors, including myeloproliferative neoplasms and prothrombotic genetic defects, have been identified. [6-15] In patients with recent thrombosis or with an underlying prothrombotic disorder, anticoagulation therapy is usually considered. [15-21] However, data on the risk and benefit of anticoagulation therapy are limited. Benefit of treatment was reported by two cohort studies. One of these studies addressed anticoagulation therapy in patients with recent PVT only. [16, 22].

The aim of this retrospective study was to assess the effect of anticoagulation therapy on gastro-intestinal (re)bleeding and thrombotic events during a long-term follow-up in patients with PVT.

PATIENTS AND METHODS

Design of the study

Patients were identified by means of a search in the computerized patient registration system of our clinic. All adult patients identified between January 1985 and November 2008 were enrolled if PVT was documented and cancer, cirrhosis, liver transplantation or combined Budd-Chiari syndrome were absent. For all patients a standardized clinical record form for detailed clinical data was completed with data obtained from the medical charts.

From a total of 241 patients with PVT 120 patients were included in this study. Patients were excluded for the following reasons: 63 patients had cirrhosis, 49 had malignancies, seven had developed PVT after liver transplantation and two had Budd-Chiari syndrome.

Follow-up started at the time of diagnosis, defined as the date of the first radiological imaging documenting PVT, and was continued to either December 2009 or death, whichever came first. Patients lost to follow-up were censored at the last visit.

Diagnostic assessment

Diagnostic criteria for PVT were partial or complete obstruction of the extrahepatic portal vein, as documented by radiological imaging, such as Doppler ultrasonography, computed tomography, magnetic resonance imaging, and venography. Risk factors for the development of PVT were divided in inherited and acquired risk factors. Inherited risk factors were: factor V Leiden mutation, prothrombin gene mutation, protein C deficiency, protein S deficiency, and

antithrombin deficiency. Inherited deficiencies of the latter three proteins were diagnosed if patients were known with this deficiency prior to PVT, when a low concentration of these proteins was confirmed at least six weeks after the onset of PVT or, in case anticoagulation therapy was given, at least six weeks after discontinuation of anticoagulation, all in the absence of hepatic dysfunction. Acquired risk factors were: a myeloproliferative neoplasm (MPN), paroxysmal nocturnal hemoglobinuria, antiphospholipid antibody syndrome, abdominal surgery, infection, inflammatory bowel disease (IBD), use of oral contraceptives or trauma. Abdominal surgery, trauma or abdominal infections were regarded as underlying cause if these events had occurred within three months prior to the development of PVT. PVT was defined recent in the absence of a portal cavernoma and gastrointestinal varices. All other cases were classified as chronic PVT. Gastroesophageal varices were graded as [23]: grade I, varices flattened by insufflation; grade 2, varices not flattened by insufflation; grade 3, confluence of varices not flattened by insufflation; grade 4, grade 3 with red marks. Anticoagulation therapy included treatment with vitamin K antagonists or therapeutic use of low molecule weight heparin or heparin. To compare the severity of bleeding between patients with and without anticoagulation therapy, we assessed the number of bleeding events per patient, the amount of units transfused red blood cells, the number of Intensive Care Unit admissions and hemoglobin level at admission for each bleeding episode in both groups.

Statistics

Kaplan Meier method was used to calculate (re)bleeding free, thrombotic free and overall survival and the log-rank test was used for comparing groups. Predictors of (re)bleeding and new thrombotic events were estimated as a hazard ratio (HR) and corresponding 95% confidence interval (CI) using Cox regression analysis adjusted for sex and age. The effect of bleeding, thrombotic events and anticoagulation treatment on survival were estimated as time dependent covariates using Cox regression analysis. Due to the small amount of thrombotic events univariate Cox regression models, stratified for the number of thrombotic events, were used to determine significant predictors of thrombosis. Since patients could stop and restart anticoagulation therapy during follow-up, we correlated new thrombotic and bleeding events to the therapy used at that time. In case of oral anticoagulation use, a period of three days was added to the stop date and considered as using anticoagulation therapy. Use of anticoagulation therapy was analysed as a time-dependent factor. Sensitivity analysis was performed for subgroups. A p-value of <0.05 was considered statistically significant. Data were expressed as median with the accompanying range and interquartile range (IQR), where appropriate. All statistical analysis were performed with the Statistical Package for Social Sciences for Windows, version 16.0 (SPSS, Chicago, IL).

Table 1. Baseline Characteristics of 120 Patients with PVT

Variable at baseline	Patients (n=120)	(%)
Sex (m/f)	43/ 77	
Age (yrs)§	44 ± 16 (16 - 87)	
History of thrombosis	14/ 118	12%
Acute/ chronic thrombosis	40/71	
Site of thrombosis		
Portal vein	64/120	53 %
Portal and splenic veins	9/120	8 %
Portal and mesenterial veins	17/120	14 %
Portal, splenic and mesenterial veins	30/120	25 %
Inherited thrombophilia	24/95	25 %
Protein C deficiency	4/82	5%
Protein S deficiency	11/83	13 %
Antithrombin deficiency	4/101	4%
Factor V Leiden	6/86	7%
Prothrombin gene mutation	1/71	1%
Acquired disorders	96/120	80 %
Myeloproliferative neoplasm	39/120	33 %
Infection	22/120	18 %
IBD	7/120	6 %
Antiphospholipid syndrome	6/88	7%
Surgery	35/120	29 %
Splenectomy	7/120	6 %
Oral contraceptive use	28/113	25 %
Smoking	28/94	30 %
Varices (at diagnosis)	61/94	65 %
Varices grade I/II/III/IV	10/12/16/16	-
Gastro-intestinal bleeding	27/98	28 %
Ascites	32/118	27 %
Blood hemoglobin (mmol/ L) §	7.3 ± 1.7 (3.9 – 13.8)	
Platelet count (x 10E9/ L) §	257 ± 194 (31 –1083)	
Bilirubin (μmol/L) §	14 ± 33 (3 – 242)	
Aspartate transaminase (U/ L) §	27 ± 73 (3 – 709)	
Alanine transaminase (U/ L) §	28 ± 59 (9 – 508)	
Serum albumin (g/ L) §	38 ± 7.0 (22- 51)	
Serum creatinin (μmol/ L) §	68 ± 55 (35 – 385)	
APTT (sec) §	34 ± 21 (3 – 144)	
PT (sec) §	15 ± 10 (10 – 66)	
PT INR§	1.3 ± 0.8 (1.0 – 4.3)	

§ Median (range). IBD, inflammatory bowel disease; APTT, activated partial thromboplastin time; PT, prothrombin time; INR, international normalized ratio.

RESULTS

A total of 120 patients, 43 males and 77 females, with a median age of 44 years (range 16- 87 years), were enrolled. Baseline characteristics are summarized in Table 1. In approximately

50% of the patients thrombosis was confined to the portal vein. In the remaining cases thrombosis was more extensive, involving the splenic and/or mesenteric veins. Two patients presented with concomitant thrombosis outside the portal venous system: deep vein thrombosis of the lower limb and sinus sagittalis thrombosis, respectively. PVT was diagnosed by ultrasound (n=69), computed tomography (n=45), venography (n=2), magnetic resonance imaging (n=3) or per-operative (n=1). Median duration of follow up was 5.5 years (range 0.1-32.5 years; IQR 2.0-10.4 years). A prothrombotic state was found in 69 patients (58%). Thirty-nine patients (33%) had MPN as determined by either bone marrow biopsy and/or JAK2 mutation. Gastro-intestinal blood loss at baseline was caused by variceal bleeding, in all except of two patients where angiomatosis of the stomach and arteriovenous malformation of the rectum were present.

Sixty-six patients - 29/40 cases with recent PVT, 33/71 cases with chronic PVT and 4/9 cases where onset of thrombosis could not be determined - were treated with anticoagulation therapy. In 59 patients therapy was instituted immediately after the diagnosis of PVT was established. In the seven other cases anticoagulation therapy was started during follow up after a median period of 3.4 years (range 1.2-15.4 years). Anticoagulation therapy was used during a median period of 1.9 years (range 0-15.8 years; IQR 0.7-5.1 years). Forty-two patients still used anticoagulants at the end of follow-up. Anticoagulants were stopped in 24 of 66 patients for the following reasons: completed intended time of treatment (n=16), iron deficiency anemia caused by menorrhagia (n=2), epistaxis (n=1), presence of varices grade ≥ 2 or variceal bleeding (n=2) or unknown (n=3).

From the total of 120 patients, 16 patients (13%) were treated with both anticoagulation therapy and an antiplatelet drug, 13 cases (11%) were on antiplatelet treatment only and 41 patients (35%) neither received anticoagulation or antiplatelet therapy. The number of patients on antiplatelet therapy was too small to assess effects on bleeding, recurrent thrombosis or survival.

Bleeding events

A total of 83 bleeding episodes (variceal bleeding n=52 and other gastro-intestinal bleeding n=31) occurred in 37 patients (31%) during follow up. Sixteen patients had a single episode of bleeding, 11 patients had two episodes and 10 patients had more than three episodes of bleeding (range 3-9 events). The median time between PVT diagnosis and a first bleeding event was seven months (range 0.1-11.5 years; IQR 0.2-3.0 years). Overall risk of gastro-intestinal bleeding was 33% (95% CI 24-41) at one year, 43% (95% CI 33-53) at five years and 46% (95% CI 36-56) at ten years. The median time to development of rebleeding was four months (range 0.01-8.6 years; IQR 0.9-8 months) after the initial bleeding episode. Overall risk of re-bleeding was 46% (95% CI 36-56) at one year, 63% at five years (95% CI 52-74) and 69% at ten years (95% CI 59-82). (Figure 1)

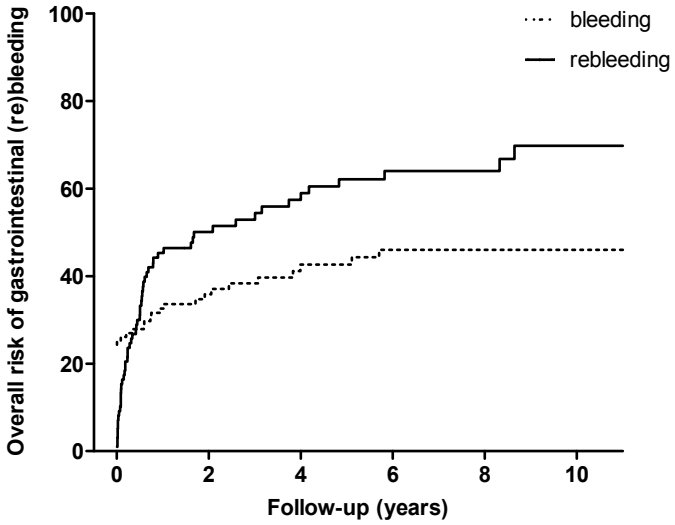

Figure 1. Kaplan-Meier survival curve showing the overall risk of bleeding and rebleeding during follow-up.

Predictors of (re)bleeding in the univariate analysis are shown in Table 2. In the multivariate analysis gastrointestinal bleeding (HR 2.1, p=<0.01), ascites at baseline (HR 2.0, p=0.01) and anticoagulation therapy at the time of bleeding (HR2.0, p=<0.01) were all significant predictors of gastrointestinal (re)bleeding. There was no significant relationship between the severity of the gastro-intestinal bleeding and the use of anticoagulants. Patients with anticoagulation therapy (n=66) had 58 bleeding episodes versus 25 events in patients without anticoagulation therapy (n=54). Median hemoglobin level at admission was 5.5 mmol/L (range 1.8-8.7) in patients with anticoagulation therapy versus 5.8 mmol/L (range 3.0-8.5) in patients without anticoagulation therapy. Median number of transfused packed red blood cells per bleeding episode was four (range 2-20) in both groups. In total there were seven admissions to the ICU in the group of patients on anticoagulation therapy versus five in the patients not on anticoagulation.

New thrombotic events

A total of 22 new thrombotic events occurred in 19 patients (venous events n=15, arterial events n=7). Sites of thrombosis were: pulmonary embolism (n=3), superior mesenteric vein (n=2), lower limb (n=4), upper limb (n=4), sinus sagittalis (n=1), mesocaval shunt (n=2), intestinal ischemia (n=2) and ischemic stroke (n=4).The median time between diagnosis of PVT and occurrence of a new thrombotic event was 5.7 years (range 0.1-19.0 years; IQR 1.8-8.6

years). Overall risk of a new thrombotic event was 3% (95% CI 0-7) at one year, 8% (95% CI 3-14) at five years and 24% (95% CI 13-36) at ten years. (Figure 2) Seventy-four percent of the new thrombotic events occurred in patients with a prothrombotic disorder. Recurrence or extension of thrombosis into the splanchnic veins was only seen in patients with a prothrombotic disorder. Sixteen new thrombotic events were found in 69 patients with a prothrombotic disorder (23%) versus six events in 51 patients without a prothrombotic disorder (11%). Use of anticoagulants tended to reduce the occurrence of a new venous thrombotic event (HR 0.2, p= 0.1). Predictors of new thrombotic events are shown in Table 2.

Table 2. Univariate analysis of variables associated with bleeding, recurrent thrombosis and survival

	Bleeding		Thrombosis		Survival	
Variable	HR (CI 95%)	P	HR (CI 95%)	P	HR (CI 95%)	P
Age (years)***	1.1 (0.9-1.2)	0.5	1.2 (0.9-1.6)	0.20	1.9 (1.5-2.4)	< 0.01**
Female gender	0.7 (0.5-1.1)	0.2	0.9 (0.4-2.4)	0.9	0.9 (0.4-2.0)	0.8
Chronic portal vein thrombosis	1.1 (0.7-1.8)	0.7	1.2 (0.5-3.0)	0.8	1.7(0.7-4.0)	0.2
Site of thrombosis						
Portal vein only	1		1		1	
Portal, splenic and mesenterial veins	1.7 (1.0-2.9)	0.04**	1.4 (0.5-3.9)	0.5	1.0 (0.4-2.6)	0.9
Varices	1.0 (0.6-1.7)	0.9	0.7 (0.3-2.0)	0.5	0.9 (0.4-2.3)	0.8
Varices grade ≥ 2 ****	4.2 (1.0-18.0)	0.02**	1.1 (0.2-5.7)	0.9	1.3 (0.3-6.1)	0.8
Gastro-intestinal bleeding at baseline	1.7 (1.0-2.7)	0.04**	1.3 (0.5-3.6)	0.6		
Gastro-intestinal (re)bleeding during follow up*					1.6 (0.7-3.5)	0.2
Ascites	2.2 (1.3-3.6)	< 0.01**	1.7(0.5-5.5)	0.4	6.1 (2.2-16.4)	<0.01**
New thrombotic event*					3.1 (1.2-8.0)	0.02**
Anticoagulation therapy *	1.7 (1.1-2.7)	0.03**	0.2 (0.0-1.9)	0.1	0.5 (0.2-1.3)	0.2
Underlying causes						
Inherited	1.0 (0.5-1.7)	0.9	1.0 (0.3-3.6)	0.9	0.3 (0.0- 2.5)	0.2
Acquired	0.7 (0.4-1.1)	0.1	0.9 (0.3-2.8)	0.9	1.4 (0.5-4.0)	0.6
MPN	1.2 (0.8-2.0)	0.4	1.9 (0.8-4.5)	0.2	2.6 (1.3-5.5)	0.01**
Infection	0.6 (0.3-1.3)	0.1	0.3 (0.8-1.5)	0.1	0.3 (0.1-1.1)	0.04**
IBD	- [1)]		1.0 (0.2-4.4)	0.9	0.8 (0.2-3.5)	0.8
Prothrombotic disorder	1.1 (0.7-1.7)	0.7	3.1(1.0-9.5)	0.03**	3.0(1.2-7.4)	0.01**

** Analysed as a time-dependent factor; ** Statistically significant; *** Ten-year increment; ****According to the Paquet Classification [23]; [1)] There were not enough bleeding events observed to assess the effect of IBD. MPN, myeloproliferative neoplasm; IBD, inflammatory bowel disease.*

Survival

Twenty-nine patients died. Median age of death was 64.2 years (range 30.4-95.3 years). Overall survival was 90% (95%CI 84-96) and 70% (95%CI 58-82) at 5 and 10 years, respectively. (Figure 3) Predictors of survival in the univariate analysis are presented in Table 2. In multivariate analysis increased age (HR 1.1, p<0.01), ascites at diagnosis (HR 4.0, p<0.01) and recurrent thrombotic events (HR 3.1, p=0.02) were the only significant factors associated with increased mortality. Gastrointestinal bleeding (HR 1.6, p=0.2) and anticoagulant use (HR 0.5, p=0.2) both had effects on survival, but this was not statistically significant. Five patients died due to a bleeding event: cerebellar hematoma (n=1), variceal bleeding (n=2) and other upper gastrointestinal bleeding (n=2). The latter two patients were both on anticoagulation therapy. Three

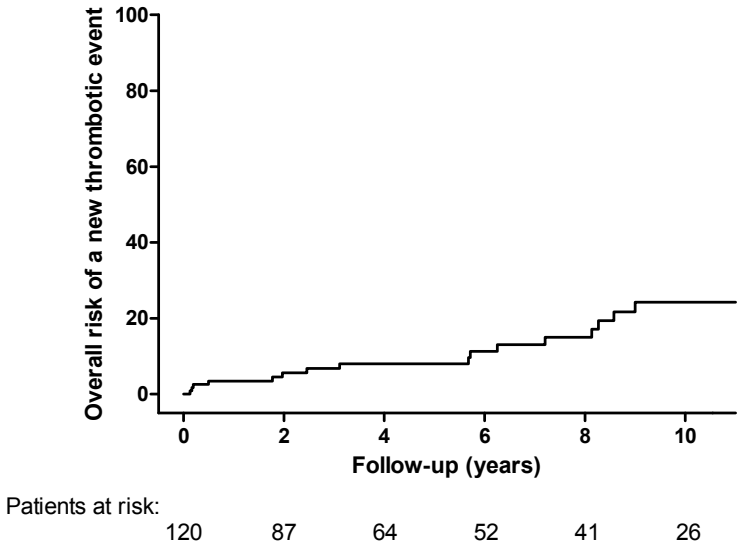

Figure 2. Overall risk of a new thrombotic event during follow-up.

patients died due to a new thrombotic event (necrotic colon due to massive arterial ischemia, ischemic cerebral vascular accident and sinus sagittalis thrombosis). The last two patients were both on anticoagulation therapy when the thrombotic events occurred. Other causes of death were progressive MPN (n= 6), infection (n=3), other causes (n= 7) and unknown (n= 5).

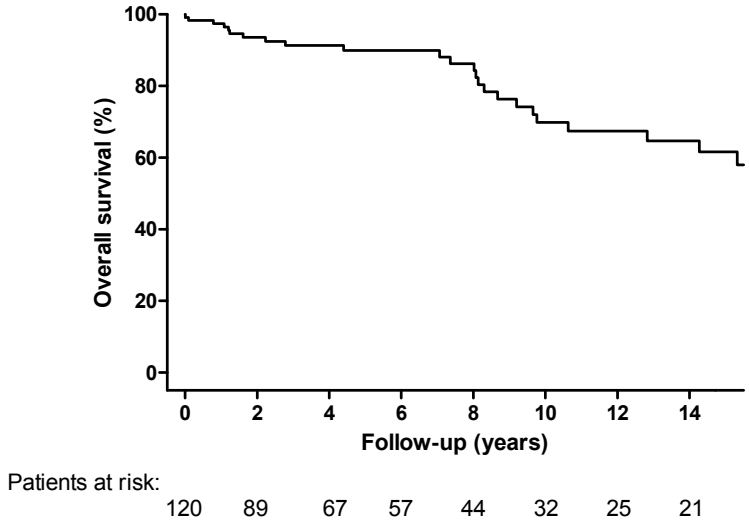

Figure 3. Kaplan-Meier curve showing overall survival of 120 patients with PVT.

DISCUSSION

In this study we investigated anticoagulation therapy and other factors associated with gastrointestinal bleeding, new thrombotic events and survival in patients with non- cirrhotic non malignant PVT. In about twenty percent of the patients with a prothrombotic disorder a new thrombotic event occurred, which was significantly associated with decreased survival. Anticoagulation therapy showed a tendency to prevent new thrombosis, however it significantly increased the risk of gastro-intestinal bleeding.

As is known from previous studies (re)bleeding is mainly determined by size of varices and initial presentation of a gastrointestinal bleeding [5, 16, 24]. In addition to these factors, we found that both extension of thrombosis into the splanchnic veins as ascites at baseline were significant factors predicting (re)bleeding. These new findings may help to identify patients with a high risk of gastrointestinal bleeding and also may influence whether to start anticoagulation therapy. Condat et al. concluded that the benefit-risk ratio favours anticoagulation therapy for most patients with PVT [16]. They made this conclusion on two major findings. Firstly, the risk of new thrombotic events was profoundly reduced by anticoagulation therapy. This reduction involved mainly thrombotic recurrence or extension within the portal venous system. Secondly, they did not find a significant correlation between the risk and severity of gastro-intestinal bleedings and the use of anticoagulation therapy. We found that patients with PVT and a prothrombotic disorder had a three-fold increased risk to develop new thrombotic events both in and outside the splanchnic veins. Extension of thrombosis into the splanchnic veins occurred only in patients with a prothrombotic disorder. These findings suggest that anticoagulation therapy is warranted in patients with a prothrombotic disorder. In our study anticoagulation therapy tended to have an overall beneficial effect on decreasing new thrombotic events and even somewhat on improving survival. On the other hand the use of anticoagulation therapy significantly increased the risk of gastrointestinal bleeding. A total of four patients (3%) died due to gastrointestinal bleeding, two were on anticoagulation therapy. Three patients (3%) died due to a thrombotic event, of which two were on adequate anticoagulation therapy.

With this study we support the current guidelines which state that anticoagulation therapy can be considered in patients with PVT and an underlying prothrombotic disorder [19, 25]. However, we also found that anticoagulation therapy is not without risk.

Our study has several limitations. Decisions about anticoagulation treatment and performing radiological imaging were made by the treating physician. This led to the fact that not all patients did have a systemic radiological imaging during follow up and that the time between radiological imaging differed among the patients. Recurrence of portal vein thrombosis or extension of thrombosis into the splanchnic veins could have occurred in some patients and remained unidentified. Especially in those cases where no clinical significant signs or symptoms were present. Patients with an extensive thrombosis are more likely to

get anticoagulation therapy than patients presenting with a gastro-intestinal bleeding. Both factors were carefully taken into account in the uni- and multivariate analysis. Furthermore during the long study period new insights and diagnostic tests have been developed. This may have led to an underrepresentation and - treatment of patients with prothrombotic disorders enrolled in the early part of the study. In parallel, these patients were initially not on anticoagulation therapy. Also patients could stop and restart anticoagulation therapy during their course. We therefore analyzed each bleeding or thrombotic event according to the therapy used at that time.

In conclusion, in patients with non-cirrhotic PVT new thrombotic events occur primarily in patients with a prothrombotic disorder and are significantly associated with decreased survival. Anticoagulation therapy can lead to prevention of recurrent thrombosis, but also significantly increases the risk of gastrointestinal bleeding. These findings imply that anticoagulation therapy on one hand is warranted in patients with PVT and an underlying prothrombotic disorder, while on the other hand it should be given with caution to those with high risk of bleeding.

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Chapter 7

LONG-TERM FOLLOW-UP OF PATIENTS WITH PORTAL VEIN THROMBOSIS AND MYELOPROLIFERATIVE NEOPLASMS

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Submitted

ABSTRACT

Myeloproliferative neoplasms (MPN) are frequently identified as an underlying cause in patients with non-cirrhotic portal vein thrombosis (PVT). The aim of this study was to describe the long-term outcome of patients with PVT and MPN. A cohort study was performed including all adults patients referred to our hospital between 1980 and 2008 with non-cirrhotic, non-malignant PVT and confirmed MPN. A total of 44 patients (70% female) were included, with a median age at PVT-diagnosis of 48 years (range 18-79). In 31 patients (70%) PVT was the first manifestation of a MPN. Additional risk factors for thrombosis were present in 20 patients (45%). Median follow-up was 5.8 years (range 0.4-21). Twenty-three patients (52%) were treated with anticoagulants after diagnosis of PVT, of whom 15 (34%) received long-term therapy. During follow-up, 17 patients (39%) experienced at least one episode of gastrointestinal bleeding. Additional thrombotic events occurred in 12 patients (27%). Eight patients (18%) had progression of the underlying MPN. Seventeen patients (39%) died at a median age of 64 years (range 30-88). Death was directly related to end-stage MPN in 8 patients (47%) and to a new thrombotic event in 3 patients (18%). No patients died resulting from gastrointestinal bleeding.

Conclusions. PVT is often the presenting symptom of an underlying MPN, highlighting the need for thorough screening for this disease. Recurrent thrombosis is a common and severe complication in patients with PVT and MPN. Mortality is primarily related to the underlying MPN and not to complications of portal hypertension.

INTRODUCTION

Development of thrombosis in the extrahepatic portal vein is an uncommon finding. The etiology of portal vein thrombosis (PVT) is heterogeneous and involves both local and systemic prothrombotic factors. [1] Liver cirrhosis and hepatobiliary malignancies are considered to be the most important local precipitating factors of PVT. [2-3] In the absence of cirrhosis or local tumors, PVT has a better survival. Often there is a prominent role of systemic thrombophilia in these patients. [4-5] The most common systemic risk factor encountered in patients with non-cirrhotic, non-malignant PVT are myeloproliferative neoplasms (MPNs). [6] MPNs encompass a group of disorders that share features of increased myeloproliferation, resulting in overproduction of mature, functional blood cells. The three main Philadelphia chromosome-negative MPNs are polycythemia vera (PV), essential thrombocythemia (ET) and idiopathic myelofibrosis (IMF). [7]

MPNs are found as an underlying cause in approximately 20-30% of the patients with non-cirrhotic non-malignant PVT. [8-9] Especially in young adults, development of PVT can be a first manifestation of an underlying MPN. However, diagnosis of MPN in these patients can be difficult because typical signs in peripheral blood (e.g. elevated hemoglobin or platelets) may be absent due to hypersplenism and other consequences of portal hypertension. [10] Although a number of studies have been published concerning the distribution of etiological factors and clinical presentation of patients with PVT, little is known about the subgroup of cases with confirmed MPN. Due to the rarity of this disease, scarce data is available on the long-term outcome of these patients, including the incidence of recurrent thrombosis, complications of portal hypertension, survival and causes of death.

To describe the natural disease course and main complications, we conducted a cohort study of patients with non-cirrhotic non-malignant PVT and an underlying MPN. Additionally, we determined long-term survival and causes of death in these patients.

METHODS

Study population

Patients with PVT and MPN were identified using our computerised hospital registration system. We selected all adult patients who were registered with a diagnosis of PVT between January 1980 and December 2008. Patients with cirrhosis, Budd-Chiari syndrome and/or overt hepatobiliary malignant disease were excluded. For all patients clinical data was collected on medical history, disease presentation, treatment, clinical events during follow-up and outcome through review of the medical charts.

PVT was defined as a partial or complete thrombotic obstruction of the main portal vein, as documented by appropriate radiological abdominal imaging (i.e. duplex ultrasound, computed tomography, magnetic resonance imaging or venography) or during laparotomy. Date of PVT diagnosis was defined as the date that imaging first demonstrated thrombosis. The presence of cirrhosis was excluded through liver biopsy, imaging and/or the absence of biochemical evidence of liver failure. Subtype of MPN was retrospectively classified according to the WHO 2008 guidelines, confirmed by an expert in the field (FWL). [11] Diagnostic work-up for MPN included peripheral blood cell counts, bone marrow morphological examination, endogenous erythroid colony (EEC) formation assessment and $JAK2^{V617F}$ mutation analysis.

Follow-up was from date of diagnosis of PVT until death, study closure in May 2009, or, in case of loss to follow-up, date of last visit. If the outcome was unknown, family physicians were contacted for further information.

Statistics

Quantitative data were expressed as median values, whereas percentages were used for qualitative data. Differences between subgroups were studied using the Mann Whitney U and Chi square test for continuous and categorical variables, respectively. Survival curves were calculated using the Kaplan-Meier method and comparison of survival functions was based on log-rank testing. Overall survival rates were measured from the date of PVT diagnosis and patient survival was censored at death or loss to follow-up. All statistical tests were 2-sided, with p-values of .05 or less denoting statistical significance. Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 16.0 software package for Windows (Chicago, IL).

RESULTS

Patient characteristics

A total of 44 patients with PVT and an underlying MPN were included in this study. Median age at diagnosis of PVT was 48 years and 70% of the patients was female. General characteristics at time of PVT diagnosis are summarized in Table 1. Based on clinical presentation and results of imaging, 13 patients (30%) presented with acute PVT, whereas the remaining 31 patients already had signs of a portal cavernoma and/or portal hypertension (e.g. gastroesophageal varices, variceal bleeding or splenomegaly) at time of diagnosis, consistent with chronic PVT.

In 30 patients (68%) diagnosis of MPNs fulfilled WHO 2008 criteria. Three other patients without overt features of MPNs were $JAK2^{V617F}$ positive, confirming MPNs in these cases.

Table 1. General characteristics of 44 patients with PVT and MPN

	Number of patients (%)
Gender – female	31 (70)
Age at PVT diagnosis (years)	48 (18-79)‡
PVT prior to MPN diagnosis	31 (70)
Acute PVT	13 (30)
Clinical manifestations at presentation¥	
Splenomegaly at imaging	33 (75)
Abdominal pain	31 (70)
Ascites	15 (34)
Variceal bleeding	8 (18)
Hepatomegaly at imaging	8 (18)
Asymptomatic	7 (16)
Localization of thrombosis	
Isolated PV	21 (48)
PV, SMV, SV	15 (34)
PV, SV	5 (11)
PV, SMV	3 (7)

PV, portal vein; SMV, superior mesenteric vein; SV, splenic vein. ‡ Median (range); ¥ at the time of PVT diagnosis, patients can have more than one clinical manifestation.

The remaining ten patients, in whom the current WHO criteria were not strictly met, had highly suspect bone marrow findings in combination with clearly elevated peripheral blood cell counts. These individuals were all diagnosed with MPN by their treating hematologists. Subtype of MPNs was classified as PV in 14 patients, ET in 12 cases, IMF in 7 patients and as unclassified in the remaining 11 patients. The JAK2 mutation was present in 26 out of 29 tested patients (90%).

In 31 patients (70%) PVT was the initial manifestation of an underlying MPN. Of 13 patients with a previous diagnosis of MPN, 11 patients had been diagnosed at least one year prior to development of PVT. Median time between diagnosis of PVT and subsequent diagnosis of MPN in patients not previously known with MPN was 7 months (range 0-175 months). In the majority of these cases laboratory values around time of PVT-diagnosis were not suggestive of an underlying MPN. An elevated hemoglobin level was present in 2 patients (6%) and 9 patients (29%) had thrombocytosis.

Of all cases, five patients (11%) had a previous history of thrombosis and ten patients (23%) had a positive family history for venous thrombosis. Analysis of local and systemic prothrombotic factors revealed that 20 patients (45%) had another etiological factor besides MPN. (Table 2) Use of oral contraceptives was a common systemic risk factor for PVT. Among women in the age group of 15 to 49 years, oral contraceptives were used at the time of diagnosis in 11 of 21 patients (52%).

Table 2. Additional prothrombotic factors identified in the study cohort

	Number of patients (%)
Systemic prothrombotic factors	
Oral contraceptive use‡	11 (35)
Natural anticoagulant deficiency¥	4 (9)
Factor V Leiden mutation	2 (5)
Antiphospholipid antibodies	1 (2)
Pregnancy	1 (2)
Local prothrombotic factors	
Abdominal surgery (< 6 months)	5 (11)
Intra-abdominal infection	2 (5)

‡ Percentage of female patients is given; ¥ Protein C, protein S or antithrombin deficiency.

Follow-up and treatment

Median follow-up time for the total cohort was 5.8 years (range 0.4-21 years). Two patients (4%) were lost to follow-up at 66 and 76 months after PVT diagnosis, respectively.

In total, twenty-three patients (52%) received anticoagulation therapy following diagnosis of PVT, consisting of either vitamin K antagonists (VKA), low-molecular-weight heparin or unfractionated heparin. Long-term anticoagulation with VKA, considered as lifelong, was given in fifteen cases after diagnosis of PVT (34%) whereas in the other eight patients anticoagulation therapy was discontinued after a median period of 9 months (range 0.5-39 months). Treatment with anticoagulation was initiated significantly more often in cases presenting with acute PVT as compared to those with chronic PVT (77% vs. 42%, respectively, p=0.034).

Treatment for the underlying MPN was given in 30 patients (68%) during follow-up after PVT. Twenty-two patients (50%) received anti-platelet therapy, consisting of either aspirin (n=19) or clopidogrel (n=3). In two patients treatment was stopped after 2 and 3 months, respectively, due to suspected gastrointestinal bleeding and intolerance for aspirin. Of the other 20 patients, 12 cases (27%) received long-term treatment with anti-platelet drugs following diagnosis of PVT. Cytoreductive therapy with hydroxyurea was initiated in 21 cases (48%), with varying dosages and duration of therapy. Other interventions consisted of regular phlebotomies (n=10), treatment with alpha-interferon (n=8), busulfan (n=3) or anagrelide (n=1).

Of the thirteen patients diagnosed with MPN prior to PVT, seven cases were being treated for MPN at the time of PVT. One patient was on hydroxyurea therapy, two patients were treated with aspirin, two patients received a combination of aspirin and hydroxyurea and in another two patients regular phlebotomies were performed. Levels of hemoglobin and platelets in these patients were all within the normal range around time of PVT.

Table 3. Complications during follow-up of 44 patients with PVT and MPN

	Number of patients (%)
Episode of gastrointestinal bleeding	17 (39)
Events per person	1 (1-5) ‡
Time between PVT and first bleeding event (yrs)¥	2.3 (0.5-5.1)‡
Additional thrombotic event	12 (27)
Venous thrombosis§	9 (20)
extension of thrombosis to SMV or SV	*3*
jugular and/or axillary vein thrombosis	*3*
pulmonary embolism	*1*
femoral vein thrombosis	*1*
sinus sagittalis thrombosis	*1*
Arterial thrombosis$	5 (11)
ischemic CVA	3
arterial thrombosis of lower extremity	1
intestinal infarction due to occlusion of celiac artery	1
Time between PVT and subsequent thrombosis (yrs)	7.5 (0-18)‡
Progression of MPN	8 (18)
Secondary myelofibrosis	*6 (14)*
Acute myeloid leukemia£	*4 (9)*
Time between MPN diagnosis and disease progression (yrs)	10.7 (3-16)‡

‡ Median (range); ¥ Only considered in patients that did not present with an episode of bleeding at diagnosis (n=9); § One patient experienced two additional episodes of venous thrombosis; $ One patient experienced two arterial thrombotic events; £ Two patients progressed to AML after developing secondary myelofibrosis.

Complications

The most common complications that occurred during follow-up are summarized in Table 3. In twelve patients (27%) at least one other thrombotic event occurred in addition to PVT with a total of 14 thrombotic events. Most frequent localizations were extension of thrombosis into the splenic and/or superior mesenteric vein (n=3) and ischemic stroke (n=3). Recurrent thrombosis occurred in 3 of 7 patients (43%) carrying additional thrombophilic factors as compared to 9 of 37 (24%) patients without thrombophilia (p=0.369). Three patients experienced a new episode of thrombosis despite treatment with VKA at that time. None of the patients that developed recurrent thrombosis were being treated with anti-platelet drugs when thrombosis developed. A schematic overview of the relationship between long-term use of VKA or antiplatelet therapy and the occurrence of new thrombotic events is given in Figure 1. After recurrent thrombosis, another 7 patients were started on lifelong treatment with either VKA (n=3) or aspirin (n=4).

Around the time of PVT diagnosis an upper gastro-intestinal endoscopy was performed in 38 patients, of whom 30 patients (79%) had esophagogastric varices. Grade III-IV esophageal varices according to Pacquet were found in 18 patients (47%). Overall, 32 documented episodes of variceal bleeding occurred in 17 patients (39%), most frequently resulting from esophageal varices. Endoscopic treatment was the first-line therapy during active bleeding

episodes. Variceal bleeding was the presenting symptom of PVT in eight patients (18%) and recurrent bleeding occurred in four of these patients. Of 36 patients that had no signs of bleeding at presentation, 9 patients (25%) experienced at least one bleeding event during follow-up and five cases had recurrent bleeding. Long-term treatment with anticoagulation did not appear to influence the risk of variceal bleeding. Of 36 patients without variceal bleeding at diagnosis, 16 cases were given long-term anticoagulation therapy. Subsequent bleeding episodes during follow-up occurred in two patients on anticoagulation (13%) and in seven patients not treated with long-term anticoagulation (35%, p=0.245).

Progression of the underlying MPN occurred in 8 patients (19%) after a median period of 10 years following MPN diagnosis. Five patients with PV and one patient with ET developed secondary myelofibrosis, of which two cases subsequently progressed to acute myeloid leukemia (AML). Additionally, two other patients developed AML for a total of four patients with AML. One patient with secondary myelofibrosis underwent successful allogenic stem cell transplantation and in one patient AML was successfully treated by intensive chemotherapy.

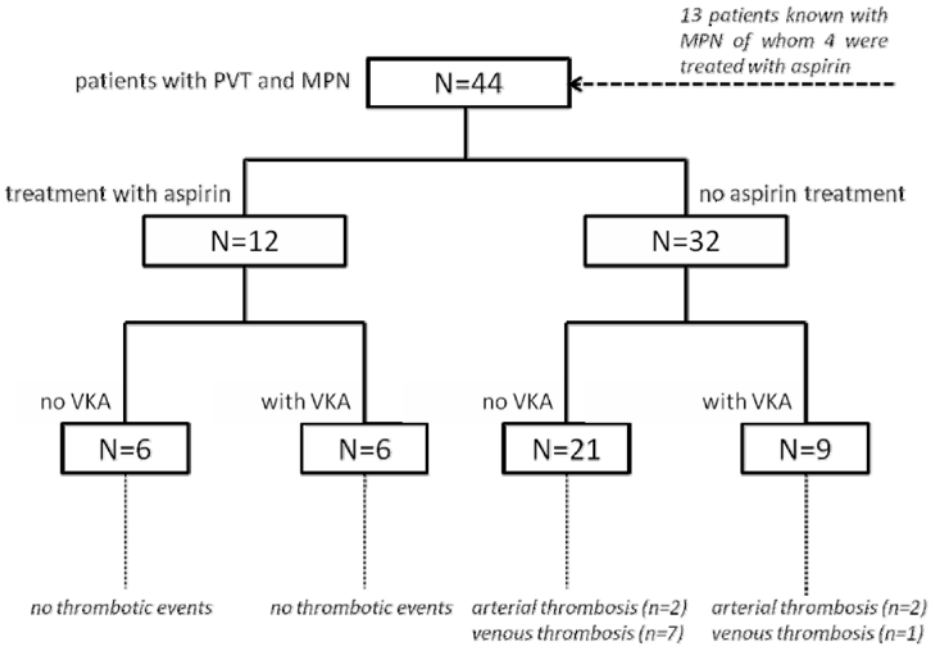

Figure 1. Flow diagram depicting the treatment with anti-platelet drugs in combination with vitamin K antagonists (VKA) during follow-up and the occurrence of additional thrombotic events.

Mortality

Seventeen patients (39%) died during follow-up, with a median age at death of 64 years (range 30-88 years). Table 4 displays the different causes of death. In 8 patients (47%) death

was directly related to end-stage MPN or AML. A new thrombotic event was the cause of death in 18% of the cases. One of these patients was receiving anticoagulation therapy at the time of death and in another patient treatment with VKA had been discontinued 3 months before death. In both patients ischemic stroke was the cause of death. No patients died as a result of variceal bleeding. The overall survival rate was 98% at one year and 88% at five years of follow-up. (Figure 2) Survival was not significantly different between patients treated with or without long-term anticoagulation with VKA (p=0.536).

Table 4. Causes of death among 18 patients with PVT and MPN

Cause of death	Number of patients (%)
End-stage myelofibrosis	5 (29)
Acute myeloid leukaemia	3 (18)
Thrombosis	3 (18)
Sepsis	1 (6)
Other causes	2 (12)
Unknown	3 (18)

DISCUSSION

In the current cohort study, we have investigated the long-term outcome and survival of patients with non-cirrhotic, non-malignant PVT related to an underlying MPN. To our knowledge, this study is the largest and most detailed assessment of these patients as yet.

The results of our study show that recurrent thrombosis is a severe and clinically significant complication. Almost one-third of the patients with PVT and MPN experienced an additional

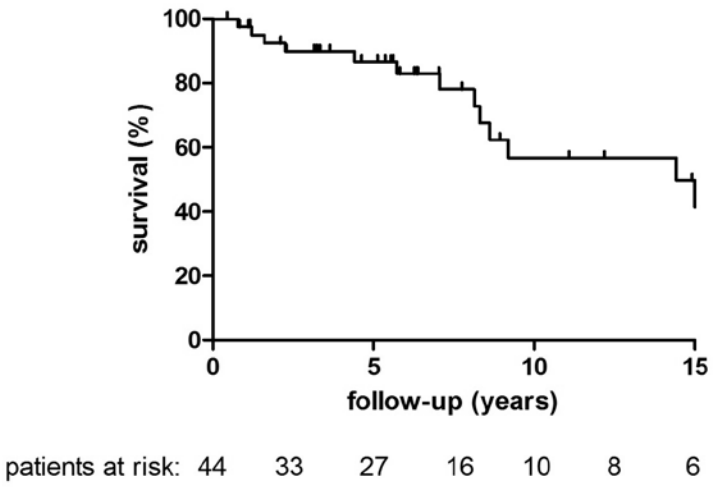

Figure 2. Kaplan-Meier survival curve, showing overall survival of 44 patients with non-malignant, non-cirrhotic portal vein thrombosis (PVT) and underlying myeloproliferative neoplasm (MPN). The number of patients at risk during follow-up is shown along the x-axis.

thrombotic event during follow-up. In MPN-patients, both arterial and venous thrombosis are well-known complications, with the highest rates reported in patients with PV. [12-14] Once affected by thrombosis, cases remain at high risk for developing new thrombotic events. [15-16] A recent study reported a rate of recurrent thrombosis among PV and ET patients of 33%, similar to that encountered in this study. [17] Extension of thrombosis into the splenic vein or superior mesenteric vein occurred relatively frequent in our cohort of patients with PVT. This is in line with earlier studies describing that PVT-patients with underlying prothrombotic conditions, such as a MPN, may be at particular risk of extension of thrombosis. [18-19] In addition to increasing morbidity during follow-up, new thrombotic events were also related to mortality. In our cohort, 18% of death causes were related to a thrombotic event and 47% of deaths were caused by end-stage MPN or AML. Mortality in patients with PVT and MPN thus appears to be primarily related to the underlying MPN and not to complications of portal hypertension. This has not been recognized previously and would support close monitoring of the haematological disease and active collaboration between hepatologists and hematologists in the care of these patients.

Although variceal bleeding is a common complication in patients with PVT [5, 20], our results suggests that it is currently well managed. No patients died as a result of variceal bleeding even though 39% of the patients experienced at least one episode of bleeding. Overall, we could not detect an association between long-term use of anticoagulation and the risk of variceal bleeding. This is in line with a previous report, which showed that treatment with anticoagulation did not increase the risk or severity of bleeding. [19] In contrast to another study, we did not find a relationship between variceal bleeding during follow-up and first presentation with an episode of bleeding. [21] Considering the high incidence of bleeding, its associated morbidity and the lack of good predictors of bleeding, regular endoscopic examinations and adequate preventive measures appear justified.

This study confirms that PVT is often the presenting symptom of an underlying MPN, highlighting the need for thorough screening for this disease. Because peripheral blood cell counts often remain in normal ranges due to hemodilution, iron deficiency or splenomegaly, routine blood cell count testing does not suffice to rule out MPN in these patients. [10] Identification of the $JAK2^{V617F}$ mutation, present in nearly all patients with PV and approximately 50% of the cases with ET or IMF [22], has significantly contributed to the detection of MPNs. [23-24] Testing for this mutation has now become an important part of the work-up of patients presenting with PVT in the absence of cirrhosis or hepatobiliary malignancies. [25-26] Still, a bone marrow biopsy is often also required as absence of the JAK2 mutation does not exclude the presence of a MPN. In patients diagnosed with PVT in the early part of our study, a complete etiological screening was not always performed. Because diagnosis of PVT dated as far back as 1980 and the JAK2 mutation was not discovered until 2005, JAK2 mutation testing was not performed in all patients. Of fifteen untested patients for JAK2, eleven patients either died prior to or in

the year 2005. Therefore, we cannot exclude that in some patients with PVT, especially those diagnosed before 2005, the diagnosis of a (latent) MPN may have been missed.

Another finding is that in approximately half of the patients, there was coexistence of MPN and other prothrombotic factors, underlining the important role of a multifactorial etiology in venous thrombosis. For this reason, even in the presence of an overt MPN, physicians should be aware of the presence of other risk factors for thrombosis and not refrain from thrombophilia screening. It has been suggested that, especially in younger patients with MPN, the risk of recurrent thrombosis is significantly higher when additional thrombophilic factors are present. [17, 27] However, we could not confirm this in the current study, but this could be due to the small number of patients with thrombophilia.

Regarding the optimal treatment of patients with PVT there is much debate, especially concerning anticoagulation therapy. Although controlled studies are not available, treatment with anticoagulation has been shown to increase the rate of portal vein recanalization in patients presenting with acute PVT [9, 28-29], and is therefore recommended in these cases. [30] In chronic PVT the role of anticoagulation is less clear as possible beneficial effects of preventing extension or recurrence of thrombosis are counterbalanced by potential risks of portal hypertension-related bleeding. Similarly, there is no clear consensus on the optimal strategy for the prevention of (recurrent) thrombosis in patients with MPN.

In this study, there was no mortality due to bleeding in patients receiving anticoagulant therapy. Adversely, three patients experienced a new thrombotic event after previous anticoagulation therapy was discontinued, which supports the use of anticoagulation in patients with PVT and MPN. Still, in three other cases thrombosis recurred despite treatment with VKA. Overall, we did not detect a significant effect on survival when we compared patients treated with VKA to those that were not. From these results it is therefore not possible to conclude whether or not long-term anticoagulation in these patients is indicated. Nevertheless, two earlier studies in patients with PVT, with or without MPN, showed that treatment with anticoagulation was associated with a lower rate of recurrent thrombosis. [19, 21]

However, for patients with MPN, treatment options do not only include oral anticoagulants but also antiplatelet drugs, cytoreductive therapy or combinations. Recently, a large retrospective study of MPN-patients with PV or ET reported that both treatment with oral anticoagulation and treatment with antiplatelet drugs were equally effective in preventing recurrence in cases with a first venous thrombosis. [17] Although the number of patients in our study is somewhat limited, there was no recurrent thrombosis in patients treated with antiplatelet drugs. In contrast, a new thrombotic event occurred in 12 of 32 cases not receiving long-term aspirin therapy. This suggests that patients with PVT and MPN may benefit from long-term treatment with antiplatelet drugs. Obviously, these findings require confirmation, if possible, in prospective studies comparing the outcome of long-term treatment with VKA to short-term anticoagulation followed by treatment with antiplatelet agents, both in terms of

bleeding risk and prevention of recurrent thrombosis. Until general treatment recommendations become available, awareness of the high rate of thrombosis recurrence in PVT-patients with MPN appears crucial and treatment for the underlying MPN should probably be actively pursued.

Although our study provides several important insights, it has some limitations that deserve comment. First, although this is the largest cohort of patients with PVT and MPN described until now, the sample size is still relatively small and data was obtained retrospectively. Another potential limitation of this study is that the observation period ranged from 1980 to 2008. Over this time period, the clinical management of patients with PVT and MPN has changed. For example, the awareness of screening for the presence of a MPN in patients with PVT has increased over the past years, especially since the discovery of the JAK2 mutation. Moreover, treatment for MPN has also changed over time. Aspirin has become a standard of treatment after landmark trials showed a reduction of thrombotic events in patients treated with aspirin. [31] Nevertheless, we did not detect a significant difference in survival when we compared patients diagnosed before 2000 to those diagnosed thereafter (data not shown). Despite these limitations, we believe that our findings contribute to the further understanding of this rare combination of disorders, especially since large, controlled studies are lacking and hardly feasible.

In conclusion, this study shows that PVT is often the presenting symptom of an underlying MPN, underlining that the presence of a MPN should always be thoroughly investigated in these patients. In addition to a high incidence of portal hypertension-related variceal bleeding, recurrent thrombosis is also a common and severe complication in patients with PVT and MPN. Treatment with antiplatelet drugs may prove to be important in the prevention of additional thrombotic events. Mortality in these patients is primarily related to the underlying MPN and not to complications of portal hypertension.

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Chapter 8

PREGNANCY IN WOMEN WITH PORTAL VEIN THROMBOSIS: RESULTS OF A MULTICENTRIC EUROPEAN STUDY ON MATERNAL AND FETAL MANAGEMENT AND OUTCOME

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ABSTRACT

Although many patients with primary portal vein thrombosis (PVT) are females of childbearing age, data on pregnancy in this patient group are scarce. Therefore, the aim of this study was to assess maternal and fetal outcome of pregnancies in women with known PVT. A retrospective European multicenter analysis was performed of pregnancies in women with non-cirrhotic PVT seen between 1986 and 2010. All pregnancies occurred 6 months or more after PVT-diagnosis. In total 24 women were included, that had 45 pregnancies, with a median age at first pregnancy of 30 years (range 17-45 years). Median duration between diagnosis of PVT and pregnancy was 54 months (range 7-370 months). Sixteen women had esophageal varices, which had ruptured prior to pregnancy in ten cases. Low molecular weight heparin (LWMH) was given during 28 of 45 pregnancies (62%). Median duration of gestation was 38 weeks (range 8-41 weeks). Nine pregnancies (20%) were lost before week 20. There were 3 very preterm births at week 26-27, 7 moderately preterm births (at 32-36 weeks) and 26 term births (after week 37). Two mothers had HELLP syndrome at week 24 and 33, respectively. In 67% of pregnancies, outcome was favorable, with live birth after 32 weeks of gestation and no serious obstetrical complications. There were 3 bleeding complications after 18 caesarean sections, and 1 case of bleeding after 18 vaginal deliveries. Three esophageal variceal bleedings occurred, in the absence of primary prophylaxis, at week 3, 18 and 38 respectively. Two thrombotic events were observed during pregnancy or post-partum. There were no maternal deaths.

Conclusions. In chronic PVT patients that become pregnant, there is a relatively high rate of miscarriages. However, in pregnancies that are not lost before 20 weeks of gestation, fetal outcome is generally favorable and maternal outcome is good. Variceal bleeding and thrombotic events are uncommon but may complicate pregnancies or the postpartum period in women with PVT.

INTRODUCTION

Portal vein thrombosis (PVT), in the absence of cirrhosis or hepatobiliary tumors, is a rare disorder characterized by the development of a thrombus in the portal vein or its left or right branches, leading to portal hypertension. [1] Extension to mesenteric venous arches carries a high risk of intestinal infarction, with has a reported mortality of up to 50%. [2] Without recanalization, a network of collateral veins develops (portal cavernoma), associated with a permanent risk of potentially fatal gastrointestinal bleeding, recurrent thrombosis, or biliary obstruction. Numerous risk factors for venous thromboembolism, including several thrombophilias have been implicated as risk factors for PVT. [3]

The prognosis of PVT presenting during pregnancy or post partum is unclear, due to the small number of reported cases. [4-14] Until now, pregnancy was often contraindicated in patients with PVT and portal hypertension. [15] In parallel, the outcome of PVT has improved over the past years, with the use of portal hypertension prophylaxis and anticoagulation. [16-18] The wish to become pregnant may become a major issue for young women with established and well-controlled PVT. However, from the limited number of available reports, it is unclear whether pregnancy should be contraindicated in patients with PVT.

In the current study we have assessed the manifestations, the associated thrombotic risk factors, and the fetal and maternal outcome in women that became pregnant during follow-up of recognized and treated PVT.

PATIENTS AND METHODS

Definitions

Date of diagnosis of PVT was considered as the date of the first imaging procedure demonstrating thrombosis of the portal vein. Patients with cirrhosis, hepatobiliary malignancy, liver transplantation or concomitant Budd-Chiari syndrome were excluded from the study. Regarding the duration of pregnancy, very preterm birth was defined as live birth before 32 completed weeks of gestation, moderately preterm birth as live birth between 32 to 36 completed weeks of gestation, and term birth as live birth at 37 or more completed weeks of gestation. Miscarriages were defined as a termination of pregnancy before 20 weeks of gestation. [19]

In order to identify factors influencing the outcome of pregnancy, we identified two types of outcome, as previously described for Budd-Chiari syndrome [20]: (a) pregnancies with relatively favorable outcome, when live birth occurred at 32 or more completed weeks of gestation, with a healthy infant and no serious obstetrical complication (intrahepatic cholestasis was not considered as a serious complication); and (b) pregnancies with a poor outcome, otherwise.

Study group

The files of female patients aged below 45 years at time of PVT diagnosis were retrospectively analyzed at one of three referral centers (Hôpital Beaujon, Clichy, France; Erasmus University Medical Center, Rotterdam, The Netherlands; Hospital Clinic, Barcelona, Spain). All women that became pregnant between 1986 and 2010 after at least 6 months of follow-up for PVT were included in the study (16 pregnancies in 10 patients from France, 14 pregnancies in 7 patients from The Netherlands, and 15 pregnancies in 7 patients from Spain). This study conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institutional review board Bichat-Claude-Bernard (Paris, France).

Work-up and management

For all patients a standardized case record form was completed using data from the medical charts. Specifically, information was collected on disease course and treatment of PVT before pregnancy, underlying etiology, number and outcome of pregnancies and treatment with anticoagulation. The following prothrombotic factors were regarded as risk factors for PVT, when tested according to previously reported methods [3, 18]: primary myeloproliferative neoplasms, factor V Leiden mutation (R506Q), prothrombin gene mutation (G20210A), deficiencies of protein C, protein S, or antithrombin, and anti-phospholipid antibodies. Deficiencies of antithrombin, protein C and protein S were only considered primary in the absence of hepatocellular insufficiency and when testing for these deficiencies was performed either before pregnancy or at least 6 weeks after the end of pregnancy. Oral contraceptive use was considered as a thrombotic risk factor when used within the three months preceding diagnosis of PVT.

Information on management of patients included treatment with oral anticoagulation, and prophylaxis and treatment of complications of portal hypertension, as recently recommended. [1, 21] In patients with thrombophilia or mesenteric vein thrombosis guidelines suggest that anticoagulation is given lifelong when there are no other contraindications.

Statistical analysis

Continuous variables were expressed as median with range, and categorical variables as absolute and relative frequencies. Comparisons between groups of continuous and categorical variables were performed using, respectively, the Mann-Whitney U test and the Chi-square or Fisher exact test. All tests were two-sided and used a significance level of 0.05. Data handling and analysis were performed with the Statistical Package for Social Sciences version 16.0 (SPSS Inc., Chicago, IL).

RESULTS

Twenty-four women, with a median age at first pregnancy of 30 years (range 17-45 years), became pregnant during the follow-up for PVT, with a total of 45 pregnancies. (Table 1) Median time period from diagnosis of PVT to first conception was 28 months (range 7-344 months). Twenty-two women (92%) had a complete obstruction of the main portal vein, including 8 patients (33%) with additional mesenteric vein (MV) thrombosis. The remaining two patients had thrombosis of the right portal vein branch, including one woman with additional MV obstruction. Regarding the etiology of PVT, four women had a myeloproliferative neoplasm (MPN), seven cases had either protein S or C deficiency, three were diagnosed with antiphospholipid syndrome and in one case each factor V Leiden en prothrombin gene mutation were identified.

Table 1. Characteristics before pregnancy of 24 female patients with portal vein thrombosis

	N (%)
Age at diagnosis (yrs)	26 (0-42) [§]
Age at conception (yrs)	30 (17-45) [§]
Extension of thrombosis	
main portal vein	22 (92)
right or left portal branch	14 (58)
mesenteric vein	9 (38)
splenic vein	3 (13)
Symptoms	
esophageal varices	16 (67)
variceal bleeding	10 (42)
splenomegaly	11 (46)
ascites	4 (17)
portosystemic collaterals	17 (71)
Etiology	
myeloproliferative neoplasm	4 (17)
factor V leiden / factor II mutation	2
antiphospholipid syndrome	3 (13)
protein S deficiency	6 (26)
protein C deficiency	1 (4)
oral contraceptive use	8 (33)
Anticoagulation before pregnancy	11 (46)
Surgical portosystemic shunt	2 (1)

§ Values expressed as median (range).

Anticoagulation therapy

Before pregnancy, treatment with anticoagulation (mostly vitamin K antagonists) was initiated in 11 patients (42%) after PVT was diagnosed. When pregnancy occurred, treatment with vitamin K antagonists (VKA) was stopped in all cases. In another 4 patients (17%), anticoagulation therapy with VKA was first started after the end of pregnancy.

Out of the 45 pregnancies after PVT-diagnosis, low molecular weight heparin (LMWH) was administered before 5 weeks of gestation in 17 pregnancies and after week 6 during 11 pregnancies (median delay of 14 weeks after conception). In the other 17 pregnancies (38%) no treatment with LMWH was given.

Gestational course and perinatal complications

All 24 women with PVT that eventually became pregnant had compensated liver disease at the time of conception. Sixteen patients (67%) had evidence of esophageal varices, which had ruptured and caused bleeding in ten cases prior to pregnancy. Eleven patients (46%) had evidence of splenomegaly and ascites was present in 4 patients (17%). One woman had moderate ascites, the other three cases had only minimal ascites. In two patients a surgical portosytemic shunt had been created before pregnancy. One patient had underwent cavernoma to caval vein derivation, and one a splenorenal shunt, 8 years and 1 month before conception, respectively

A total of 7 women had one or more pregnancies before diagnosis of PVT, of whom two cases had experienced miscarriages. In one patient, PVT developed during her first pregnancy. Of all 45 pregnancies occurring after PVT-diagnosis, the median duration of gestation was 38 weeks (range 8-41 weeks). As shown in Table 2, miscarriages occurred in 9 pregnancies (20%). Out of 36 pregnancies reaching 20 weeks of gestation, term birth, moderately preterm and very preterm birth occurred in 26, 7, and 3 pregnancies, respectively. Complications that occurred during pregnancy were intrahepatic cholestasis in one patient, preeclampsia and HELLP in two patients at 24 and 33 weeks of gestation, respectively, and placenta praevia in one patient.

Table 2. Gestational course and perinatal complications in 45 pregnancies occurring during the follow-up of women with portal vein thrombosis

Nº of weeks of gestation	Nº of pregnancies	Mode of delivery	Infant condition
≥ 37	26	caesarean: 12 vaginal: 14	all healthy
32-36	7	caesarean: 4 vaginal: 3	1 infant with cardiac malformation (wk36)
20-31	3	caesarean: 3	1 infant with hyaline membrane disease and ischemic colitis (wk 26) 2 infants with no complications apart from hypotrophy and prematurity (wk 26&27)
< 20	9	vaginal: 8	

Considering only the 33 term and moderately preterm deliveries (≥ 32 weeks of gestation), delivery was vaginal in 17 cases (52%) and a caesarian section was performed in the other 16 cases. Of these 33 pregnancies, one infant born at 36 weeks of gestation underwent emergency surgery for a cardiac malformation. In another case, an emergency caesarean

section was performed due to fetal suffering at 38 weeks. Infant outcome was favorable but ultrasound and placenta analyses later showed a diminished outflow of the placental veins. Of the three very preterm deliveries, all caesarean sections, one infant born at 24 weeks of pregnancy had hyaline membrane disease and necrotic colitis, both with favorable outcome. All other infants were healthy and had a good outcome. Median birth weight was 2915 grams (range 2270-3930 grams).

According to the above described definitions, pregnancy outcome was classified as good in 30 instances (67%), and as unfavorable in 15 cases (33%). During the postpartum period, 4 women developed bleeding complications, including intraperitoneal bleeding after caesarian section in 3 cases and genital bleeding after delivery in 1 case.

Impact of pregnancies on PVT and outcome

During pregnancy, three women experienced an episode of esophageal variceal bleeding, at 5, 20 and 38 weeks of gestation, respectively, all in the absence of primary prophylaxis. In the first patient, pregnancy was diagnosed during hospitalization for variceal bleeding. She was treated with sclerotherapy and during the remainder of the pregnancy no further complications occurred. The second patient was treated successfully with endoscopic band ligation and until delivery at 41 weeks of gestation, no further events occurred. In the third patient, variceal bleeding was treated with both band ligation and sclerotherapy. This patient subsequently underwent an emergency caesarian section. Additionally, two other patients also underwent endoscopic treatment of esophageal varices during pregnancy but did not experience an episode of bleeding. Treatment with beta-blockers was given during 14 pregnancies (31%). In three women, moderate ascites developed during pregnancy, all without further complications. During one pregnancy, there was a suspicion for a transient ischemic attack (TIA). In another patient, post-partum splenic infarction occurred, in the absence of anticoagulation therapy. No other thrombotic events occurred during pregnancy or the postpartum period. There were no maternal deaths and all women were doing well after a median follow-up of 27 months (range 0.2-243) after their last delivery.

When maternal complications such as post-partum bleeding, variceal bleeding during pregnancy and thrombotic events were also included in the definition, 23 pregnancies (51%) were considered as having a good outcome without any complications. Table 3 shows the difference in several maternal and therapeutic factors between pregnancies with an uncomplicated course and pregnancies with complications or an unfavorable outcome. There were no significant differences between both groups.

Table 3. Comparison between uncomplicated pregnancies and pregnancies with complications or an otherwise unfavorable outcome

	Uncomplicated pregnancy (n=23)	Complication or unfavorable outcome (n=22)	p
Age at conception	31 (20-45) §	32 (17-42) §	0.838
Thrombophilia	11 (48)	8 (36)	0.436
Myeloproliferative neoplasm	3 (13)	5 (25)	0.440
History abortion	8 (35)	5 (23)	0.372
Extended thrombosis	13 (57)	10 (47)	0.458
Presence of varices	14 (61)	18 (82)	0.121
Ascites at diagnosis	2 (9)	5 (23)	0.243
LMWH during pregnancy	16 (70)	12 (55)	0.299
Use of betablockers	5 (22)	9 (41)	0.165
Conception before 2000	6 (26)	8 (36)	0.457

§ Values expressed as median (range).

DISCUSSION

Portal vein thrombosis (PVT) frequently affects young women of childbearing age. Pregnancy has for long been thought to be a relative contraindication in these patients as little was known on thrombotic recurrence and bleeding risk in this context. Available literature data is minimal and only based on a few case reports. [4-14] This is the largest study until now reporting on pregnancy outcome in women with PVT.

The incidence of pregnancy-related venous thromboembolism ranges from 0.71 to 1.75 per 1000 deliveries. In a large American study aiming to estimate the incidence, risk factors and mortality of pregnancy-related venous thromboembolism, the incidence was 1.72 per 1000 deliveries with 1.1 deaths per 100,000. [22] Medical conditions associated with a significantly increased risk of venous thromboembolism during pregnancy included thrombophilia, (OR 51.8), a history of thrombosis (OR 24.8), and the antiphospholipid syndrome (OR 15.8). [22] In our series of PVT-patients, more than half of the women had evidence of thrombophilia, and in 13% an antiphospholipid syndrome was present. Thrombotic complications occurring in our patients were HELLP syndrome in 2 patients with visible placental infarcts on placenta examination, one case of a suspected TIA and post-partum splenic vein thrombosis in another patient. In three of these cases, thrombotic complications occurred in insufficiently anticoagulated patients.

In addition to the risk of extension of thrombosis in the splanchnic vessels, patients with PVT may develop complications of portal hypertension. Little is known about the impact of pregnancy on the occurrence or progression of these complications. In previous reports, variceal bleeding seemed to be particularly frequent, occurring in approximately 15% of pregnancies in patients with non-cirrhotic portal hypertension. [23-24] In our survey, portal hypertensive complications were well controlled: variceal bleeding occurred during 3 pregnancies (7%) and moderate ascites also developed during 3 pregnancies (7%).

Another important issue to consider is bleeding complications, as patients with PVT are often treated with anticoagulation. Out of 33 pregnancies reaching 20 weeks of gestation, bleeding occurred in 3 of 16 women that underwent caesarean sections versus only 1 of 17 vaginal deliveries. As caesarean section is otherwise known to carry a substantially increased risk of thromboembolic disease and may be hazardous in patients with portal hypertension, it seems safe to recommend vaginal delivery whenever possible, caesarean being restricted to obstetric indications. [25]

Concerning fetal outcome, miscarriage was found to be common, complicating 20% of pregnancies. This rate appears higher than the frequency of miscarriage in a healthy female population, estimated to be around 10-15%. [26] However, this may be primarily related to the large number of women with underlying thrombophilia. Preterm delivery occurred in 21% of pregnancies reaching 20 weeks of gestation. Still, only one infant, born at 26 weeks of gestation, suffered severe complications of prematurity, but eventually with favorable outcome. Another fetal complication was a cardiac malformation in one infant, which was not related to the mother's disease nor to thrombosis during pregnancy, according to cardiologist expertise. After emergency surgery, outcome was also favorable in this infant.

Although this is the largest reported series of pregnancies in patients with PVT, due to the retrospective nature of the study, management and follow-up was not uniform and based on each treating physician's decisions. The outcome and timing of specific treatment and interventions should therefore be interpreted with caution. Still, our results clearly show a favorable outcome in this patient group with only a limited number of fetal and maternal complications in pregnancies that progress to 20 weeks of gestation.

As both bleeding and thrombosis can occur in patients with PVT, the role of anticoagulation remains somewhat controversial. Given the placental passage of coumarin derivatives and the associated teratogenic risk and fetal loss, these drugs should not be given during pregnancy. Instead, in women treated with anticoagulation, it is advised that vitamin K antagonists are substituted by LMWH when patients express their desire for pregnancy, or as soon as pregnancy is diagnosed otherwise. After delivery, LMWH can again be replaced by vitamin K antagonists. [27] Due to the scarcity of data there are no specific guidelines for the management of pregnancy occurring in patients with PVT. Based on the results of this study it is not possible to obtain a definite answer as to whether or not patients with PVT should be treated with anticoagulation during pregnancy. A good outcome was seen both in women with and without anticoagulation treatment during pregnancy. Still, in patients with proven thrombophilia or extensive thrombosis anticoagulation during pregnancy should probably be recommended. [21] Moreover, to minimize the risk of variceal bleeding during pregnancy, careful screening for the presence of esophageal varices is warranted and, whenever possible, eradication of large varices should be performed before pregnancy. More studies are needed to elucidate the potential role of bleeding prophylaxis with beta-blockers during

pregnancy in these patients. Given the rarity of this disorder, the potential risks associated with pregnancy and the scarcity of available data, management of pregnancy in women with PVT obviously requires a multidisciplinary approach with close cooperation between hepatologists, hematologists and obstetricians.

In conclusion, in female patients with PVT, pregnancy is possible with controllable complications, and generally favorable fetal and maternal outcome. Still, the frequency of miscarriage seems somewhat higher than in the general population. Variceal bleeding and thrombotic events may occur during pregnancy or post-partum but appear uncommon. Pre-partum screening and management of portal hypertensive complications, careful anticoagulation monitoring with brief interruption around delivery and close surveillance of HELLP syndrome symptoms may further reduce the rate of bleeding and thrombotic complications in these patients.

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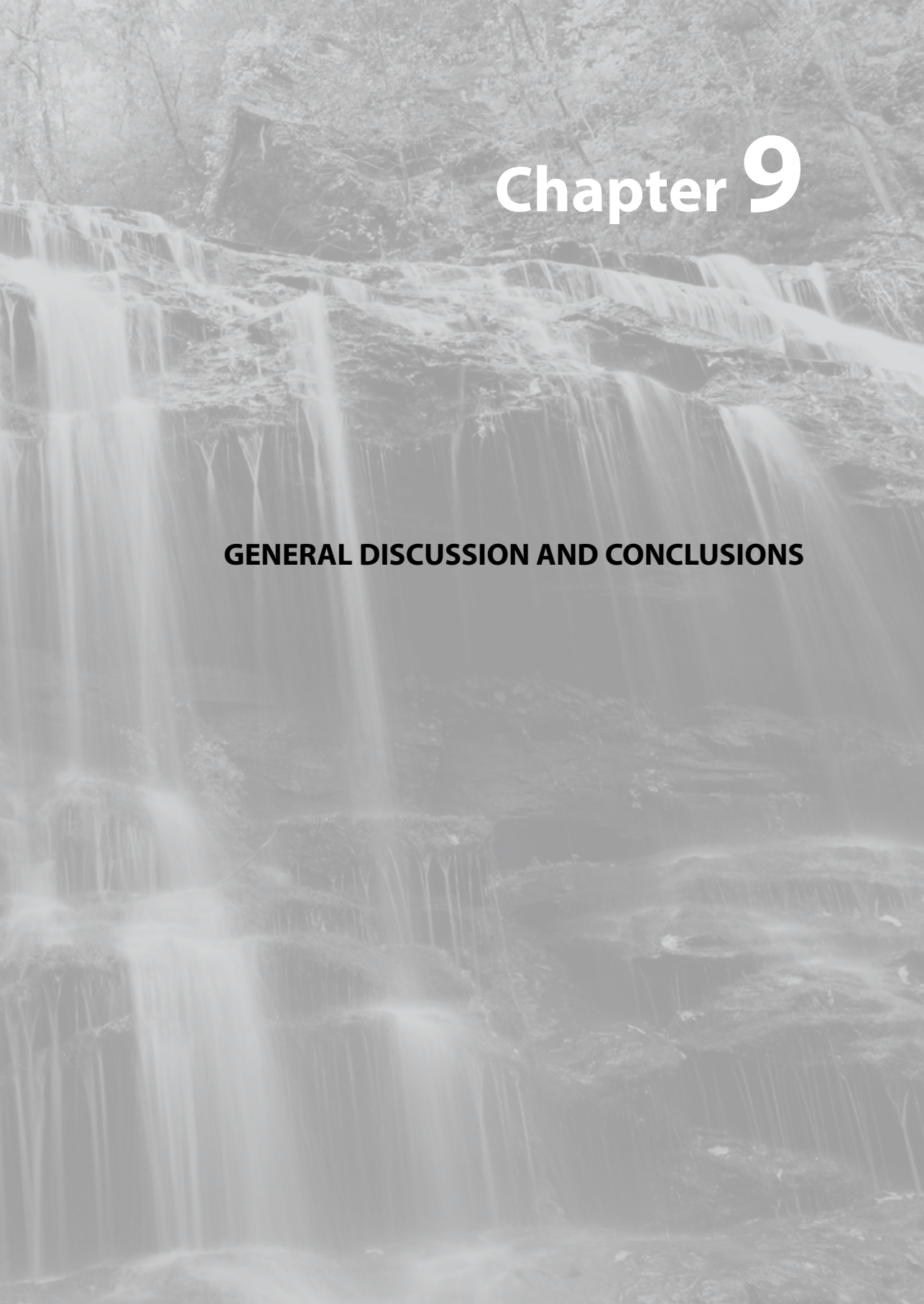

Chapter 9

GENERAL DISCUSSION AND CONCLUSIONS

Budd-Chiari syndrome (BCS) and non-cirrhotic portal vein thrombosis (PVT) are both considered vascular disorders of the liver. Although rare, both diseases can lead to significant morbidity and mortality, especially when diagnosis is delayed, work-up is incomplete or disease-specific treatment is not initiated. Most current evidence on diagnosis and management of BCS and PVT stems from case studies, retrospective patient series and several prospective cohort studies. In recent years, much knowledge has been gained regarding treatment strategies and the underlying risk factors for thrombosis in patients with BCS or non-cirrhotic PVT. Nevertheless, there are still many patients in whom the etiology of thrombosis remains unclear. Moreover, many aspects of therapy, such as the role and duration of anticoagulation in PVT, have not been fully elucidated.

This thesis aims to provide further insights into the etiology and disease course of patients with BCS and patients with PVT. The preceding chapters present a selection of studies focusing on differences in risk factors underlying BCS or PVT, potential, previously unknown prothrombotic factors in BCS and the long-term clinical outcome of patients with non-cirrhotic PVT. Most studies are based on patient data from the EN-Vie study, a European cohort study of patients with BCS and PVT, and data from a retrospective cohort of Dutch patients with PVT. In the EN-Vie study (European Network for Vascular Disorders of the Liver), newly diagnosed patients with BCS or PVT were included from nine different countries and extensive data was collected on clinical presentation, etiology and events during follow-up.

SITE-SPECIFIC THROMBOSIS

To identify factors that may determine the site-specificity of thrombosis in either BCS or non-cirrhotic PVT, we evaluated the distribution of etiologic factors in the two patient cohorts from the EN-Vie study. In Chapter 2 we show that the profile of underlying risk factors for thrombosis clearly differs between patients with BCS and patients with PVT. The results of this study confirm that the Factor V Leiden (FVL) mutation is significantly associated with BCS, whereas the FII (prothrombin) mutation does not appear to be related to the presence of BCS. Remarkably, the opposite is true for PVT, where the G20210A mutation in the prothrombin gene but not the FVL mutation poses a greatly increased risk of thrombosis. Several studies have suggested that the prothrombin gene mutation, in contrast to the FVL mutation, is associated with an increased risk of ischemic stroke, coronary heart disease or peripheral arterial disease. [1-3] However, these findings were not confirmed in a large meta-analysis studying the role of prothrombotic mutations in arterial thrombosis. [4] Given that both FVL and FII mutation induce hypercoagulability in a similar way, it could be hypothesized that other, vascular bed-specific factors are responsible for the susceptibility to thrombosis in either the hepatic or portal veins, but this remains to be elucidated.

Another interesting finding in this study was that the type of myeloproliferative neoplasm (MPN) seemed to differ between the two patient groups. While polycythemia vera seemed to be the major underlying form in BCS-cases, essential thrombocythemia was more common in PVT. Involvement of the microcirculatory system is a common manifestation in patients with essential thrombocythemia that may manifest as erythromelalgia, transient ischemic attacks, headache or visual and hearing defects. These symptoms arise as a result of platelet thrombi that form in small vessels of the end-arterial circulation and typically occur in patients with thrombocytosis. [5-6] Along these lines, one could speculate that essential thrombocythemia may predispose to PVT through platelet-mediated microvascular thrombosis of small portal venules, ultimately leading to overt thrombosis of the portal vein.

Although there remains a clear overlap in the spectrum of etiologic factors associated with thrombosis in BCS and PVT, our findings that some factors appear related to thrombosis at a certain location, support the idea of vascular bed-specific homeostasis.

ETIOLOGY OF BUDD-CHIARI SYNDROME

Hypofibrinolysis and thrombosis

In patients with deep venous thrombosis it has recently been shown that an impaired plasma fibrinolytic potential is a risk factor for thrombosis. [7-8] To investigate whether hypofibrinolysis could be a previously unknown risk factor for BCS we performed a case-control study. For this study plasma samples were used of BCS-patients from the EN-Vie study and a cohort of healthy, sex- and age-matched controls. The results in Chapter 3 show that an impaired fibrinolytic potential can potentially contribute to the development of BCS. Levels of individual components of the fibrinolytic pathway were clearly altered in patients with BCS as compared to the group of healthy controls. In patients, plasma concentrations of plasminogen activator inhibitor-1 (PAI-1) activity were significantly higher than in controls. As a measure of overall plasma fibrinolytic potential an in vitro plasma clot lysis assay was performed. Although median clot lysis times (CLTs) were not significantly different between BCS-patients and controls, a subset of patients had a clearly prolonged CLT. Further characterization of these patients that had signs of hypofibrinolysis showed that they had significantly higher plasma levels of PAI-1. Thrombin activatable fibrinolysis inhibitor (TAFI) did not seem to contribute to the observed hypofibrinolysis as TAFI levels in plasma of patients with BCS were actually lower than in controls. Activity levels of plasmin inhibitor were also decreased in the cases with BCS.

In the patient group studied, there were no major correlations between several measures of liver function and the plasma levels of different factors involved in fibrinolysis. Therefore, the observed differences in plasma fibrinolytic parameters between BCS-patients and healthy controls cannot be explained by disturbances in liver synthetic function alone. Some studies

have suggested that fibrinolysis may be impaired in patients with an underlying MPN, such as polycythemia vera or essential thrombocytosis. [9-10] Although evidence is scarce, one study has also shown that levels of plasmin inhibitor were significantly decreased in patients with chronic MPN. [11] The low levels of TAFI and plasmin inhibitor found in this study may, in part, have been influenced by the presence of an underlying MPN.

Based on the results from this study, hypofibrinolysis may prove to be a previously unknown risk factor for the development of BCS.

Role of apolipoprotein A1

To further explore potential new factors that may be associated with venous thrombosis in BCS, a proteomic approach was applied in Chapter 4 to investigate differences in proteins that are bound to a plasma clot. Using plasma samples from patients with BCS and healthy controls, our study showed that there are significant differences in the protein composition of in vitro formed plasma clots. With 2-dimensional difference gel electrophoresis (2D-DIGE) as a detection technique [12], 26 protein spots were identified that differed in abundance between BCS-patients and controls. Apolipoprotein A1 (apo A1) was detected using mass spectrometry as the protein with the most significant decrease in abundance between patients and controls. In plasma samples from the EN-Vie study apo A1 levels were also significantly decreased in BCS-patients as compared to controls, confirming this finding.

Apo A1 is the major structural protein component of HDL. Accordingly, levels of HDL in plasma of patients with BCS were also found to be significantly lower than in healthy, matched controls. Because apo A1 is synthesized by the liver, it is difficult to distinguish whether low apo A1 levels are a cause or a result of liver disease. However, there was no major correlation between apo A1 and liver function tests, BCS-patients with normal albumin levels also displayed lower apo A1 levels and the duration of symptoms did not appear to affect apo A1 levels. Therefore, it is unlikely that our findings can be attributed solely to an impaired liver synthetic function. Hence, the results from this study provide the first evidence that apo A1 may be involved in the development of venous thrombosis in BCS.

Obviously, these findings need confirmation by other studies and further research is required to examine the mechanism(s) that link low levels of apo A1 to venous thrombosis and, specifically, the development of BCS. Both plasma levels of HDL and apo A1 are known to be inversely related with the risk of arterial thrombosis. [13-14] Recently, several studies have suggested that the same inverse association may exist for venous thrombosis. [15-16] This is in line with the view that arterial and venous thrombosis, although often considered as separate disease entities, do share certain common risk factors. [17] HDL, and also apo A1, have diverse biological functions, some of which could potentially exhibit protective effects against the development of venous thrombosis. Among others, HDL has been shown to reduce platelet aggregation and promote protein C mediated anticoagulant effects. [18-19] It has also been

demonstrated that apo A1 can stimulate fibrinolysis. [20] Still, future studies are needed to unravel the exact causative role of apo A1 in BCS, and the possible influence of clot-binding herein.

Paroxysmal nocturnal hemoglobinuria

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare clonal disorder of the bone marrow associated with a markedly increased risk of venous thrombosis which can occur both at usual as well as unusual sites, such as the hepatic veins. [21-22] In Chapter 5 we describe the largest series of consecutive patients with BCS caused by underlying PNH. With data from 163 BCS-patients included in the EN-Vie study, we found that PNH is responsible for approximately 6-8% of all cases of BCS. Because PNH can manifest itself in a variety of clinical scenarios, the disease may be difficult to recognize and therefore all patients presenting with BCS should be tested for PNH. Moreover, the introduction of eculizumab, a monoclonal antibody directed against complement C5, has revolutionized the treatment of PNH. Eculizumab is the first specific therapy that targets the underlying disease process and treatment has been shown to significantly reduce transfusion requirements, improve quality of life and reduce the risk of thrombosis in patients with PNH. [23-24] Given the potential impact of eculizumab therapy, failure to diagnose PNH may thus result in potentially life-saving treatment being withheld.

In patients with BCS, the presence of PNH was associated with a significantly higher rate of additional thrombosis of the portal, superior mesenteric or splenic vein. Furthermore, more than 25% of the patients experienced new thrombotic events. These findings highlight the marked predisposition to thrombosis encountered in patients with PNH. [21, 25] Although the etiology of the hypercoagulable state in PNH is thought to be multifactorial, studies have shown that the risk of thrombosis increases significantly with every 10% increase in PNH clone-size. [26] In this respect, prophylactic use of anticoagulants has been advocated for patients with large PNH-clones (>50% of granulocytes affected). [27] In our study, none of the patients developing BCS received prophylactic treatment with anticoagulation despite the fact that many had a large PNH-clone. Still, treatment with anticoagulation could not prevent the development of new thrombotic events in all patients.

Our results indicate that once BCS is diagnosed, subsequent management and short-term survival are not affected by the presence of underlying PNH. Placement of a transjugular intrahepatic portosystemic shunt (TIPS) could be successfully performed in the majority of cases that required hepatic decompression. Although spontaneous remission of PNH has been reported, to date, the only curative treatment for PNH is stem cell transplantation. Despite the severity of thrombosis associated with BCS, hematopoietic stem cell transplantation appears to be a feasible therapeutic intervention in patients with PNH and BCS.

Advances in therapeutic options for patients with BCS and PNH have most likely contributed to the decreased mortality rates observed over time. [28] Additional improvements in

treatment strategies will hopefully contribute to a further improvement of clinical outcome in these patients.

PORTAL VEIN THROMBOSIS

Anticoagulation in portal vein thrombosis

According to the current consensus, anticoagulation therapy for PVT should be considered in patients with a recent thrombosis or in patients with PVT and an underlying prothrombotic disorder. [29-30] In Chapter 6 we have investigated the role of anticoagulation therapy and studied factors associated with gastrointestinal bleeding, new thrombotic events and survival in patients with non-cirrhotic non-malignant PVT. For this study, data was used from a retrospective cohort of Dutch patients with PVT. In more than 20% of the patients with an underlying prothrombotic disorder a new venous thrombotic event occurred during follow-up, which was significantly associated with an impaired survival. Treatment with anticoagulation tended to prevent new thrombotic events but also significantly increased the risk of gastrointestinal bleeding. As is known from previous studies the risk of gastrointestinal (re) bleeding is mainly determined by the size of esophageal varices and an initial presentation with an episode of gastrointestinal bleeding. [31-33] In addition to these factors, we found that both ascites and treatment with anticoagulation were significant predictors of variceal (re) bleeding in patients with PVT. These findings imply that anticoagulation therapy in patients with PVT should be individualized according to the onset of thrombosis and the presence of a prothrombotic disorder.

Treatment in patients with myeloproliferative neoplasms

To study the natural course of disease in patients with PVT and an underlying MPN a retrospective cohort study was performed. Chapter 7 shows that recurrent thrombosis is a severe and clinically significant complication in patients with MPN. Almost one-third of the patients with PVT experienced an additional thrombotic event during follow-up. It is well-known that patients with MPN, once affected by thrombosis, remain at high risk for developing new thrombotic events. [34-35] In addition to increasing morbidity during follow-up, new thrombotic events were also related to mortality. In our cohort, 18% of death causes were related to a thrombotic event and 47% of deaths were causes by end-stage MPN or acute myeloid leukemia. Mortality in patients with PVT and MPN thus appears to be primarily related to the underlying MPN and not to complications of portal hypertension.

A second finding of our study was that PVT is often the presenting symptom of an underlying MPN, highlighting the need for thorough screening for this disease. Because peripheral

blood cell counts often remain in normal ranges due to hemodilution, iron deficiency or splenomegaly, routine blood cell count testing does not suffice to rule out MPN in these patients. [36]

Regarding the role of anticoagulation therapy, there is no clear consensus on the optimal strategy for the prevention of (recurrent) thrombosis in PVT-patients with MPN. Overall we did not detect a significant effect on survival when we compared patients treated with vitamin K antagonists to those that were not. Nevertheless, for patients with MPN, treatment options do not only include oral anticoagulants but also antiplatelet drugs, cytoreductive therapy or combinations. Aspirin has become a standard of treatment after landmark trials showed a reduction of thrombotic events in patients treated with aspirin. [37] Although the amount of patients in our study was somewhat limited, there was no recurrent thrombosis in patients treated with anti-platelet drugs. Obviously, these findings require confirmation, if possible, in prospective studies comparing, for example, the outcome of long-term treatment with vitamin K antagonists to short-term anticoagulation followed by treatment with antiplatelet agents, both in terms of bleeding risk and prevention of recurrent thrombosis.

Pregnancy in patients with portal vein thrombosis

PVT can affect both children and adults. A significant number of patients with non-cirrhotic PVT are women of childbearing age. In these patients, the desire to become pregnant and questions concerning its risks and possibilities may arise during follow-up of the disease. However, due to the low incidence, little is known about the outcome and optimal management of pregnancy in this specific patient group. To assess the risks and outcome of pregnancy in women with established PVT, we performed a multi-center cohort study. In Chapter 8 we found that in women with well-controlled PVT and compensated liver disease, pregnancy generally has a good outcome for both mother and child with manageable complications. Although the rate of miscarriage appears somewhat higher than encountered in the general population, pregnancy should thus not be considered contraindicated in female patients with PVT. Still, both complications of bleeding and thrombosis were encountered and physicians and patients should be aware of the potential risks associated with pregnancy. Since no controlled studies have been performed, guidelines regarding treatment with anticoagulation during pregnancy are not available. However, given the fact that pregnancy itself is also associated with an increased risk of thrombosis [38], treatment with anticoagulation (low molecular weight heparin) should always be considered, particularly in patients with underlying thrombophilia. [29] To diminish the risk of bleeding, anticoagulation probably needs to be interrupted shortly around the time of delivery. Furthermore, adequate measures should be taken to minimize the risk of bleeding related to portal hypertension. This includes awareness of the presence of gastroesophageal varices and, ideally, prophylactic treatment with endoscopic band ligation or sclerotherapy when large varices are present. Nevertheless,

further studies are required to identify which patients are at the highest risk of developing pregnancy-related complications and to define the optimal role of anticoagulation therapy and treatment to prevent gastrointestinal bleeding.

DIRECTIONS FOR FUTURE RESEARCH

The preceding chapters have focused on different aspects of the etiology and clinical outcome of Budd-Chiari syndrome (BCS) and non-cirrhotic portal vein thrombosis (PVT). When studying rare diseases like these two vascular liver disorders, international cooperation is indispensable. The collaboration of nine European countries in the European Network for Vascular Disorders of the Liver (EN-Vie) has resulted in a unique patient cohort providing valuable clinical information.

Although we have demonstrated that in addition to the coagulation cascade, the fibrinolytic system may also be involved in the etiology of thrombosis in BCS, this should be further explored. More studies are needed to evaluate the exact impact of hypofibrinolysis on thrombosis risk and potential interactions with other established risk factors. Future, long-term follow-up studies will hopefully provide further insight into the interplay between different etiologic factors and the effects of specific risk factors, such as myeloproliferative neoplasms (MPNs), on prognosis and disease course in BCS-patients.

Interestingly, our studies have shown clear differences in the distribution of etiologic factors between patients with BCS and those with non-cirrhotic PVT. This provides evidence for the more general hypothesis that different factors may promote thrombosis in different vascular systems and should prompt further research into the site-specificity of thrombosis.

Patients with PVT, especially those with underlying thrombophilia, are at increased risk of both thrombotic and bleeding complications, and therefore, treatment with anticoagulation may lead to both risks and benefits. Until now, most data on anticoagulation therapy in PVT-patients stems from retrospective cohort studies. Randomized, controlled studies will be needed to fully establish the place of oral anticoagulation in the treatment of PVT. Moreover, for the subgroup of PVT-patients with an underlying MPN the role of aspirin treatment in the prevention of thrombotic events seems a very promising area for further studies.

CONCLUDING REMARKS

Budd-Chiari syndrome (BCS) and portal vein thrombosis (PVT) are usually not the first two things that come to mind when liver disorders are discussed. However, due to their low prevalence and corresponding scarcity of clinical data, these two intriguing vascular liver disorders often form a great challenge for treating physicians. Over the past years, treatment strategies

and prognosis have significantly improved and progress has been made in unraveling the underlying risk factors of these disorders. The studies presented in this thesis contribute to the further understanding of the etiology of thrombosis and effects of anticoagulation in these complex vascular liver disorders. These insights may provide clinicians with tools for further optimizing therapy and clinical management of patients with BCS or non-cirrhotic PVT.

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SUMMARY

SAMENVATTING

SUMMARY

The liver is a highly vascularised organ that plays an important role in various metabolic processes and removal of toxic factors. Blood is supplied to the liver through both an arterial (hepatic artery) and a venous (portal vein) system. Through three liver veins, blood is drained from the liver and flows back to the heart through the inferior vena cava. Vascular liver diseases encompass a group of diseases involving the blood vessels of the liver that cause changes in the hepatic blood flow. Such changes in blood flow to and from the liver can lead to life-threatening complications. (**Chapter 1**)

Budd-Chiari syndrome is a rare vascular liver disorder characterized by an occlusion of the hepatic venous outflow tract. In the majority of cases, the obstruction is caused by thrombosis of the hepatic veins, with or without extension of thrombosis into the inferior vena cava. Budd-Chiari syndrome (BCS) usually presents between the 3rd and 5th decade of life. Previous studies have shown that known risk factors for deep venous thrombosis are also present in patients with BCS. Common risk factors include Factor V Leiden mutation, antiphospholipid antibodies and oral contraceptives. However, myeloproliferative neoplasms are shown to be the main underlying cause of BCS, and are present in approximately half of the patients. Moreover, in many patients more than one prothrombotic factor can be identified, illustrating that the etiology of thrombosis is multifactorial. Despite increasing knowledge regarding the etiology of BCS, there are still patients in whom no underlying factor is found.

In portal vein thrombosis (PVT), another vascular liver disease, there is an occlusion of the portal vein. As a result, congestion of blood occurs leading to an increased blood pressure in veins flowing from other abdominal organs towards the liver, so-called portal hypertension. One of the most feared complications of portal hypertension is bleeding from varices that can develop in the esophagus or stomach. Liver cirrhosis and hepatobiliary tumors are the two main causes of PVT. It has become clear that in the group of patients with PVT without underlying cirrhosis or hepatobiliary malignancies, there are often prothrombotic factors present leading to an increased risk of thrombosis, as in BCS.

The various chapters of this thesis describe studies aimed at further unraveling the etiology of thrombosis and describing the disease course of patients with BCS and patients with non-cirrhotic, non-malignant PVT. An important part of the results is derived from a European collaboration (EN-Vie, European Network for Vascular Disorders of the Liver) in which 9 countries participated. In this study prospective data was collected from patients newly diagnosed with BCS or non-cirrhotic, non-malignant PVT, resulting in two unique patient cohorts.

In **Chapter 2**, data from the EN-Vie database is used to compare the underlying risk factors for thrombosis present in patients with BCS or PVT. The prevalence of several genetic thrombophilic factors is also compared with a group of healthy controls. The results show that there are clear differences in the risk factor profile found in patients with BCS and in

patients with PVT. Factor V Leiden mutation is significantly more common in patients with BCS as compared to healthy controls but does not seem to be a strong risk factor for the development of PVT. Conversely, factor II (prothrombin) mutation is strongly associated with PVT, whereas its prevalence in patients with BCS is not significantly different from the prevalence in healthy controls. Myeloproliferative neoplasms (MPN) are a common cause of both BCS and non-cirrhotic PVT but the distribution of the three main subtypes of MPN appears to differs between these patient groups. Polycythemia vera is the most common MPN-subtype in BCS-patients. In contrast, essential thrombocythemia and idiopathic myelofibrosis are more often found in patients with PVT. These differences in etiologic factors support the hypothesis that there are specific risk factors for the development of thrombosis at distinct sites.

Using stored plasma samples from both patients with BCS from the EN-Vie study and a group of healthy, sex- and age-matched controls, potentially unknown risk factors for thrombosis have been explored. In **Chapter 3** the role of the fibrinolytic system is studied. Several factors involved in fibrinolysis are determined in plasma and as a measure of overall fibrinolytic potential a clot lysis assay is performed. Plasma levels of plasminogen activator inhibitor-1 (PAI-1) are shown to be significantly higher in patients with BCS. A prolonged clot lysis time (CLT), corresponding to hypofibrinolysis, is also more common in BCS-patients as compared to the healthy controls. A decreased fibrinolytic potential thus seems to be a previously unknown risk factor that may contribute to the development of thrombosis in BCS.

Differences in clot binding proteins between patients with BCS and controls are studied with a proteomic approach in **Chapter 4**. Using 2D-DIGE (2-Dimensional Difference Gel Electrophoresis), all clot-bound proteins from an in-vitro formed plasma clot are separated on a gel. The results of this study show that there are significant differences between patients and controls in the composition of proteins bound to a fibrin clot. The amount of clot-bound apolipoprotein A1 (Apo A1) is lower in patients with BCS. The same also applies to the level of Apo A1 in plasma, which is also significantly lower in patients as compared to controls. These data suggest that reduced levels of Apo A1 may play a role in the development of thrombosis in BCS.

In addition to MPN, there is also another hematologic disease associated with BCS, namely paroxysmal nocturnal hemoglobinuria (PNH). This condition is the result of an acquired mutation of hematopoietic stem cells that causes, among others, hemolytic anemia and leads to an increased risk of thrombosis. Interestingly, patients with PNH are at markedly increased risk of developing cerebral or abdominal vein thrombosis, including BCS. **Chapter 5** describes the results of a cohort study focusing on the presentation, disease progression and survival of BCS-patients with PNH and BCS-patients without PNH. The data show that patients with BCS and underlying PNH often have an additional thrombosis of the portal vein, splenic vein or superior mesenteric vein at the time of diagnosis of BCS. During the course of the disease treatment strategies are similar between BCS-patients with and without PNH and short-term

survival is not significantly different. Thus, the presence of PNH does not seem to influence the outcome and short-term prognosis of patients with BCS.

In **Chapter 6**, the disease course of patients with PVT is further examined using data from a cohort of Dutch patients with non-cirrhotic, non-malignant PVT. In a retrospective, long-term follow-up study, the effects of anticoagulation therapy on the occurrence of bleeding, new thrombotic events and survival are studied. The results demonstrate that an additional thrombotic event mainly occurs in PVT-patients with an underlying prothrombotic risk factor. Treatment with anticoagulation appears to reduce the risk of recurrent thrombosis but is also associated with an increased risk of bleeding events. Based on these data, anticoagulation therapy should be initiated with caution in patients with PVT and an individual assessment of underlying etiology and the risk of bleeding seems warranted.

Approximately one-third of the cases of non-cirrhotic, non-malignant PVT is caused by an underlying MPN. The study described in **Chapter 7** focuses on the clinical presentation and outcome of this specific patient group. In the majority of the patients, development of PVT is the first manifestation of an underlying MPN. The occurrence of new thrombotic events is a frequent complication encountered during follow-up in patients with PVT and MPN. In addition to anticoagulation therapy with vitamin K antagonists, treatment with antiplatelet drugs may also be important in the prevention of recurrent thrombosis. Monitoring and treatment of the hematological disorder appears crucial as the prognosis of these patients is mainly determined by the underlying MPN and not by complications of portal hypertension.

Another subgroup among patients with PVT are women of childbearing age. **Chapter 8** describes the outcome of pregnancies in women diagnosed with PVT from three European countries. The results show that the course of pregnancy in these patients is generally favorable, although the rate of miscarriages seems a little higher than that of the normal, healthy population. Still, in pregnancies that are not lost before 20 weeks of gestation, fetal outcome is almost always good. Maternal complications that may occur during or after pregnancy are bleeding from gastroesophageal varices, post-partum hemorrhage and thrombosis. Appropriate prophylaxis of variceal bleeding, close monitoring during the course of pregnancy and puerperium and short-term cessation of anticoagulation around the time of birth seems indicated in these patients to further reduce the risk of complications.

Summarizing, the results presented in this thesis provide further insight into the etiology, the clinical course and the role of anticoagulation in the treatment of patients with BCS or PVT. Expanding the knowledge of these two rare vascular liver contributes to the prevailing medical understanding of these diseases and offers means to optimize treatment and monitoring of these patients.

SAMENVATTING

De lever is een sterk doorbloed orgaan dat een belangrijke rol speelt bij onder andere de stofwisseling en ontgifting van het lichaam. De aanvoer van bloed is tweeledig en vindt plaats door de leverslagader (arterieel systeem) en de poortader (veneus systeem). Via een drietal leveraderen wordt het bloed vanaf de lever weer teruggevoerd naar het hart door de onderste holle ader (vena cava inferior). Vasculaire leverziekten omvatten een groep ziektebeelden waarbij de bloedvaten van de lever zijn aangedaan en er veranderingen in de doorbloeding van de lever optreden. Dergelijke veranderingen in de bloedtoevoer of -afvoer van de lever kunnen leiden tot levensbedreigende complicaties. (**Hoofdstuk 1**)

Het syndroom van Budd-Chiari is een zeldzame aandoening waarbij er een obstructie ontstaat in de afvoerende vaten van de lever. In vrijwel alle gevallen behelst deze obstructie een trombose van de leveraderen, zich soms uitbreidend tot in de onderste holle ader. Budd-Chiari syndroom (BCS) wordt meestal gediagnosticeerd tussen het 20e en 45e levensjaar. Uit eerder onderzoek is gebleken dat diverse risicofactoren voor het ontstaan van een diepe veneuze trombose (c.q. trombosebeen) ook aantoonbaar zijn bij patiënten met BCS. Voorbeelden hiervan zijn Factor V Leiden mutatie, antifosfolipiden-antistoffen en pilgebruik. De belangrijkste onderliggende oorzaak voor BCS blijken echter myeloproliferatieve aandoeningen te zijn, welke aanwezig zijn bij ongeveer de helft van de patiënten. In veel gevallen is zelfs meer dan één risicofactor voor trombose aantoonbaar en is er sprake van een multifactoriële etiologie van trombose. Ondanks voortschrijdende inzichten ten aanzien van de etiologie van BCS zijn er evenwel nog steeds patiënten bij wie geen oorzakelijke factor kan worden aangetoond.

Bij vena portae trombose (VPT), een andere vasculaire leverziekte, is er sprake van een trombose in de poortader. Als gevolg hiervan treedt er stuwing van bloed op richting de overige buikorganen, zogeheten portale hypertensie. Eén van de meeste gevreesde complicaties van portale hypertensie is het optreden van bloedingen uit spataderen in de slokdarm of maag, die kunnen ontstaan door de verhoogde bloeddruk in het portale systeem. Levercirrose en kwaadaardige tumoren van de lever en galwegen zijn de twee belangrijkste oorzaken van VPT. In de groep patiënten met VPT zonder onderliggende cirrose of maligniteit (non-cirrhotische, non-maligne VPT), blijken net als bij BCS vaak afwijkingen in de bloedstolling aanwezig te zijn die leiden tot een verhoogd risico op trombose.

De verschillende hoofdstukken van dit proefschrift beschrijven studies die gericht zijn op het nader in kaart brengen van de etiologie van trombose en het ziekteverloop bij patiënten met BCS en patiënten met non-cirrhotische, non-maligne VPT. Een belangrijk deel van de resultaten komt voort uit een Europees samenwerkingsverband (EN-Vie; European Network for Vascular Disorders of the Liver) waarbij er vanuit 9 landen prospectief patiëntengegevens

zijn verzameld van nieuwe gevallen van BCS en non-cirrhotische, non-maligne VPT, resulterend in een tweetal unieke patiëntencohorten.

In **Hoofdstuk 2** worden gegevens van de EN-Vie database gebruikt om een vergelijking te maken tussen de onderliggende risicofactoren voor trombose bij patiënten met BCS of VPT. De prevalentie van een aantal genetische trombogene factoren wordt eveneens vergeleken met een groep gezonde controles. Uit de resultaten blijkt dat er duidelijke verschillen bestaan in het profiel van onderliggende etiologische factoren tussen patiënten met BCS en patiënten met VPT. Factor V Leiden mutatie komt significant vaker voor bij patiënten met BCS vergeleken met gezonde controles maar lijkt geen sterke risicofactor te zijn voor het ontstaan van VPT. Andersom is factor II (protrombine) mutatie sterk geassocieerd met VPT, terwijl de prevalentie bij BCS-patiënten niet significant verschilt van die bij gezonde controles. Myeloproliferatieve aandoeningen (MPN, 'myeloproliferative neoplasms') zijn een veel voorkomende oorzaak van zowel BCS als VPT, maar de verdeling van de drie belangrijkste subtypen lijkt te verschillen tussen deze patiëntengroepen. Zo is polycythemia vera het meeste voorkomende MPN-type bij BCS-patiënten. Essentiële trombocytose en idiopathische myelofibrose blijken daarentegen vaker voor te komen bij VPT. De beschreven verschillen in etiologische factoren ondersteunen de hypothese dat er mogelijk specifieke risicofactoren zijn voor het ontstaan van trombose op bepaalde locaties in het vaatbed.

Met behulp van opgeslagen plasmamonsters van zowel patiënten met BCS uit de EN-Vie studie als een groep gezonde controles overeenkomend in leeftijd en geslacht, is onderzoek gedaan naar mogelijk nog onbekende risicofactoren voor trombose. In **Hoofdstuk 3** is de rol van het fibrinolytische systeem in kaart gebracht. Hiervoor zijn verschillende factoren die betrokken zijn bij de fibrinolyse gemeten in plasma en als algemene maat voor de fibrinolytische potentie is een zogenaamde 'clot-lysis tijd' (CLT) bepaald. Waarden van het eiwit 'plasminogen activator inhibitor-1' (PAI-1) blijken significant hoger te zijn bij patiënten met BCS. Een verlengde CLT, overeenkomend met hypofibrinolyse, komt daarnaast vaker voor bij de patiënten vergeleken met de gezonde controles. Een verminderde fibrinolytische capaciteit lijkt daarom een vooralsnog onbekende risicofactor te zijn voor het ontstaan van trombose bij patiënten met BCS.

Met behulp van proteomics is in **Hoofdstuk 4** gekeken naar verschillen in stolselbindende eiwitten tussen BCS-patiënten en controles. Door middel van 2D-DIGE (2-Dimensional Difference Gel Electrophoresis) zijn alle stolselgebonden eiwitten op een gel van elkaar gescheiden. De resultaten laten zien dat er significante verschillen zijn tussen patiënten en controles in de samenstelling van eiwitten die gebonden zijn aan een fibrinestolsel. De hoeveelheid stolselgebonden apolipoproteine A1 (Apo A1) is lager bij patiënten met BCS. Ditzelfde blijkt ook te gelden voor de concentratie Apo A1 in plasma. Deze gegevens suggereren dat verlaagde Apo A1-waarden wellicht een rol zouden kunnen spelen bij het ontstaan van trombose bij BCS.

Naast myeloproliferatieve aandoeningen is er ook nog een andere hematologische ziekte die geassocieerd is met BCS, namelijk paroxysmale nachtelijke hemoglobinurie (PNH). Bij dit ziektebeeld is er sprake van een verworven mutatie van hematopoietische stamcellen die onder andere leidt tot hemolytische anemie en een verhoogd risico op trombose. Opvallend is dat patiënten met PNH een sterk verhoogde kans hebben op trombose van bloedvaten in de hersenen of in de buik, waaronder BCS. **Hoofdstuk 5** beschrijft de resultaten van een cohortstudie waarbij de presentatie, het ziekteverloop en de overleving van BCS-patiënten met PNH wordt vergeleken met die van BCS-patiënten zonder PNH. Hieruit komt naar voren dat patiënten met BCS en onderliggend PNH op het moment van diagnose vaker een additionele trombose van de vena portae, de vena lienalis of de vena mesenterica superior hebben. Gedurende het ziekteverloop verschilt de behandeling echter niet evident van die bij BCS-patiënten zonder PNH en zijn er ook geen significante verschillen in de korte-termijns overleving. PNH lijkt daarmee de prognose van patiënten met BCS niet te beïnvloeden.

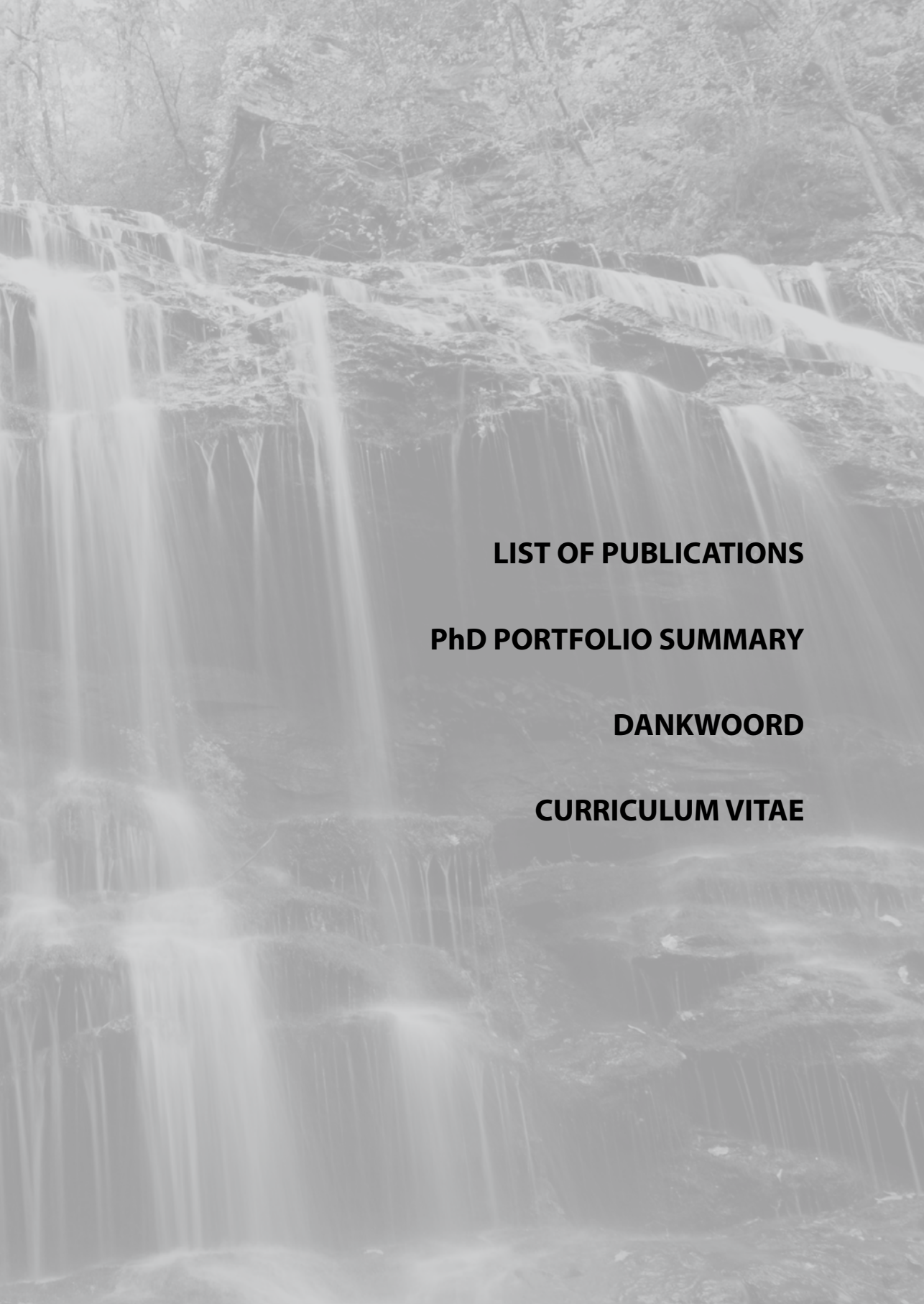
In **Hoofdstuk 6** wordt het ziekteverloop van patiënten met VPT nader bestudeerd met behulp van gegevens van een cohort Nederlandse patiënten met non-cirrhotische, non-maligne VPT. In een retrospectieve, lange-termijns vervolgstudie is gekeken naar de rol van behandeling met antistolling op het optreden van bloedingen, het ontstaan van nieuwe trombose en de overleving. De resultaten tonen aan dat een nieuwe trombose vooral optreedt bij VPT-patiënten met een onderliggende risicofactor voor trombose. Behandeling met antistolling lijkt het risico op het optreden van een nieuwe trombose te verlagen, maar is daarentegen ook geassocieerd met een verhoogde kans op bloedingen. Op basis van deze gegevens is behandeling met antistolling niet zonder meer aangewezen bij patiënten met VPT. Een individuele afweging op basis van onderliggende etiologie en het risico op bloedingen lijkt hierbij van belang.

Bij ongeveer één derde van de gevallen van non-cirrhotische, non-maligne VPT is een MPN aantoonbaar als onderliggende oorzaak. De studie beschreven in **Hoofdstuk 7** gaat specifiek in op het ziekteverloop en de behandeling van deze groep patiënten. Bij de meerderheid van de patiënten blijkt het optreden van VPT de eerste uiting te zijn van een onderliggend MPN. Het optreden van nieuwe trombose is een veel voorkomende complicatie gedurende het ziekteverloop van patiënten met VPT en MPN. Naast behandeling met antistolling is er voor de preventie van trombose mogelijk ook een belangrijke rol weggelegd voor behandeling met aspirine. Aandacht en behandeling van de onderliggende hematologische aandoening is van groot belang aangezien de prognose van patiënten met VPT en MPN met name wordt bepaald door de myeloproliferatieve ziekte en niet zozeer door complicaties van portale hypertensie.

Een andere subgroep binnen de patiënten met VPT zijn jonge vrouwen met een kinderwens. **Hoofdstuk 8** beschrijft de uitkomst van zwangerschappen bij vrouwen met VPT uit drie Europese landen. De resultaten laten zien dat het verloop van zwangerschap bij deze patiënten over het algemeen gunstig is, hoewel het percentage miskramen wel wat hoger

lijkt te liggen dan die van de normale, gezonde populatie. Bij zwangerschappen die echter niet in een vroeg stadium verloren gaan komen vrijwel alle kinderen gezond ter wereld. Complicaties die bij de moeder kunnen optreden gedurende of na de zwangerschap zijn bloedingen uit spataderen in de slokdarm, bloedingen na de bevalling en het optreden van trombose. Derhalve lijken adequate profylaxe voor bloedingen uit spataderen in de slokdarm, intensieve medische begeleiding gedurende en na de zwangerschap en kortdurend staken van antistolling rondom de bevalling geïndiceerd bij deze groep patiënten om het risico op complicaties nog verder te verlagen.

Samenvattend bieden de bovenbeschreven resultaten van dit proefschrift verder inzicht in de etiologie, het klinisch beloop en de rol van antistolling bij de behandeling van patiënten met BCS en VPT. Verbreding van de kennis over deze twee zeldzame vasculaire leverziekten draagt bij aan de heersende medische inzichten en verschaft behandelaars zo de mogelijkheid om de behandeling en begeleiding van deze patiënten te optimaliseren.



LIST OF PUBLICATIONS

PhD PORTFOLIO SUMMARY

DANKWOORD

CURRICULUM VITAE

PUBLICATIONS

1. A. Grefhorst, J. Hoekstra, T.G. Derks, D.M. Ouwens, J.F. Baller, R. Havinga, L.M. Havekes, J.A. Romijn, F. Kuipers. *Acute hepatic steatosis in mice by blocking beta-oxidation does not reduce insulin sensitivity of very-low-density lipoprotein production.* Am J Physiol Gastrointest Liver Physiol 2005 Sep;289(3):G592-8.
2. F.R. Van der Leij, V.W. Bloks, A. Grefhorst, J. Hoekstra, A. Gerding, K. Kooi, F. Gerbens, G. te Meerman, F. Kuipers. *Gene expression profiling in livers of mice after acute inhibition of beta-oxidation.* Genomics 2007 Dec;90(6):680-9.
3. J. Hoekstra, H.L.A. Janssen. *Behandeling van vena portae trombose.* Ned Tijdschr Hematologie 2008; 5(1):23-7.
4. J. Hoekstra, H.L.A. Janssen. *Vascular Liver Disorders (I): Diagnosis, Treatment and Prognosis of Budd-Chiari Syndrome.* Neth J Med 2008;66:334-9.
5. J. Hoekstra, H.L.A. Janssen. *Vascular Liver Disorders (II): Portal Vein Thrombosis.* Neth J Med 2009;67:46-53.
6. J. Hoekstra, F.W.G. Leebeek, A. Plessier, S. Raffa, S. Darwish Murad, J. Heller, A. Hadengue, C. Chagneau, E. Elias, M. Primignani, J.C. García-Pagán, D.C. Valla, H.L.A. Janssen. *Paroxysmal Nocturnal Hemoglobinuria in Budd-Chiari Syndrome: Findings from a Cohort Study.* J Hepatol 2009 Oct;51(4):696-706.
7. J. Hoekstra, A.H.C. Guimaraes, F.W.G. Leebeek, S. Darwish Murad, J.J.M.C. Malfliet, A. Plessier, M. Hernandez-Guerra, P. Langlet, E. Elias, J. Trebicka, M. Primignani, J.C. García-Pagán, D.C. Valla, D.C. Rijken, H.L.A. Janssen. *Impaired Fibrinolysis as a Risk Factor for Budd-Chiari Syndrome.* Blood 2010 Jan;115(2):388-95.
8. S. Talens, J. Hoekstra, S.P.G. Dirkx, S. Darwish Murad, J. Trebicka, E. Elias, M. Primignani, J.C. García-Pagán, D.C. Valla, H.L.A. Janssen, F.W.G. Leebeek, D.C. Rijken. *Proteomic analysis reveals that apolipoprotein A1 levels are decreased in patients with Budd-Chiari syndrome.* J Hepatol 2010 *accepted for publication.*

PHD PORTFOLIO SUMMARY

Name PhD student:	Jildou Hoekstra
Erasmus MC Department:	Gastroenterology and Hepatology
Supervisors:	Prof.dr. H.L.A. Janssen
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PhD period:	March 2007 – April 2010

Summary of PhD training activities

(Inter)national conferences

2007 Annual meeting of the Dutch Society of Gastroenterology and Hepatology (NVGE); *October 4-5, Veldhoven, the Netherlands.*
58th Annual meeting of the American Association for the Study of Liver Diseases (AASLD); *November 2-6, Boston, MA, United States of America.*
22nd Erasmus Liver Day; *December 6, Rotterdam, the Netherlands.*

2008 43rd Annual meeting of the European Association for the Study of the Liver (EASL); *April 23-27, Milan Italy.*
Annual meeting of the Dutch Society of Gastroenterology and Hepatology (NVGE); *October 2-3, Veldhoven, the Netherlands.*
59th Annual meeting of the American Association for the Study of Liver Diseases (AASLD); *October 31-November 4, San Francisco, CA, United States of America.*
23rd Erasmus Liver Day; *November 27, Rotterdam, the Netherlands.*

2009 Annual meeting of the Dutch Society of Gastroenterology and Hepatology (NVGE); *October 8-9, Veldhoven, the Netherlands.*
60th Annual meeting of the American Association for the Study of Liver Diseases (AASLD); *October 30-November 3, Boston, MA, United States of America.*
24th Erasmus Liver Day; *November 19, Rotterdam, the Netherlands.*

2010 Annual meeting of the Dutch Society of Gastroenterology and Hepatology (NVGE); *October 7-8, Veldhoven, the Netherlands.*
61st Annual meeting of the American Association for the Study of Liver Diseases (AASLD); *October 29-November 2, Boston, MA, United States of America.*

Oral and poster presentations

2007 Paroxysmal Nocturnal Hemoglobinuria in Budd-Chiari Syndrome: Findings from the EN-Vie Study. *NVGE, Veldhoven, the Netherlands (oral) & AASLD, Boston, MA, United States of America (poster).*

2008 Etiology of Budd-Chiari Syndrome: the Role of Multiple Underlying Risk Factors. *NVGE, Veldhoven, the Netherlands (oral) & AASLD, San Francisco, CA, United States of America (poster).*
Impaired Fibrinolysis as a Risk Factor for Budd-Chiari Syndrome. *NVGE, Veldhoven, the Netherlands (oral) & AASLD, San Francisco, CA, United States of America (poster).*
Gastrointestinal Bleeding in Patients with Budd-Chiari Syndrome. *NVGE, Veldhoven, the Netherlands (oral) & AASLD, San Francisco, CA, United States of America (poster).*

2009 Apolipoprotein A1 Levels are Decreased in Patients with Budd-Chiari Syndrome. *NVGE, Veldhoven, the Netherlands (oral) & AASLD, Boston, MA, United States of America (poster).*

2010 Etiologic Factors underlying Budd-Chiari Syndrome and Portal Vein Thrombosis: Clues for Site-specific Thrombosis. *NVGE, Veldhoven, the Netherlands (oral) & AASLD, Boston, MA, United States of America (poster).*

Courses

Introduction to medical statistics. *Novartis, June 2007*
Regression analysis. *Netherlands Institute for Health Sciences (NIHES), Erasmus Winter Program, January 2008*
Survival analysis. *Netherlands Institute for Health Sciences (NIHES), Erasmus Winter Program, January 2009*

Teaching and supervising activities

Supervision of master student Esmay Bresser. (2009-2010)
Vascular Liver Disorders. *Lecture for 2nd year medical students participating in a 4-week course on Blood Coagulation (January 2010).*

Memberships

Dutch Society of Gastroenterology
Dutch Society of Hepatology

DANKWOORD

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Jildou Hoekstra
Rotterdam, oktober 2010

CURRICULUM VITAE

Jildou Hoekstra werd geboren op 4 december 1981 te Amstelveen. Na een aantal jaren op een internationale school in Saoedie Arabië te hebben gezeten, volgde zij het middelbaar onderwijs aan het Keizer Karel College te Amstelveen. In juni 2000 behaalde zij haar gymnasiumdiploma om vervolgens in september van dat jaar te beginnen met de studie Geneeskunde aan de Rijksuniversiteit Groningen. Bij het Laboratorium Kindergeneeskunde van het Universitair Medisch Centrum Groningen deed zij voor haar wetenschappelijke stage onderzoek naar de vetstofwisseling van de lever. Vervolgens liep zij nog vijf maanden stage bij de afdeling Pathologie van de University of Cincinnati te Cincinnatti, Ohio (VS). Vanaf januari 2005 startte zij met haar coschappen in het Medisch Spectrum Twente te Enschede. Op de afdeling Maag-, Darm- en Leverziekten van het Erasmus MC te Rotterdam liep zij haar keuzecoschap. In januari 2007 behaalde zij cum laude haar artsendiploma. Vanaf maart 2007 begon zij aan haar promotieonderzoek bij de afdeling Maag-, Darm- en Leverziekten van het Erasmus MC (afdelingshoofd prof.dr. E.J. Kuipers) onder begeleiding van prof.dr. H.L.A. Janssen en prof.dr. F.W.G. Leebeek (afdeling Hematologie). Na 3 jaar fulltime aan haar onderzoek te hebben gewerkt is zij in mei 2010 gestart met de opleiding tot Maag-Darm-Leverarts aan het Erasmus MC (opleider dr. R.A. de Man). De vooropleiding Interne Geneeskunde volgt zij gedurende twee jaar in het Sint Franciscus Gasthuis te Rotterdam (opleider drs. A. Rietveld). Zij woont samen met Niels Agatz in Rotterdam.