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Non-malignant portal vein thrombi in patients with cirrhosis consist of intimal fibrosis with or without a fibrin-rich thrombus

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Key words: portal vein thrombosis, cirrhosis, anticoagulation, fibrin, intimal fibrosis

Abbreviations: CT, Computed Tomography; DOACs, Direct Oral Anticoagulants; DVT, Deep Vein Thrombosis; EVG, Elastic Von Gieson; H&E, Hematoxylin and Eosin; LMWH, Low Molecular Weight Heparin; MSB; Martius Scarlet Blue; PVT, Portal Vein Thrombosis; SEM, Scanning Electron Microscopy; VKA, Vitamin K Antagonist; VWF, Von Willebrand Factor

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Background and aim Portal vein thrombosis (PVT) is a common complication of cirrhosis. The exact pathophysiology remains largely unknown and treatment with anticoagulants does not lead to recanalization of the portal vein in all patients. A better insight in the structure and composition of portal vein thrombi may assist in developing new strategies for the prevention and treatment of PVT.

Methods Sixteen prospectively and 63 retrospectively collected non-malignant portal vein thrombi from cirrhotic patients who underwent liver transplantation were included. Histology, immunohistochemistry and scanning electron microscopy were used to assess structure and composition of the thrombi. Most recent computed tomography (CT) scans were reanalysed for thrombus characteristics. Clinical characteristics were related to histological and radiological findings.

Results All samples showed a thickened, fibrotic tunica intima. Fibrin-rich thrombi were present on top of the fibrotic intima in 9/16 prospective cases and in 21/63 retrospective cases. A minority of the fibrotic areas stained focally positive for fibrin/fibrinogen (fg, 16% of the cases), Von Willebrand Factor (VWF, 10%) and CD61 (platelets, 21%), while most of the fibrin-rich areas stained positive for those markers (fg, 100%; VWF, 77%; CD61, 100%). No associations were found between clinical characteristics including estimated thrombus age and use of anticoagulants and presence of fibrin-rich thrombi.

Conclusion Here we demonstrated that PVT in cirrhotic patients consists of intimal fibrosis with an additional fibrin-rich thrombus in only one-third of the cases. We hypothesize that our observations may explain why not all portal vein thrombi in cirrhotic patients recanalise by anticoagulant therapy.

INTRODUCTION

Portal vein thrombosis (PVT) is a rare condition in the general population but is common in patients with cirrhosis. The prevalence of PVT in cirrhotic patients increases with disease severity and varies from 5% - 26% in patients with advanced disease.[1] The exact pathophysiology of PVT is still unknown, but decreased portal flow velocity and severity of liver disease are important risk factors for PVT in cirrhotic patients.[2–4] Although it has been suggested that hypercoagulability may also be a risk factor for PVT development[5,6], multiple recent studies did not find congenital thrombophilia to be related to PVT risk.[2,7,8] Notably, it has been debated whether PVT contributes to liver disease progression or whether it is merely a marker of severity of disease.[4,7,9] For example, a systematic review showed an increased risk of mortality and hepatic decompensation in cirrhotic patients with PVT[10], while other studies showed no independent association between the presence of PVT and progression of cirrhosis.[4,7] It is, however, generally accepted that PVT can cause technical challenges during liver transplantation that may result in decreased graft survival rates and increased morbidity.[11,12]

PVT is often asymptomatic and incidentally found as part of routine imaging.[2] Only a minority of patients present with symptoms such as acute abdominal pain or gastrointestinal bleeding, which is presumably a result of a portal vein thrombus that has recently been formed. For the majority of thrombi, however, the exact timing of thrombus formation is unknown, although it may be estimated by its appearance on radiological imaging or examination of sequential images over time. The age of the thrombus might be of importance for the decision whether or not to treat PVT, as more recently formed portal vein thrombi seem to respond better to anticoagulant therapy than older thrombi.[13,14] Given the lack of precision in determination of thrombus age in the majority of patients, the distinction between acute (or recent) and chronic PVT may be considered arbitrary. In addition, whether anticoagulant therapy is indicated for both recent and chronic PVT remains a matter of debate, since 1) some of the thrombi resolve spontaneously[7,15], 2) anticoagulant therapy does not always result in recanalization of the portal vein [13,16–18], 3) bleeding may complicate treatment with anticoagulants[19,20], 4) therapy may not benefit the patient, particularly if the patient is not a liver transplant candidate.[18,20,21] Therefore, some experts suggest that anticoagulant therapy is not always indicated, and observation with serial imaging every three months, for example to monitor thrombus extension, is a reasonable alternative.[2,5,17,20,22]

Once the decision is made to start anticoagulants, the pharmacological treatment for PVT resembles treatment strategies used in patients with deep vein thrombosis (DVT). Given the

difficulties with using vitamin K antagonists (VKA) in patients with cirrhosis, low molecular weight heparin (LMWH) is the most commonly used anticoagulant, but direct oral anticoagulants (DOACs) are gaining popularity for this indication despite the lack of randomized studies of these agents in patients with cirrhosis.[20,23] Whether these therapeutic strategies are optimal for treatment or (secondary) prevention of PVT remains uncertain. Importantly, the portal venous system is not directly comparable to deep venous systems: 1) the portal vein does not drain blood to the heart, but to hepatic sinusoids in the liver[24]; 2) the portal vein does not have venous valves, which are important in the development of DVT.[25] These features suggest that portal thrombi could be different in composition and structure compared with deep venous thrombi and might therefore require different treatment strategies.

Although the composition of venous and arterial thrombi has been studied extensively[26–29], to the best of our knowledge the composition of portal vein thrombi has not been studied in detail. Better understanding of the composition and structure of portal vein thrombi will be crucial to improve treatment and preventive strategies for PVT in cirrhotic patients and can reveal important information on the pathophysiology of PVT. Therefore, in this study, we used histology, immunohistochemistry and electron microscopy to define the structure and composition of portal vein thrombi in patients with cirrhosis at the time of liver transplantation.

MATERIALS AND METHODS

Study population

Sixteen portal vein thrombi were prospectively collected from adult (≥18 years old) patients with cirrhosis who underwent liver transplantation at King's College Hospital (KCH) (n=5), London, United Kingdom, University Medical Center Groningen (UMCG) (n=5), the Netherlands, or Hospital Clinic Barcelona, Spain (n=6), between October 2018 and October 2020. All patients gave informed written consent for participation in this study. Ethical approval from the Health Research Authority and Health Care and Research Wales, Study Number 19/LO/0920, from the UMCG local Institutional Review Board, Study Registry Number 201800967, and from the Hospital Clinic local Institutional Review Board (HCB/2018/0546), was obtained. The study protocol was approved by the Health Research Authority and Health Care and Research Wales, and the Research and Innovation department at KCH; good clinical practice guidelines were followed. The thrombi were excised by the surgeon with preservation of the entire vessel in the specimen. Only samples with vessel wall components attached to the thrombus material were included. Eight of the thrombi were cut into two even, representative samples, of which one half was fixed in formalin and embedded in paraffin for histological assessment, and the other half was washed in a cacodylate buffer (50 mM sodium cacodylate, pH 7.4, 150 mM NaCl) and then fixed in 2% glutaraldehyde in cacodylate buffer for scanning electron microscopy (SEM). The remaining eight thrombi were fixed in formalin and embedded in paraffin only.

For the retrospective part of this study, adult (≥18 years old) cirrhotic patients from KCH (n=30) and UMCG (n=33), who underwent liver transplantation between 2009 and 2018, were included. PVT was diagnosed prior to transplantation by routine imaging procedures, during liver transplantation by the liver transplant surgeon, or by the pathologist during examination of the explant. Patients with tumorous infiltration in the portal vein were excluded. Formalin-fixed, paraffin embedded liver tissue with portal vein thrombus in sections taken at the liver hilum of the explant were collected from local pathology archives. Medical history and relevant clinical data were collected from patient's medical records in an anonymised electronic database. This study was approved by the local Institutional Review Boards (KCH liver biobank approval number A19WBYZ11; UMCG registry number 201900748). We also included hilar explant liver tissue from 15 patients with cirrhosis without PVT, and from 5 patients with acute liver failure and no PVT (KCH liver biobank approval number A19WBYZ11), and samples from 5 donor livers that were offered to and transplanted in the UMCG (METC number M14.152454).

Histology and immunohistochemistry

The paraffin-embedded portal vein thrombi were cut into 3 – 3,5 µm thick sections using a microtome and fixed on adhesive glass slides. Sections from each patient were stained with hematoxylin and eosin (H&E), Elastic van Gieson (EVG) and with Martius Scarlet Blue (MSB) stain. In addition, portal vein thrombi sections were stained with antibodies to fibrin/fibrinogen (Abcam; Ab58207), Von Willebrand Factor (VWF) (Dako; A0082) and CD61 (Leica; PA0308) on an automated staining machine (Bond Max autostainer; Leica). Images were acquired using a light microscope (Olympus; BX51) and digital camera (Olympus; DP26) and were analysed by an experienced liver pathologist (YZ). Intimal thickness was quantified using imaging software (CellSens; Olympus). The intimal thickness in prospectively collected portal vein thrombi samples was measured in an area where the outline of the blood vessel, with a clear distinction between intima and media was recognized (Supplementary Figure 1). In samples in which only intimal tissue was recognized, we recorded the maximal thickness of the intima present in the sample.

Scanning electron microscopy (SEM)

Small pieces of prospectively collected portal vein thrombi were rinsed with saline and fixed in 2% glutaraldehyde in 50 mM cacodylate buffer containing 150 mM NaCl (pH 7.4). The fixed thrombi were washed in 50 mM sodium cacodylate, 150 mM NaCl (pH 7.4), and dehydrated in ascending concentrations of ethanol (30-100 v/v%), dried using hexamethyldisilazane, and sputter-coated with gold-palladium. High-definition images were obtained from randomly chosen areas of each thrombus to eliminate selection bias using a FEI Quanta 250FEG scanning electron microscope (FEI, Hillsboro, OR, USA).

Radiology analysis

From each patient, most recent computed tomography (CT) scans before liver transplantation were analysed by radiologists from KCH (SG, PK) or from the UMCG (RdH) to specifically estimate portal vein thrombus age and the degree of portal vein occlusion. Assignment as being acute or chronic thrombus considers the time interval between imaging where no thrombus was apparent and that when first detected. Specific morphological features also suggest an acute 'fresh' thrombus: for example, specific vessel location, central thrombus position, smooth surface and vessel occlusion, whilst other features such as a laminar appearance, the presence of chords or calcification suggest a more chronic thrombosis. It is however evident that in practice this is not absolute binary categorisation and both acute and chronic radiologic features may be present simultaneously.

Statistical analysis

Descriptive statistics were used to summarise demographics and clinical characteristics of the study population. Continuous variables were reported as means and standard deviations. Categorical variables were reported as numbers and percentages, and variables of the retrospective study population were compared with a Chi-squared test, Cramer's V test or Fisher's exact test as appropriate. All analyses were done using IBM SPSS Statistics 23, with a two-sided significance level of .05.

RESULTS

Cirrhotic portal vein thrombosis consists of intimal fibrosis with or without a fibrin-rich thrombus

First, we assessed the structure and composition of 16 prospectively collected portal vein thrombus samples of cirrhotic patients (37.5% female, mean age 53 ± 12 years) that were obtained by the surgeon during liver transplantation. All samples showed a thickened, fibrotic tunica intima of the portal vein on H&E and MSB staining. The median thickness of this intimal fibrosis was 2325 µm (IQR 1728 – 3695 µm). This thickened vessel wall occluded the lumen for more than 50% in 5 of the 16 cases. Nine of the 16 samples also contained a fibrin thrombus as evidenced by (orange to red) MSB staining. Figure 1 shows 2 representative light microscopy images of the portal vein thrombi samples. Figure 1A demonstrates a focally thickened fibrotic tunica intima of the vessel wall with some haemorrhage but without a fibrin-rich thrombus. Figure 1B shows a circumferential thickened fibrotic tunica intima of the portal vein, with a fibrin thrombus within the lumen of the vessel. Images of all 16 prospectively collected thrombi are shown in Supplementary Figure 2, demonstrating heterogeneity of the thrombi in terms of structure and fibrin/fibrinogen content. To study the components of the portal vein thrombi in more detail, the samples were analysed using immunohistochemistry for fibrin/fibrinogen, VWF, and CD61 (platelets), as shown in Supplementary Figure 3. The fibrotic intima stained focally positive for fibrin/fibrinogen in 6 of the 16 cases and for VWF in 4 cases. None of the samples stained positive for CD61 in the fibrotic intima of the portal vein. The 9 intravascular fibrin-rich thrombi all stained positive for fibrin/fibrinogen, 4 stained positive for VWF and 4 for CD61.

The prospectively collected portal vein thrombus samples were also analysed using SEM. In the samples only containing a fibrotic intima, bundles of collagen with red blood cells in the typical biconcave shape (Figures 2A and 2B) were observed. In the nine samples that also contained a fibrin thrombus on MSB staining, branched fibrin networks with entrapped red blood cells, platelet remnants and cell debris were observed (Figures 2C and 2D). Notably, polyhedral erythrocytes, which are a hallmark of contracted clots commonly seen in patients with venous and arterial thrombosis[30–32], were only rarely observed.

Next, we assessed the structure and composition of retrospectively collected portal vein thrombus samples of 63 patients with cirrhosis (27% female; mean age 53 ± 13 years). Alcoholic steatohepatitis (32%) was the most prevalent aetiology of disease. The majority of patients had moderate to severe liver disease according to the Child Pugh score (i.e. Child Pugh B (51%) or Child Pugh C (41%)) at time of liver transplantation. Median duration between diagnosis of

cirrhosis and liver transplantation was 6 years (range 0-28 years) and median duration between diagnosis of PVT and liver transplantation was 5 months (range 0-125 months). In 21 patients (33%), PVT had not been diagnosed on radiological imaging prior to liver transplantation. Anticoagulant therapy was used by 19 of the 63 patients (30%) at time of liver transplantation. Two patients had been on anticoagulant therapy that was stopped 21 months and 9 months prior to liver transplantation. The reason to stop the therapy was chronic minor oral bleeding in the first case, and unknown in the second case. At time of liver transplantation, LMWH was used by 8 patients (12.7%), VKA by 11 patients (17.5%) and 2 patients (3.2%) used acetylsalicylic acid. Patient characteristics are summarized in Table 1.

All 63 paraffin-embedded samples showed a thickened, fibrotic tunica intima of the portal vein on H&E, EVG and MSB staining. The median thickness of the tunica intima was 2406 µm (IQR 1349 – 3346 µm). The intimal thickening occupied more than 50% of the vessel lumen in 48 patients (76%). To ascertain that a fibrotic tunica intima is specific for patients with PVT and not a general feature of a portal vein in patients with liver disease, we studied retrospectively collected tissue from cirrhotic patients that did not have PVT. Clinical characteristics of these patients are outlined in Table 1. In hilar liver tissue from 15 patients with cirrhosis, 5 patients showed very mild intimal fibrosis, which was almost an order of magnitude thinner compared to the intimal thickness in patients with PVT (median thickness 358 µm (IQR 294 – 378 µm)). In 5 samples from patients with acute liver failure no intimal fibrosis was found. Figure 3 shows typical examples of the thickness of the tunica intima in liver donors, patients with acute liver failure and cirrhotic patients without PVT. From the 63 samples of patients with PVT, 21 (33%) contained a fibrin-rich thrombus. Of these 21 samples, the fibrin structures occupied the vessel lumen for more than 50% in 7 patients (33%). The median time between diagnosis of PVT and liver transplantation was 5 months (range 0-125 months) for patients who had a fibrin-rich thrombus based on histology results, and 3.5 months (range 0-81 months) for patients who did not have a fibrin-rich thrombus based on histology results. Representative images of H&E, EVG, MSB and immunostained hilar liver tissue samples are shown in Figures 4 and 5.

Immunohistochemical analysis of the fibrotic tunica intima of the portal vein vessel wall showed focal positive staining for fibrin/fibrinogen in 10 samples (16%), VWF in 6 samples (10%), and CD61 (platelets) in 13 samples (21%). The immunoreactivity to those markers was mostly restricted to small vessels or haemorrhagic foci of the thickened intima, while the sclerotic stroma was negative (Figure 4). From the 21 samples that contained a fibrin-rich thrombus, all (100%) stained positive for fibrin/fibrinogen and CD61 (platelets), and 16 (76%) stained positive for VWF.

Fibrin/fibrinogen was abundantly present in the fibrin-rich thrombi, while platelet and VWF staining was less abundant (Figure 5).

Clinical characteristics and thrombus age do not determine whether the portal vein thrombus contains fibrin

Next to histological assessment, PVT was assessed by radiological imaging. Experienced hepato-pancreato-biliary (HPB) radiologists reanalysed the most recent CT scans prior to liver transplantation from each retrospectively included patient. The median time between the most recent CT scan and liver transplantation was 4 months (range 0 – 39 months). The time between the most recent CT scan and liver transplantation was less than or equal to 6 months in 40 cases (64%). Although PVT was not diagnosed prior to liver transplantation in 21 patients (33%), reanalysis of their CT scans, with a specific focus on PVT, showed a thrombus in 12 of these 21 patients (57%). Thus, after close radiological inspection, only 9 of the 63 (14%) thrombi were not recognized on radiological imaging. Of these 9 patients, 5 had a fresh fibrin thrombus based on histological analyses. The most recent CT scan prior to liver transplantation of these 9 patients was older than 6 months in 7/9 (78%) patients. Of the 54 portal vein thrombi that were recognized on the most recent CT scan, the location of the thrombus was both intra- and extrahepatic in 34 (62%) cases, only intrahepatic in 9 cases (16%) and only extrahepatic in 12 cases (22%). Occlusion of the portal vein was more than 50% in 18 (37%) cases based on radiological examination. Of the 49 PVT cases that were analysed by radiologists, 34 thrombi were classified as chronic whereas 10 were classified as acute. No classification could be given for the remaining 5 thrombi.

We then compared the radiological classification as chronic or acute PVT with the histological analyses. Of the 54 portal vein thrombi identified on radiology imaging, 16 patients (30%) had an intravascular fibrin-rich thrombus based on histology results. Of the 34 patients from whom the location of the thrombus was both intra- and extrahepatic on the most recent CT scan prior to liver transplantation, 12 had a fibrin-rich thrombus (35%). Of the 9 patients who only had intrahepatic PVT on the most recent CT scan, 2 had a fibrin-rich thrombus (22%), and of the 12 patients who only had extrahepatic PVT on the most recent CT scan, 4 had a fibrin-rich thrombus based on histology results (33%). Of the 34 patients from whom the portal vein thrombus was classified as chronic by the radiologist during reanalysis of the images, 9 (26%) contained a fibrin thrombus within the lumen of the vessel. Of the 10 patients from whom the portal vein thrombus was classified as acute, 5 (50%) contained a fibrin thrombus within the lumen of the vessel. Supplementary Figures 4-7 show typical examples of radiological images

from patients whom thrombus was classified as chronic or acute, compared with representative H&E stained sections from these patients. Of note, the median time between the most recent CT scan and transplantation was 3 months (range 0-14) in the thrombi classified as acute by the radiologists. Of the 5 patients from whom the thrombus could not be classified as chronic or acute by radiologists, 2 (40%) contained a fibrin thrombus within the lumen of the vessel. Of the 9 patients from whom PVT was not recognized on radiological imaging, 5 patients (55%) had fibrin-rich thrombi within the lumen of the vessel based on histology results. Importantly, the most recent CT scan from these patients was older than 6 months in 4/5 (80%) cases with a fibrin thrombus, and in 5/9 (56%) cases without a fibrin thrombus (median time between most recent CT scan and liver transplantation was 10 months, range 2-29 months).

Lastly, we related our histological and radiological findings to clinical characteristics. There was no association between the presence of a fibrin-rich thrombus and aetiology of disease, the use of anticoagulants, degree of occlusion of the portal vein, severity of disease based on the MELD score and the presence of collaterals and varices (Table 2). We did find that patients with a smaller portal vein diameter more often had no fibrin thrombus within the lumen of the portal vein ($p < .05$). No associations were found between patient characteristics and the time between PVT diagnosis and liver transplantation (Table 2). Nineteen patients used anticoagulant therapy at the time of liver transplantation. There were no significant differences in age, BMI, aetiology of disease, MELD score, presence of ascites, hepatic encephalopathy, HCC, smoker/non-smoker, diabetes mellitus, time between diagnosis of PVT and liver transplantation, diameter of the portal vein or degree of occlusion of the portal vein between patients who used anticoagulants or did not use anticoagulants (data not shown). In 5 of the 19 patients who were treated with anticoagulants, the size of the thrombus reduced after starting anticoagulant therapy, based on radiological imaging. From these 5 patients, 0 had a fibrin-rich thrombus at the time of liver transplantation. In the other 14 patients who used anticoagulants, the size of the thrombus increased, did not change, or is unknown because PVT was not diagnosed or no imaging was available between start of the treatment and liver transplantation. From these 14 patients, 6 had a fibrin-rich thrombus at time of liver transplantation. The use of anticoagulants was not associated with a decrease in the proportion of patients with a fibrin-rich thrombus (Table 2). In addition, the intimal thickness was similar in patients who did or did not use anticoagulants at the time of liver transplantation (2371 μm vs. 2465 μm, $p=0.80$). Seventeen of the 42 (40.5%) patients who used a beta-blocker at time of transplantation had a fibrin-rich thrombus. No significant differences were observed between patients who used beta-blockers and patients who did not use beta-

blockers on presence of a fibrin-rich thrombus, degree of portal vein occlusion, disease severity, portal vein diameter, and presence of collaterals (data not shown).

DISCUSSION

Here we described the composition and structure of non-malignant cirrhotic portal vein thrombi that were collected during liver transplantation. We demonstrated that all portal vein thrombi consist, at least in part, of tunica intima thickening of the portal vein vessel wall, specifically in an appearance resembling intimal fibrosis. Only one-third of the thrombi examined contained a fibrin-rich thrombus in addition to a fibrotic intima, and the presence or absence of such material appeared unrelated to thrombus age as estimated by radiological imaging, clinical characteristics or previous anticoagulant therapy. The apparent absence of polyhedral erythrocytes, which define contracted venous and arterial thrombi[30–32], reinforces the notion that the portal vein thrombus is a unique entity. As two-thirds of portal vein thrombi at the time of transplantation are devoid of fibrin, our results may redefine the biological basis of PVT. We propose that the absence of fibrin in part of the thrombi may explain why not all patients with PVT show recanalization by anticoagulant therapy.

Histological assessment of both prospectively and retrospectively collected portal vein thrombus samples showed that a portal vein thrombus in part or in its entirety is a thickened, fibrotic tunica intima of the portal vein. This was observed as circumferential as well as focal thickening of the vessel wall (Figure 1). These observations suggest that the term ‘portal vein thrombosis’ may be a misnomer, and that ‘portal vein stenosis’ or ‘non-malignant portal vein occlusion’ may be more appropriate. The origin of the intimal fibrosis of the portal vein wall cannot be derived from our study with certainty, but we propose two possible mechanisms by which portal vein intimal fibrosis develops.

First, it may be that the intimal fibrosis develops as a consequence of initial fibrin-rich thrombus formation, which organizes into a fibrotic structure that re-endothelialises over time. In this scenario, a portal vein thrombus starts as a fibrin-rich thrombus that matures and in which fibrin is replaced by a collagen and cell-rich structure over time. This process has been shown to occur in other vascular beds after profound injury to the vascular wall leading to thrombus formation with subsequent development of intimal fibrosis.[33] In patients with cirrhosis, damage to the portal vein wall by for example altered shear stress related to portal hypertension and inflammation may initiate thrombus formation, which is further facilitated by the hypercoagulable features of patients with cirrhosis.[5,34–36]

A second, intriguing hypothesis is that the intimal fibrosis develops in the absence of overt initial fibrin formation. This intimal hyperplasia can occur following all types of vascular reconstructive procedures including coronary artery bypass surgery (particularly using veins or

synthetic grafts), angioplasty, vascular stenting, endarterectomy, and vascular access grafting[37]. These procedures may lead to vascular endothelial cell stress[37,38], platelet aggregation, leukocyte chemotaxis, and endothelial proliferation.[39] This initial response to injury ultimately leads to proliferation of mesenchymal cells (e.g., myofibroblasts, vascular smooth muscle cells) and deposition of extracellular matrix components.[33,37] Interestingly, activation of coagulation and in particular the generation of thrombin is a well-recognized contributor to intimal hyperplasia after vascular injury, which is in part mediated by activation of smooth muscle cells by thrombin.[40–42] In addition, tissue factor is an important driver of intimal hyperplasia[40,43], and tissue factor expression on smooth muscle cells or fibrocytes may drive local thrombin generation. Indeed, local or systemic inhibition of thrombin or tissue factor decreased intimal hyperplasia in animal models.[40,41,44] This mechanism could also apply to the development of PVT. For example, one study has demonstrated that prophylactic anticoagulation profoundly reduces development of PVT[9], and it may be that the effects of anticoagulation in this study are not primarily due to prevention of clot formation, but by inhibition of local thrombin generation that drives intimal hyperplasia.

In patients with DVT and pulmonary embolism, the thrombus consists of fibrin, platelets, and blood cells.[27] However, in animal models of venous thrombosis[33], a thickening of the vessel wall is observed over time, a phenomenon referred to as 'vein wall fibrosis'. This post-thrombosis vein wall remodelling has been proposed to contribute to post-thrombotic syndrome, and therapeutic strategies to reduce post-thrombosis vein wall fibrosis have been proposed.[45] One study in humans has shown thickening of the vein wall by high-resolution ultrasound both in patients with DVT in the acute phase and in patients with post-thrombotic syndrome.[46] Whether these post-DVT vein wall changes have a similar pathogenesis compared to the fibrotic lesion in PVT requires additional study.

Intravascular fibrin-rich thrombi were present in one-third of the cases within the retrospective cohort in this study. In contrast to the PVT consisting solely of intimal fibrosis, these thrombi may be susceptible to anticoagulant therapy. Fibrin-specific MRI contrast agents have been developed and tested in humans, and such agents may be helpful in determining which patients may benefit from anticoagulant therapy.[47] The notion that PVT can be recurrent, with spontaneously resolving and returning thrombi[7,15], may also explain why patients did not have detectable fibrin-rich thrombi at the time of transplantation. Anticoagulants may be helpful in preventing 'reappearance' of fibrin-rich structures. Such cyclic behaviour may explain why there was no difference in the proportion of fibrin-containing thrombi between thrombi that were radiologically classified as acute or as chronic. In addition, calcification and re-endothelialisation

of thrombi can already start a few days after initiation of thrombosis.[48,49] Thrombi that were not classified as fibrin-rich on histology within this study could thus have been formed recently, but have developed into an organized thrombus at time of transplantation. We tried to define the chronicity of thrombosis in the retrospective study population based on the most recent CT scan, but no recent imaging (≤6 months) was available from 36% of the patients within the retrospective cohort, making comparisons between radiology imaging and the possibly fast-changing thrombus unreliable.

Our results thus suggest that part of the PVT might not always develop from a fibrin thrombus, but could instead be a vascular disease resulting from cirrhosis and its complications in the portal vein. The observation that a much milder intimal thickening of the portal vein is present in part of the patients with cirrhosis without PVT supports this proposed mechanism, and it is tempting to speculate that alterations in portal flow as a consequence of portal hypertension is the trigger of intimal fibrosis development. Indeed, alterations in shear stress have been shown to promote intimal hyperplasia in other settings.[50,51] It is conceivable that the composition of a portal vein thrombus has implications for treatment strategies and the effect of therapeutic interventions. Although anticoagulant therapy may be beneficial to prevent development of de novo fibrin-rich thrombi, it will likely not be effective in recanalising PVT that only consists of fibrotic tissue. Anticoagulants may prevent the development of fibrin-rich thrombi on top of a fibrotic portal vein wall structures, and may thereby prevent thrombus extension. Strategies to prevent portal venous intimal fibrosis in patients with cirrhosis should be the focus of future studies.

We acknowledge some limitations to our study. First, this study is largely based on a retrospective study population. We had access to samples taken at the liver hilum from pathology archives. We only included samples in which thrombus was detected in the hilum. These sections of the thrombus may be the center of the thrombus for example in those patients with intrahepatic thrombus only, but may also have been in the periphery of the thrombus in those patients that also had thrombus in the extrahepatic portal vein. We thus cannot ascertain whether these samples were representative for the entire thrombus. However, the thrombus composition in extrahepatic portal vein thrombi samples from the prospective cohort of this study was remarkably similar to the composition of the thrombi samples from the retrospective cohort of this study, which suggests the hilar thrombus samples to be representative of the entire thrombus. Future studies with prospective analysis of portal vein thrombi sampled at different sites of the thrombus are required to confirm our present findings. Second, the most recent CT scans were older than 6 months in 36% of the patients in the retrospective study population, which complicates comparison of the radiological imaging results with histology results. In addition, we

have compared the histological appearance of the thrombus at the liver hilum, with radiological appearance of the entire thrombus, which frequently was present both intra- and extrahepatically.

In conclusion, this study is the first to describe the composition and structure of non-malignant portal vein thrombi in cirrhotic patients. All thrombi analysed showed intimal fibrosis of the portal vein wall, and only one-third of the patients had a fibrin-rich thrombus. We propose that, as it is unlikely that non-fibrin portal vein occlusions recanalise by anticoagulant therapy, our findings may have direct consequences for the management of cirrhotic PVT. Also, our findings may lead to new strategies for prevention of PVT.

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Table 1. *Demographics and characteristics of cirrhotic patients with PVT at time of liver transplantation, the control cohort of cirrhotic patients without PVT, and patients with acute liver failure without PVT.*

	Prospective cohort. N=16.	Retrospective cohort. N=63.	Control cohort, cirrhotic patients without PVT. N=15.	Control cohort, patients with acute liver failure without PVT. N=5.
Gender (female)	6 (37.5)	17 (27.0)	9 (45.0)	3 (60.0)
Age (years) at time of LT	53 ± 12	53 ± 13	48 ± 13	40 ± 10
BMI	25.2 ± 4.0	26.4 ± 5.1	28.5 ± 5.0	No data
MELD score	17.8 ± 4.5	18.5 ± 6.0	12.8 ± 6.8	N/A
Child Pugh score				
A	1 (6.3)	5 (7.9)	2 (13.3)	N/A
B	9 (56.3)	32 (50.8)	8 (53.3)	N/A
C	6 (37.5)	26 (41.3)	5 (33.3)	N/A
Aetiology of liver disease				
ASH	2 (12.5)	20 (31.7)	5 (33.3)	0 (0.0)
NASH	2 (12.5)	9 (14.3)	2 (13.3)	0 (0.0)
PBC	1 (6.3)	5 (7.9)	1 (6.7)	0 (0.0)
PSC	2 (12.5)	9 (14.3)	5 (33.3)	0 (0.0)
Viral	3 (18.8)	2 (3.2)	1 (6.7)	0 (0.0)
Auto-immune	2 (12.5)	9 (14.3)	1 (6.7)	1 (20.0)
Cryptogenic	1 (6.3)	2 (3.2)	0 (0.0)	0 (0.0)
Drug Induced	0 (0.0)	0 (0.0)	0 (0.0)	3 (60.0)
Other	3 (18.8)	7 (11.1)	0 (0.0)	1 (20.0)
Hepatic encephalopathy				

No	11 (68.8)	32 (52.5)	15 (100.0)	0 (0.0)
Grade 1-2	5 (31.3)	26 (42.6)	0 (0.0)	0 (0.0)
Grade 3-4	0 (0.0)	3 (4.9)	0 (0.0)	5 (100.0)
Ascites				
No	5 (31.3)	13 (20.6)	9 (60.0)	4 (80.0)
Slight	6 (37.7)	8 (12.7)	1 (6.7)	1 (20.0)
Moderate	4 (25.0)	16 (25.4)	2 (13.3)	0 (0.0)
Severe	1 (6.3)	26 (41.3)	3 (20.0)	0 (0.0)
Smoker, currently or stopped (yes)	6 (37.5)	28 (50.4)	1 (6.7)	0 (0.0)
Diabetes (yes)	5 (31.3)	25 (42.4)	4 (26.8)	0 (0.0)
Previous abdominal surgery	4 (25.0)	24 (42.9)	No data	0 (0.0)
HCC	5 (31.3)	11 (17.5)	1 (6.7)	0 (0.0)
Thrombophilic disease	0 (0.0)	0 (0.0)	N/A	N/A
Medication at time of LT				
Use of LMWH	6 (37.5)	8 (12.7)	0 (0.0)	0 (0.0)
Use of VKA	6 (37.5)	11 (17.5)	0 (0.0)	0 (0.0)
Use of acetylsalicylic acid	1 (6.3)	2 (3.2)	0 (0.0)	0 (0.0)
Use of Beta-blockers	9 (56.3)	42 (66.7)	4 (26.8)	0 (0.0)
Hormonal influence (female)	1/6 (16.7)	2/17 (11.8)	0/9 (0.0)	0/3 (0.0)

NOTE The results are presented as mean ± SD for continuous variables, and number (percentage) for categorical variables of available data. LT, liver transplantation; BMI, body mass index; MELD, model for end-stage liver disease; ASH, alcoholic steatohepatitis; NASH, non-alcoholic steatohepatitis; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis; HCC, hepatocellular carcinoma; LMWH, low-molecular weight heparins; VKA, vitamin K antagonists.

Table 2. *Associations between the presence of a fibrin-rich thrombus based on histological analysis or estimated age of thrombus based on radiological imaging and patient parameters.*

		Presence of a fibrin-rich thrombus based on histology (n=63)			Radiological diagnosis of PVT prior to liver transplantation (n=42)		
		Yes (n=21)	No (n=42)	P-value	≤ 6 months (n=14)	> 6 months (n=28)	P-value
Presence of a fibrin-rich thrombus	Yes	-	-	-	7 (50.0)	9 (32.1)	0.26
	No	-	-		7 (50.0)	19 (67.9)	
Aetiology of disease	Cholestatic	6 (28.6)	8 (19.0)	0.43[a]	2 (14.3)	6 (21.4)	0.17[a]
	NASH	4 (19.0)	5 (11.9)		4 (28.6)	2 (7.1)	
	Other	11 (52.4)	29 (69.0)		8 (57.1)	20 (71.4)	
Use of anticoagulants (at time of LT)	Yes	6 (28.6)	13 (31.0)	0.85	6 (42.9)	13 (46.4)	0.83
	No	15 (71.4)	29 (69.0)		8 (57.1)	15 (53.6)	
Occlusion of portal vein (based on radiology)	≤ 50%	9 (56.3)	22 (66.7)	0.48	8 (66.7)	13 (52.0)	0.40
	> 50%	7 (43.8)	11 (33.3)		4 (33.3)	12 (48.0)	
Disease severity (based on MELD score)	≤ 15	5 (23.8)	14 (33.3)	0.44	5 (35.7)	6 (21.4)	0.46[b]
	> 15	16 (77.2)	28 (66.7)		9 (64.3)	22 (78.6)	
Portal vein diameter (based on radiology)	≤ 15 mm	6 (31.6)	25 (65.8)	**0.015**	7 (58.3)	14 (51.9)	0.71
	> 15 mm	13 (68.4)	13 (34.2)		5 (41.7)	13 (48.1)	
Presence of collaterals	Yes	20 (95.2)	36 (85.7)	0.41[b]	12 (85.7)	26 (92.9)	0.59[b]
	No	1 (4.8)	6 (14.3)		2 (14.3)	2 (7.1)	
Use of beta-blockers (at	Yes	17 (81.0)	25 (59.5)	0.16	12 (85.7)	20 (71.4)	0.45[b]

time of LT)	No	4 (19.0)	17 (40.5)		2 (14.3)	8 (28.6)	
Presence of HCC	Yes	3 (14.3)	8 (19.5)	0.74[b]	2 (15.4)	5 (17.9)	1.00[b]
	No	18 (85.7)	33 (80.5)		11 (84.6)	23 (82.1)	

NOTE Data are presented as frequency (%); p-values are based on Chi-Square test, unless stated otherwise. HCC, hepatocellular carcinoma; MELD, Model for End stage Liver Disease; NASH, Non-alcoholic steatohepatitis; LT, liver transplantation; PVT, portal vein thrombosis. [a]Cramer's V; [b]Fisher's Exact Test.

FIGURE LEGENDS

Figure 1. MSB-stained sections of extrahepatic portal vein samples removed during liver transplant surgery. These are representative images of the 16 prospectively collected thrombi. A; A portal vein thrombus consisting of a focally thickened intimal layer of the vessel wall with some haemorrhage but without a fibrin-rich thrombus. B; A portal vein thrombus consisting of a circumferential thickened intimal layer of the vessel wall with a fibrin-rich thrombus on top.

Abbreviations: MSB, Martius Scarlet Blue

Figure 2. Representative scanning electron microscopy images from eight prospectively collected portal vein thrombus samples. A&B; Collagen bundles and some biconcave shaped red blood cells. Fibrin is focally present. C&D; Fibrin networks with mostly biconcave shaped red blood cells.

Figure 3. Representative images of the portal vein wall in H&E stained sections from hilar liver tissue samples from human donor livers, acute liver failure patients, and cirrhotic patients without PVT. The tunica intima at the liver hilum in human donor liver and acute liver failure patients consists of a flat lining of endothelial cells and is almost unrecognizable, and therefore not measurable (A, B). The portal vein vessel wall at the liver hilum in cirrhotic patients without PVT shows a thickened tunica intima (C).

Abbreviations: H&E, Hematoxylin and Eosin; PVT, portal vein thrombosis.

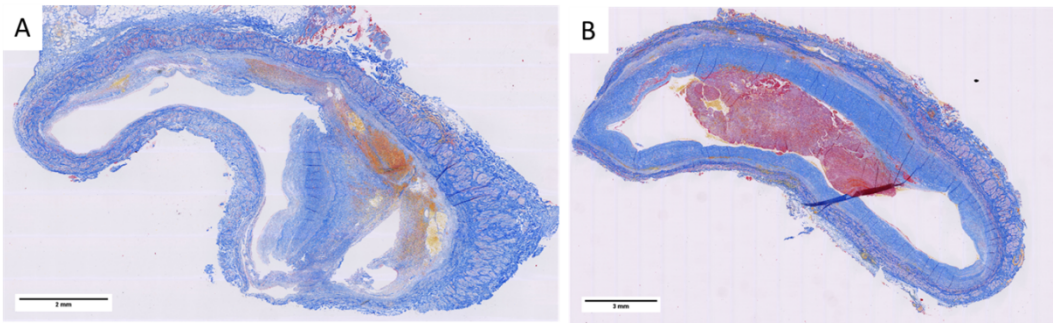
Figure 4. Histopathology of PVT at the hilar region of explanted livers. Representative examples of the thickened tunica intima of the portal vein. A crescent-shaped lamellar fibrosis of the intima is observed (a; x20). EVG staining highlights intimal thickening (b; arrows indicating the tunica media; x20). In the thickened intima, some parts show spindle shaped cells arranged in a lamellar fashion (c; x200), while others consist of hypocellular densely collagenized fibrosis (d and e; both x200). It is only focally immunoreactive to fibrin/fibrinogen (f), VWF (g) and CD61 (h; all x200).

Abbreviations: PVT, portal vein thrombosis; EVG, Elastic Van Gieson; VWF, Von Willebrand Factor

Figure 5. Histopathology of PVT at the hilar region of explanted livers. Representative examples of intimal fibrosis with fibrin thrombus of the portal vein. A fibrin thrombus overlying the

thickened intima is observed (a; x20). The fibrin thrombus consists of aggregated eosinophilic materials and blood contents (b; x200), and these parts are stained in orange to red by MSB, indicating fibrin (c; x200). The fibrin thrombus is immunoreactive to fibrin/fibrinogen, VWF and CD61, and fibrin/fibrinogen is most widely positive (d-f; x200).

Abbreviations: PVT, portal vein thrombosis; VWF, Von Willebrand Factor



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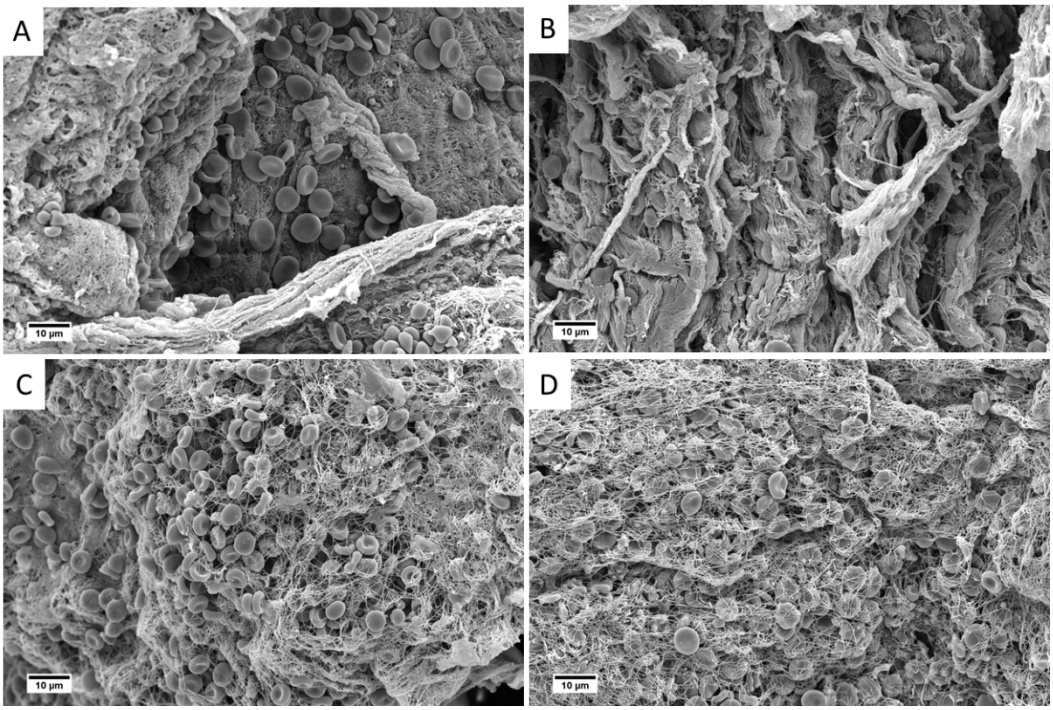

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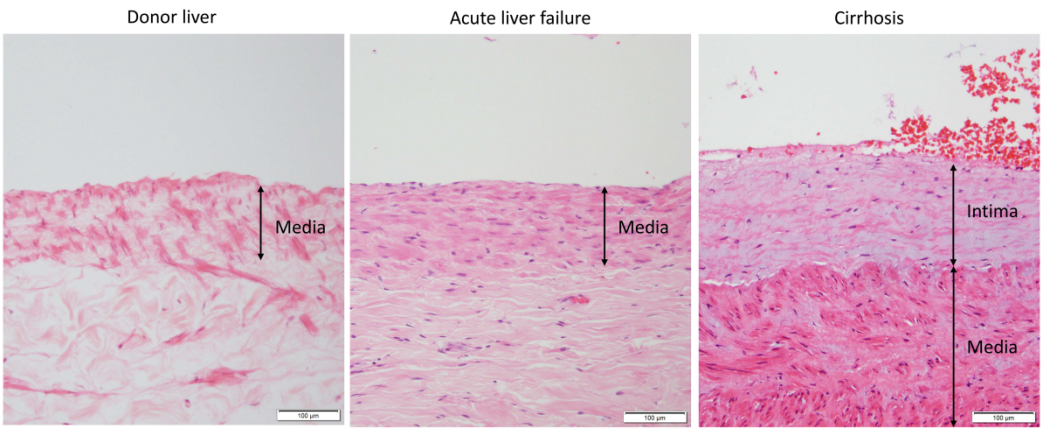

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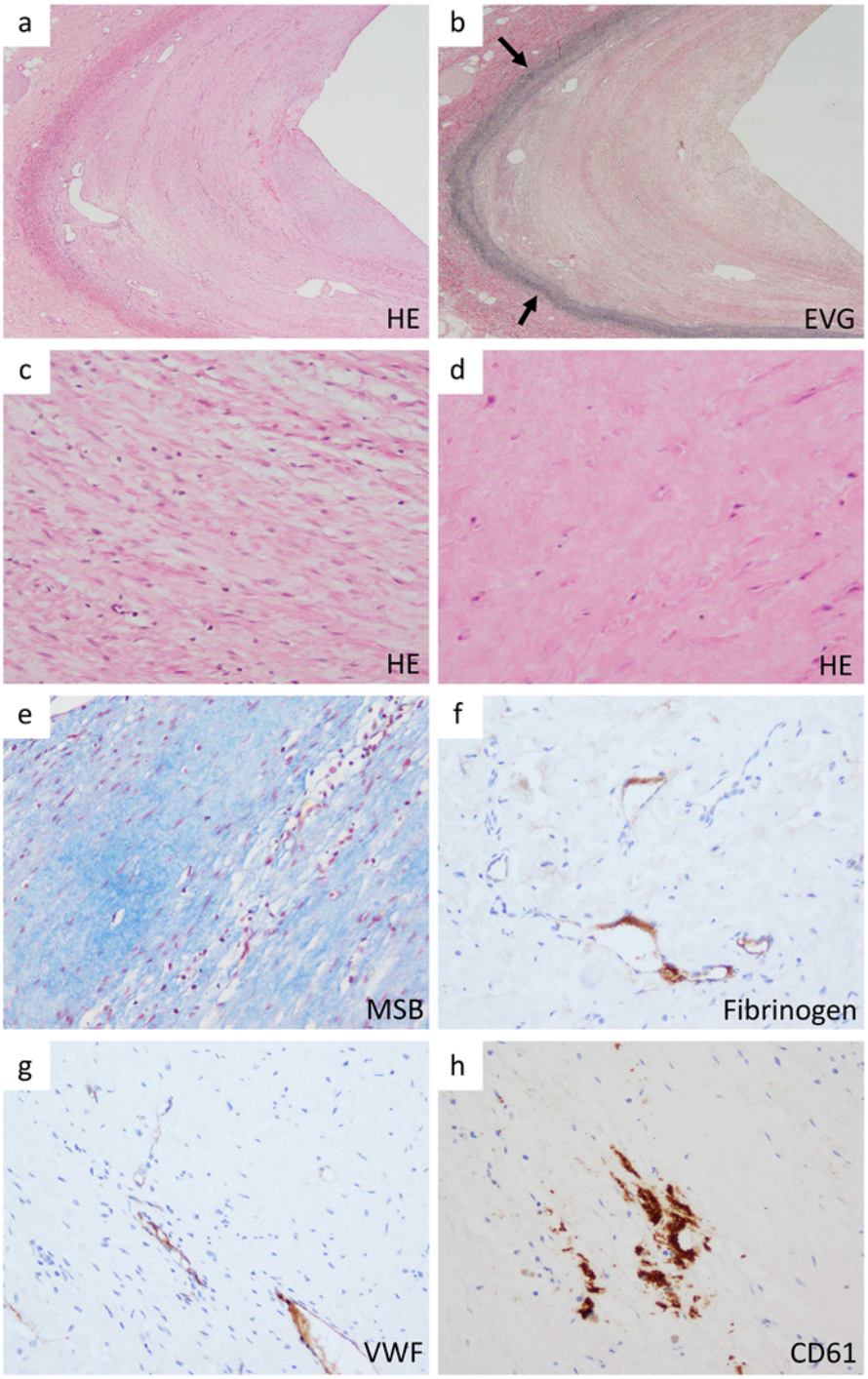

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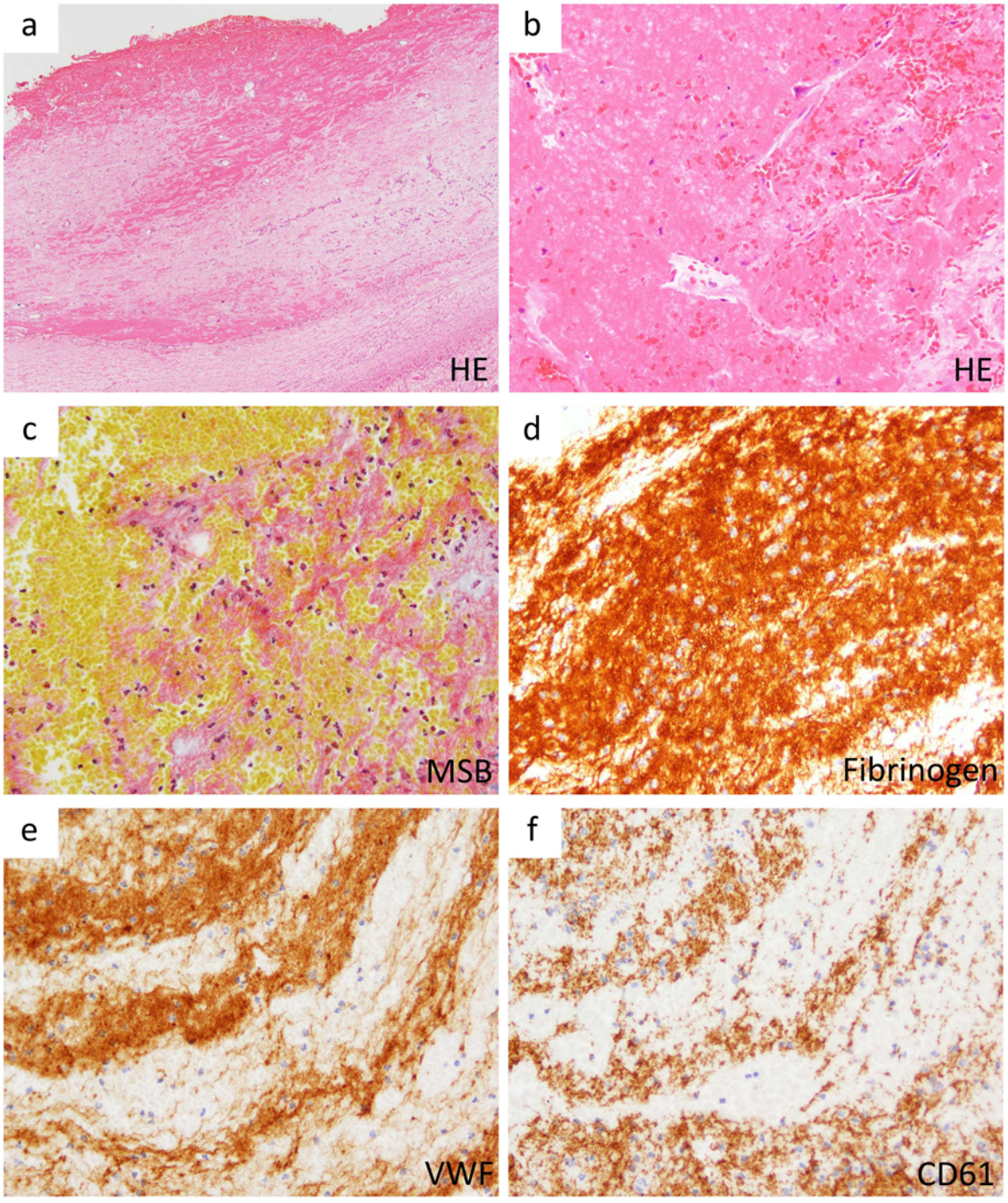

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