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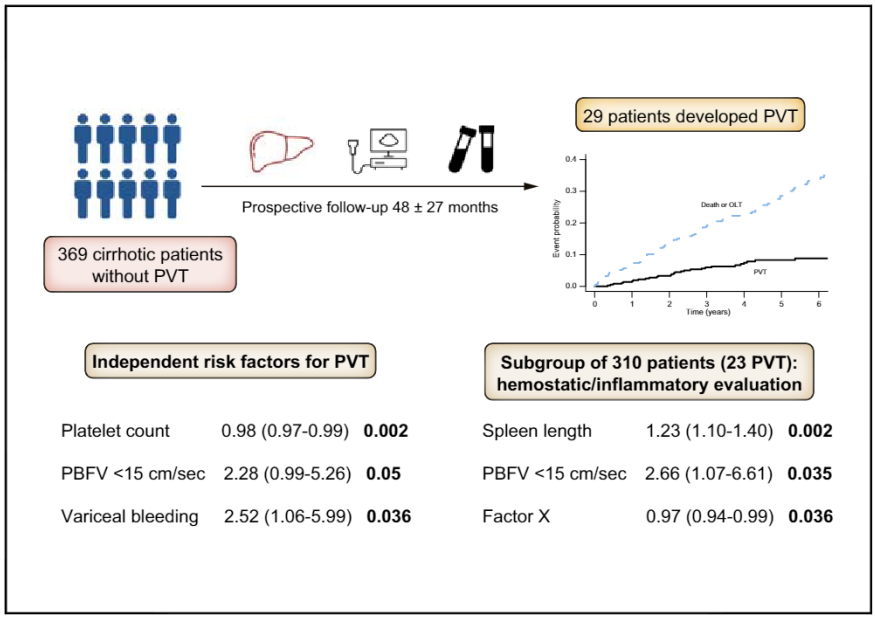
Graphical abstract



Highlights

- Factors related to more severe portal hypertension are associated with higher risk of PVT in cirrhosis.
- Acquired and inherited alterations of coagulation do not predict PVT development during follow-up.
- Cirrhosis-associated inflammation or generation of NETs are not relevant factors predicting PVT development.

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Lay summary

Patients with cirrhosis and more severe portal hypertension are at higher risk of non-tumoral portal vein thrombosis development. Acquired or inherited hemostatic disorders, as well as inflammatory status, do not seem to predict the development of portal vein thrombosis in patients with cirrhosis.

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Predicting portal thrombosis in cirrhosis: A prospective study of clinical, ultrasonographic and hemostatic factors

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Background & Aims: Portal vein thrombosis (PVT) is a relatively frequent event in patients with cirrhosis. While different risk factors for PVT have been reported, such as decreased portal blood flow velocity (PBFV) and parameters related with severity of portal hypertension, these are based on retrospective studies assessing only a discrete number of parameters. The aim of the current study was to evaluate the incidence and risks factors for non-tumoral PVT development in a large prospective cohort of patients with cirrhosis.

Methods: We performed an exhaustive evaluation of clinical, biochemical, inflammatory and acquired/hereditary hemostatic profiles in 369 patients with cirrhosis without PVT who were prospectively followed-up. Doppler ultrasound was performed at baseline and every 6 months or whenever clinically indicated. PVT development was always confirmed by computed tomography.

Results: Twenty-nine patients developed non-tumoral PVT, with an incidence of 1.6%, 6% and 8.4% at 1, 3 and 5 years, respectively. Low platelet count, PBFV <15 cm/sec and history of variceal bleeding were factors independently associated with a high PVT risk. No relationship between PVT development and any other clinical biochemical, inflammatory and acquired or hereditary hemostatic parameter was found.

Conclusions: In patients with cirrhosis, the factors predictive of PVT development were mainly those related to the severity of portal hypertension. Our results do not support the role of hemostatic alterations (inherited or acquired) and inflammatory markers in the prediction of PVT in patients with cirrhosis.

Lay summary: Patients with cirrhosis and more severe portal hypertension are at higher risk of non-tumoral portal vein thrombosis development. Acquired or inherited hemostatic disorders, as well as inflammatory status, do not seem to predict the development of portal vein thrombosis in patients with cirrhosis.

Introduction

Cirrhosis is no longer considered a condition associated with a low risk of developing thrombotic events. Indeed, several studies have shown that patients with cirrhosis are at higher risk of developing splanchnic and extrasplanchnic vein thrombosis.[1,2] Actually, development of non-tumoral portal vein thrombosis (PVT) is a relatively frequent event in patients with cirrhosis; the estimated annual incidence ranges from 4.6 to 26%,[2–6] with the highest incidence in patients with more advanced liver disease.

Most studies evaluating risk factors for PVT are retrospective and transversal, comparing clinical variables between patients with and without PVT. Only a few of these studies are prospective, evaluating the incidence of PVT during follow-up.[3,4,6–8] From these studies, a decreased portal blood flow velocity (PBFV) below 15 cm/sec[4,9] has been described as a major risk factor for PVT development. Additional risk factors for PVT are those related with liver disease severity and the presence of portal hypertension: low platelet count,[4,6,10] low albumin,[11] large esophageal varices[3,7,12] and previous sclerotherapy,[13] previous liver decompensation[6] or presence of large portosystemic collaterals.[5] More recently, it has been suggested that non-selective beta-blockers (NSBBs) may play a role in PVT development.[7,12,14] However, the potential confounding effect of other recognized risk factors for PVT was not adequately evaluated in these studies.

The hemostatic balance in patients with cirrhosis is fragile[15]; it is characterized by a decrease in procoagulant but also anticoagulant

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factors that are synthesized by hepatocytes, together with an increase in endothelial-derived factors such as Factor VIII (FVIII) and von Willebrand factor (VWF). It has been proposed that some hypercoagulable characteristics such as an increased FVIII to protein C ratio (FVIII/PC),[16] increased endogenous thrombin potential (ETP),[17] high FVIII,[18] low ADAMTS13[19] or increased plasma levels of VWF[16] may represent risk factors for developing cirrhosis-associated PVT. Additionally, inherited thrombophilia, notably Factor V Leiden (FVLeiden) and the prothrombin G20210A mutation, has also been suggested to be more frequent in cirrhotic patients with PVT than in those without,[20] but this has not been confirmed in other studies.[3,21] A close relationship between alterations in coagulation and inflammation exists.[22] Neutrophil extracellular traps (NETs) are a web-like structure composed of neutrophil expelled DNA and proteins that have recently been described as a link between inflammation and coagulation and have been implicated in thrombotic diseases.[23] The role of NETs and systemic inflammation in the pathogenesis of PVT in patients with cirrhosis has not been evaluated.

Although clinical, hemostatic and inflammatory parameters may all be involved in development of PVT, all proposed mechanisms continue to be debated and a prospective systematic analysis of all these mechanisms in predicting PVT development has not yet been performed.

The aim of the current study is to evaluate the incidence and risks factors for PVT development in a large cohort of patients with cirrhosis who were prospectively followed-up and in whom an exhaustive clinical, biochemical, inflammatory and acquired/hereditary hemostatic profile was obtained.

Materials and methods

Study population

This is a prospective single center study including consecutive patients with cirrhosis submitted to an abdominal Doppler ultrasound (Doppler-US) for the screening of hepatocellular carcinoma (HCC) between December 2010 and April 2013. Patients between 18 and 80 years with cirrhosis demonstrated by liver biopsy and/or with compatible clinical, laboratory and imaging data were considered eligible for the study. Exclusion criteria were as follows: known HCC, pregnancy, previous orthotopic liver transplantation (OLT), refused to provide informed consent, use of anticoagulation, previous surgical or transjugular intrahepatic portosystemic shunt (TIPS).

Baseline data

Baseline abdominal Doppler-US was performed by 5 ultrasound experts with more than 10 years of experience at our center following a standard protocol[24] and evaluating the patency of the main portal vein trunk and both intrahepatic portal branches, portal blood flow velocity (PBFV – measured as time averaged maximal velocity), portal blood flow direction, portal vein trunk diameter, presence of porto-systemic collaterals, hepatic vein patency, spleen length, splenic artery pulsatility and resistance index and patency of the superior mesenteric and splenic vein if technically possible. For each patient, baseline clinical and laboratory data were collected. Blood samples at baseline were also stored at the Hospital Clinic Biobank facilities for an exhaustive evaluation of hemostatic and inflammatory parameters.

Follow-up

Patients were prospectively followed until February 2019 or until OLT, death, TIPS placement, start of anticoagulation for any reason, evidence of tumoral PVT or hepatic resection surgery. Additionally, because of the potential impact on the natural history of PVT in cirrhosis, patients with HCV who received antiviral treatment during follow-up were censored when sustained virological response (SVR) was achieved. Doppler-US was repeated every 6 months or whenever clinically indicated.

Portal vein thrombosis

PVT was diagnosed by demonstrating the presence of endoluminal material compatible with non-tumoral thrombosis in the portal vein and/or its branches. PVT diagnosis and its extension were always confirmed by computed tomography or magnetic resonance imaging. PVT was defined as occlusive when there was an absence of blood flow in the vein or partial when the lumen was only partially occluded and flow was still present.

Coagulation and inflammation evaluation

Hemostasis and fibrinolysis tests: Coagulation factors II, V, VII; VIII, IX, X, XI, XII, XIII, protein C, S and antithrombin activity, VWF, ADAMTS-13, fragment 1+2 (F1+2), activated factor VII (FVIIa), plasmin-antiplasmin complexes, D-dimer, plasminogen, plasminogen activator inhibitor-1, soluble P-selectin, soluble CD40L were determined as described previously.[25–27] The plasma capacity to generate thrombin was measured using a continuous thrombin-generation assay in an automated system as previously described.[28]

Microparticles were measured by ELISA capture assay based on the ability of annexin V (bonded onto plastic plates) to bind phosphatidylserine on circulating microparticles deposited in pellets (Hyphen BioMed, Neuville-sur-Oise, France). Functional assays were performed to measure the procoagulant activity of microparticles through thrombin generation, as previously described.[29] The measure was based on the activation of prothrombinase activity on the surface of the microparticles following the addition of activated bovine factors X and V and purified human prothrombin. After incubation, the thrombin generated was quantified using a specific chromogenic substrate by measuring absorbance at 405 nm. Microparticles were determined in duplicate and expressed as equivalents of nanomolar of phosphatidylserine (nM PS eq). The laboratory detection limit of the technique was 0.05 nM PS eq. and the intra-assay and inter-assay coefficient of variation were 5% and 8%, respectively.

NETs: complexes of myeloperoxidase and DNA (MPO-DNA) were quantified using a capture ELISA as previously described,[30] using commercially available antibodies (anti-MPO monoclonal antibodies from Sanbio, Uden, Netherlands, and peroxidase-labelled anti-DNA monoclonal antibodies from the cell death detection ELISA kit (Sigma Aldrich, Zwijndrecht, Netherlands). Cell-free DNA (cfDNA) concentration in plasma was measured using the Picogreen Quant-it kit (Fisher Scientific, Landsmeer, Netherlands).

Clot lysis time (CLT): The fibrinolytic capacity of a plasma sample was quantified by CLT assays. CLT was determined by measuring turbidity changes during clot formation and subsequent lysis of the clot, as described before.[31]

Clot retraction: Clot retraction with subsequent red cell extrusion from the clots was performed as described[32] in baseline samples of all PVT cases and 53 randomly selected non-PVT patients. Plasma was mixed with isolated platelets and red blood cells (RBCs) from healthy blood donors with blood group O to

obtain reconstituted blood containing 20,0000 platelets/μl and a hematocrit of 40%.[33] This reconstituted blood was clotted with human alpha-thrombin (Sekisui Diagnostics, Stamford, CT, USA, 0.1U/ml final concentration) and calcium chloride (10 mM final concentration) in siliconized wells for 2 hours at 37 °C. Clots were weighed after removing adherent liquid, and the hemoglobin level in the supernatant (diluted with PBS) was estimated by absorbance measurements at 575 nm. The percentage of extruded RBCs was calculated by comparing the absorbance of reconstituted blood and the supernatant after clot formation and retraction. By mixing patient plasma with healthy blood cells, this assay specifically quantifies the contribution of plasma fibrinogen to clot retraction and thus provides a measure for the functional properties of the fibrinogen molecule that is known to be altered in patients with liver disease.[34]

Permeability: Permeation of fibrin clots from PVT cases and from randomly selected non-PVT patients (n = 53) was measured using a liquid permeation assay as described previously.[35] The permeability coefficient Ks was calculated following Darcy's law. This assay provides a direct measure of fibrin clot quality; the relation between decrease permeability of fibrin clots and thrombotic risk has been well established,[36] as has the increased permeability of plasma clots in patients with cirrhosis.[34]

Fibrinogen concentration was determined on an automated coagulation analyser (ACL TOP 300, Werfen, Barcelona, Spain) with reagents from Werfen (Barcelona, Spain).

Concentrations of C-reactive protein, interleukin 6 (IL-6) and tumor necrosis factor-α were assessed with ELISA kits obtained from R&D Systems (Minneapolis, MN,USA).

Genotyping FVLeiden and Prothrombin 20210A: DNA isolated from whole blood samples was used to genotype using commercially available probes (Thermo Fisher Scientific, Waltham, MA, USA).

An inherited deficiency of protein C, S or antithrombin was excluded by establishing a ratio of protein C, S or antithrombin to (factor II+factor X)/2 of greater than 0.7 and by the study of first degree relatives whenever possible.[37]

Statistical analysis

Data are expressed as frequencies (%) for categorical variables and as mean ± standard deviation for continuous variables. Fisher's exact test was used for categorical variables and the paired Student's *t* test for continuous variables or paired non-parametric test when assumptions of normality could not be verified.

The (event-free) survival of patients was evaluated with Fine-Gray competing risk survival analysis. We estimated the cumulative incidence functions from competing risks data across groups: PVT (event of interest) or competing events (death, OLT, tumoral-PVT or TIPS). The predictors of PVT development were estimated by regression modeling of sub-distribution functions in competing risks analysis. Variables that showed a statistically significant effect on (event-free) survival in univariate analyses (p <0.10) or that were clinically relevant were entered into multivariate models, which were evaluated using clinical criteria and log-likelihood ratio test. For continuous variables, cut-offs were selected either by using the Youden method or based on already validated cut-offs in the literature. The number of variables that could enter the multivariate analysis was limited using the m/10 rule to prevent over-fitting. In addition, in the multivariate analysis, individual parameters were not considered when including scores that contain them (*i.e.* Child-Pugh). Similarly, variables that focus on the same specific hemostatic process were not evaluated together. Hemostatic parameters that were not evaluated in the entire cohort (clot retraction and permeability) were not included in the multivariate analysis.

Additionally, a time-dependent covariate analysis was performed to determine the potential role of NSBB use in PVT development, in a time-varying model adjusted by variceal hemorrhage and presence of large esophageal varices as longitudinal potential confounders. These variables were recorded every 6 months in the scheduled revisions during follow-up.

The level of significance was established at the 2-sided 5% level. Statistical analysis was performed using SPSS 23.0 (SPSS Inc. Chicago,IL) and R software for Windows version 3.6.1 (R project for Statistical Computing; Vienna, Austria).

Ethical aspects

All patients included in the study gave signed written informed consent to participate. The ethical committee of Hospital Clinic Barcelona approved the current study (HCB/2010/6107) in accordance with the International Guideline for Ethical Review of Epidemiological Studies and principles of the Declaration of Helsinki.

Results

Baseline Doppler-US was performed in 437 consecutive patients with cirrhosis initially considered eligible by 1 of the 5 ultrasound experts participating in the study. Thirty-seven patients had ≥1 exclusion criteria. Twenty-three patients (5.8%) had PVT at baseline (21 partial, 2 occlusive). Characteristics of patients with and without baseline PVT are described in Table S1. Eight of the 377 patients without PVT at baseline had no further follow-up at our institution. Thus, finally, 369 patients with cirrhosis without PVT were included in the prospective cohort and were followed-up for a mean of 48 ± 27 months (Fig. 1). 59% were male with a mean age of 59 ± 10 years. The most frequent etiologies of cirrhosis were HCV (56%) and alcohol (27%); 72% of patients had Child-Pugh A and 22% Child-Pugh B cirrhosis. One-hundred forty-five (39%) patients had large varices, 59 (16%) had a history of variceal hemorrhage and 148 (40%) had ascites. Table 1 shows the baseline characteristics of the study population.

During follow-up, 30 (8.1%) patients underwent an OLT, 7 (1.9%) underwent TIPS placement, 4 (1.1%) started anticoagulation, 4 (1.1%) required a large hepatic resection, 60 (16%) developed HCC and 9 (2.4%) tumoral-PVT. Additionally, 100 patients (27.1%) initiated antiviral therapy for HCV and their follow-up was stopped after 45 ± 14months. Twenty patients (5.4%) were lost to follow-up after 28 ± 16 months, mainly due to changing their reference hospital. Seventy-six patients died during follow-up and the overall OLT-free survival was 93.7%, 83% and 73.1% at 1, 3 and 5 years, respectively (Fig. 2).

PVT development

Twenty-nine patients developed non-tumoral PVT during follow-up. The cumulative incidence of PVT was 1.6%, 6% and 8.3% at 1, 3 and 5 years, respectively (Fig. 2). Thrombosis was occlusive in 3 patients and partial in 26. Table S2 provides details on the location and degree of thrombosis. Twenty-one patients (72%) were asymptomatic (2 occlusive; 19 partial) and the PVT was detected on scheduled Doppler-US. The remaining 8 patients (28%) were diagnosed during an extra Doppler-US performed at

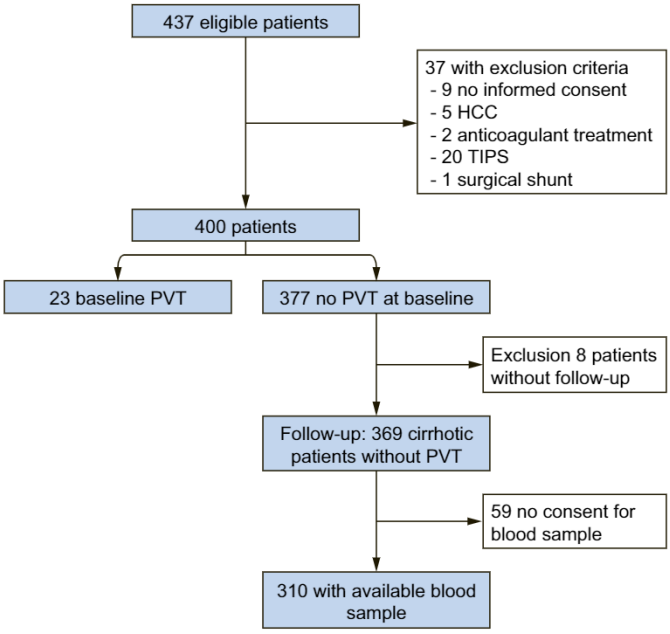

Fig. 1. Inclusion flow chart. PVT, portal vein thrombosis; TIPS, transjugular intrahepatic portosystemic shunt.

hospitalization: Seven with partial PVT presenting with variceal hemorrhage (n = 1), first ascites decompensation (n = 1), jaundice (n = 3), Salmonella gastroenteritis (n = 1), cholecystitis (n = 1) and 1 with occlusive PVT presenting with intestinal ischemia. All patients who developed PVT had a Doppler-US within the 6 previous months with no evidence of PVT. In those patients undergoing OLT without reaching the PVT endpoint, the surgical report was reviewed and no additional PVT was found. None of the 29 patients who developed PVT had HCC at the time of PVT diagnosis. After PVT diagnosis, 10 patients started anticoagulation, 1 received TIPS (as treatment for concomitant variceal bleeding) and 18 were in observation. Among these 18 non-anticoagulated patients, spontaneous recanalization occurred in 2, progression in 5 and PVT maintained stable in the other 11. Regarding their outcome, 12 patients died 22 ± 27 months after PVT and 6 patients underwent OLT after 18 ± 23 months, while the remaining 11 were alive at the end of follow-up.

Table 1. Baseline characteristics of the study population.

Variables	Mean ± SD/n(%)
Age,years	59 ± 10
Sex, male	217 (59%)
Body mass index, Kg/m^2	27.6 ± 4.4
Etiology:	
HCV	209 (56%)
HBV	17 (4.6%)
Alcohol	101 (27%)
MAFLD	13 (3.5%)
Platelets, 10^9/L	116 ± 59
INR	1.23 ± 0.24
Albumin, g/L	37 ± 6
Bilirubin, mg/dl	1.6 ± 1.5
Creatinine, mg/dl	0.88 ± 0.41
MELD	10 ± 4
Child-Pugh score	6 ± 2
Child-Pugh class	
A	264 (72%)
B	82 (22%)
Varices	230 (62%)
Large varices	145 (39%)
Any previous decompensation*:	177 (48%)
Ascites	148 (40%)
Spontaneous bacterial peritonitis	15 (4%)
Variceal bleeding	59 (16%)
Hepatic encephalopathy	51 (14%)
Endoscopic band ligation	46 (12.5%)
NSBBs	149 (40%)
Secondary prophylaxis	51 (13%)
Transient elastography, kPa (n = 253; 22 PVT)	29 ± 17
Spleen length, cm	14.5 ± 2.6
Portal vein diameter, mm (n = 361; 28 PVT)	12.5 ± 2.5
PBFV, cm/sec (n = 357; 28 PVT)	17.7 ± 5.6
PBFV <15 cm/sec	107 (29%)
Splenic artery resistance index	0.71 ± 0.1
Splenic artery pulsatility index (n = 348; 28 PVT)	1.3 ± 0.31
Porto-systemic collaterals (n = 330; 27 PVT)	144 (39%)
HVPG (n = 103;6 PVT)	15.3 ± 5
HVPG ≥10	94 (91%)
HVPG ≥16	45 (12%)
HVPG ≥20	21 (6%)

HVPG, hepatic venous pressure gradient; INR, international normalized ratio; MAFLD, metabolic dysfunction-associated fatty liver disease; MELD, model for end-stage liver disease; NSBBs, non-selective beta-blockers; PBFV, portal blood flow velocity; PVT, portal vein thrombosis.
*Some patients had more than 1 decompensation.

Risk factors for PVT

Factors associated with PVT development at univariate competing risk analysis are shown in Table 2. Multivariate analyses, including those variables significant at univariate analysis with a p value <0.10 identified 2 models: (model 1) platelet count, PBFV <15 cm/sec and variceal bleeding and (model 2) spleen length, PBFV <15 cm/sec and variceal bleeding. MELD and Child-Pugh score were not significant at multivariate analyses (Table 2). Model 1 had the best log-likelihood ratio and a PVT risk score was created by combining the sum of these 3 factors according to their HR to categorize patients based on their risk of developing PVT (Fig. S1).

Additionally, considering that PBFV is a variable not commonly assessed in clinical practice, we explored multivariate models excluding PBVF. The best model excluding PBFV was the combination of history of variceal bleeding (p = 0.0041; HR 3.09; 95% CI 1.43–6.68) and platelet count (p = 0.0026; HR 4.05; 95% CI 1.63–10.05).

Although NSBB use was not identified as a risk factor for PVT in the different multivariate analyses, and because it has been suggested to be involved in PVT development in some studies, an additional time-dependent analysis was performed considering changes in this treatment during follow-up, with evaluations every 6 months according to scheduled medical visits. Indeed, 24 patients started NSBB treatment and 13 patients discontinued treatment during follow-up. The model was adjusted by the confounders variceal bleeding and presence of large esophageal varices. Also, in this time-dependent analysis, NSBB use was not associated with PVT development (p = 0.71; HR 0.745; 95% CI 0.154–3.60]).

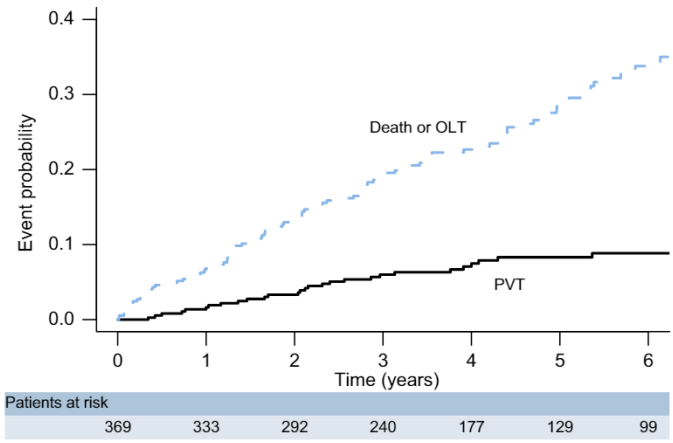

Fig. 2. Cumulative incidence for primary endpoint (PVT) and competitive event. OLT, orthotopic liver transplantation; PVT, portal vein thrombosis.

Hepatic venous pressure gradient evaluation

One-hundred and three patients had a hepatic venous pressure gradient (HVPG) measurement performed within 12 months before inclusion. Six of the 103 patients with an available HVPG developed PVT during follow-up. Median HVPG at baseline was higher in the 6 patients who developed PVT (19.4 ± 4.5 mmHg) compared to the 97 patients who did not (15.4 ± 5 mmHg), although this was not statistically significant ($p = 0.13$). All patients who developed PVT had clinically significant portal hypertension (HVPG above 10 mmHg) at baseline. Interestingly, 4 out of 6 patients (67%) who developed PVT had an HVPG above 20 compared with 17/97 (17%) who did not develop PVT ($p = 0.015$). In other words, 4/21 (19%) patients with HVPG >20 mmHg developed PVT while this only happened in 2/82 (2.4%) of those with an HVPG <20 mmHg. However, HVPG was not included in the multivariate analysis because it was only available in 103 patients.

Evaluation of the hemostatic and inflammatory profile

Three hundred and ten (84% of the included population) patients without PVT at baseline had a blood sample stored at the Biobank in which an exhaustive evaluation of hemostatic and inflammatory factors was performed. Twenty-three of them developed PVT during follow-up. The remaining 16% of patients did not consent to blood sampling.

Hemostatic and inflammatory markers associated with PVT development at univariate competing risk analysis are detailed in Table 3. As shown, patients developing PVT had lower levels of coagulation factors synthesized by the liver (either prothrombotic or antithrombotic factors) and a lower ETP with or without TM. However, these patients had significantly higher levels of soluble P-selectin, F1+2, VIIa, microparticles and a higher FVIII/PC ratio. No differences in NETs or other inflammatory markers were observed. Patients that developed PVT did not have an increased thrombogenicity of fibrin clots generated *in vitro*, except for a slight, but non-significant increase in CLT.

In the multivariate analysis, the variables that independently predicted PVT development were PBFV <15 cm/sec ($p = 0.035$; HR 2.66; 95% 1.07–6.61), spleen length ($p = 0.002$; HR 1.23; 95% CI 1.10–1.40) and levels of the pro-coagulant Factor X ($p = 0.036$; HR 0.97; 95% CI 0.94–0.99).

Table 2. Univariate and multivariate competing risk analysis for PVT development.

Univariate analysis			Multivariate analysis				
Variable	**sHR (95% CI)**	***p* value**		**Variables**	**sHR (95% CI)**	***p* value**	**Log-likelihood ratio test**
Body mass index, Kg/m^2	0.98 (0.89–1.09)	0.79	**Model 1**				
MAFLD	3.09 (0.98– 9.8)	**0.05**		Platelets	0.98 (0.97–0.99)	**0.002**	27
Platelets, 10^9/L	0.98 (0.97–0.99)	**<0.001**		PBFV <15 cm/sec	2.28 (0.99–5.26)	**0.05**	
INR	1.94 (1–3.07)	**0.049**		Variceal bleeding	2.52 (1.06–5.99)	**0.036**	
Albumin, g/L	0.93 (0.89–0.98)	**0.008**	**Model 2**				
Bilirubin, mg/dl	1.10 (0.97–1.24)	0.12		Spleen length	1.26 (1.11–1.42)	**<0.001**	25
Creatinine, mg/dl	0.25 (0.03–1.93)	0.18		PBFV <15 cm/sec	2.31 (1.02–5.26)	**0.046**	
MELD	1.05 (1–1.1)	**0.047**		Variceal bleeding	2.37 (0.99–5.67)	**0.05**	
Child-Pugh score	1.13 (0.99–1.28)	0.062	**Model 3**				
Child-Pugh class B/C	2.36 (1.14–4.88)	**0.021**		Child–Pugh score	1.00 (0.86–1.69)	0.94	22
Large varices	3.61 (1.64–7.94)	**0.001**		PBFV <15 cm/sec	2.92 (1.37–6.19)	**0.005**	
Previous decompensation	4.3 (1.77–10.5)	**0.001**		Platelets	0.98 (0.97–0.99)	**0.002**	
Variceal bleeding	3.37 (1.60–7.13)	**0.001**	**Model 4**				
Ascites	1.89 (0.91–3.96)	0.089		MELD	1.00 (0.93–1.06)	0.86	22
NSBBs	3.44 (1.57–7.53)	**0.002**		Variceal bleeding	2.91 (1.38–6.16)	**0.005**	
Primary prophylaxis	1.47 (0.68–3.16)	0.32		Platelets	0.98 (0.97–0.99)	**0.002**	
Secondary prophylaxis	3.54 (1.65–7.6))	**0.001**					
Spleen length, cm	1.28 (1.15–1.43)	**<0.001**					
Portal vein diameter, mm	1.10 (1.01–1.21)	**0.031**					
PBFV, cm/sec	0.91 (0.81–1.03)	0.15					
PBFV <15 cm/sec	2.70 (1.29–5.68)	**0.008**					
Porto-systemic collaterals	1.05 (0.57–1.91)	0.87					
HVPG, mmHg	1.10 (0.97–1.24)	0.13					
HVPG ≥20	8.08 (1.50–43.6)	**0.015**					

HVPG, hepatic venous pressure gradient; INR, international normalized ratio; MAFLD, metabolic dysfunction-associated fatty liver disease; MELD, model for end-stage liver disease; NSBBs, non-selective beta-blockers; PBFV, portal blood flow velocity; PVT, portal vein thrombosis; sHR, subdistribution hazard ratio.
Values in bold denote statistical significance.
Predictors were estimated by regression modeling of subdistribution functions in competing risks scenarios. The statistical test used to define these variable associations is the Wald test, which under the null hypothesis follows an asymptotic $\chi 2$-distribution with 1 degree of freedom.

Inherited thrombophilic disorders

The prothrombin G20210A mutation and FVLeiden were tested in 266 patients, 23 of them developing PVT during follow-up. None of these 23 patients had the prothrombin G20210A variant or were carriers of FVLeiden. Of the 243 patients that did not develop PVT, 5 patients were heterozygous and 1 was homozygous for the prothrombin 20210A variant, whereas 9 patients were heterozygous FVLeiden carriers.

Additionally, in the 21 patients who were excluded because of PVT at baseline, only 1 was heterozygous for the prothrombin 20210A variant and none were FVLeiden carriers. None of the 310 patients had a confirmed hereditary deficiency of protein C, S or antithrombin.

Discussion

This is the first study to prospectively address, in a large cohort of patients with cirrhosis, the risk factors for non-tumoral PVT development including a comprehensive number of clinical and ultrasonographic parameters together with an exhaustive evaluation of coagulation and inflammatory markers.

The observed cumulative incidence of PVT is in the lower range of that previously published[2–6] and our 5 year incidence of

Table 3. Competing risk univariate analysis of hemostatic and inflammatory evaluation.

Variables	PVT (n = 23)	No PVT (n = 287)	sHR (95% CI)	*p* value
Hemostatic proteins				
Primary hemostasis				
VWF (Ag,%)	120.9 ± 32	125.6 ± 27.3	0.99 (0.97–1.01)	0.54
VW ristocetin co-factor (functional,%)	108.8 ± 22.4	119.1 ± 26.3	0.98 (0.97–1)	0.051
ADAMTS13,%	98.6 ± 18.5	96.9 ± 17.3	1 (0.98–1.03)	0.69
Secondary hemostasis				
sTF,ng/ml	135.9 ± 25.5	143.5 ± 27.9	0.99 (0.98–1)	0.19
Factor II, %	52.8 ± 15	62.9 ± 16.6	0.96 (0.94–0.99)	**0.001**
Factor V, %	52 ± 12.9	62.2 ± 15.8	0.95 (0.93–0.98)	**0.001**
Factor VII, %	43.9 ± 12.6	52.6 ± 15.6	0.96 (0.95–0.99)	**0.002**
Factor VIII, %	125.8 ± 19.1	135.2 ± 23.3	0.981 (0.96–0.99)	**0.036**
Factor IX, %	57.5 ± 18.8	59.8 ± 19.1	0.99 (0.97–1.01)	0.49
Factor X, %	50.4 ± 12.1	61.2 ± 15.3	0.95 (0.92–0.97)	**<0.001**
Factor XI, %	62.5 ± 17	76.9 ± 19.6	0.96 (0.95–0.98)	**<0.001**
Factor XII, %	58.4 ± 14.4	65 ± 15.1	0.97 (0.95–0.99)	**0.024**
Factor XIIIa, %	61.2 ± 28.5	75.0 ± 36.1	0.99 (0.97–1.01)	0.18
Fibrinogen, mg/ml	2.24 ± 0.71	2.47 ± 0.84	0.68 (0.39–1.17)	0.16
Protein C, %	60.3 ± 20.7	79.0 ± 23.9	0.97 (0.95–0.98)	**<0.001**
Protein S, %	66.7 ± 17.9	81.5 ± 20.4	0.97 (0.95–0.98)	**<0.001**
Antithrombin, %	75.5 ± 17.9	84.1 ± 18.8	0.98 (0.96–0.99)	**0.02**
Fibrinolysis				
Plasminogen, %	53.7 ± 11.5	60.8 ± 13.1	0.96 (0.94–0.99)	**0.002**
PAI-1, ng/ml	26.7 ± 7.6	23.3 ± 7.1	1.05 (1.01–1.09)	**0.017**
Markers of activation of hemostasis				
Soluble P-Selectin, ng/ml	73.7 ± 18	63.3 ± 24.2	1.02 (1–1.03)	**0.012**
Soluble CD40L, ng/ml	98.3 ± 19.7	104.5 ± 21.9	0.98 (0.97–1)	0.14
Fragment 1+2, nmol/ml	1.8 ± 0.8	1.4 ± 0.5	2.51 (1.47–4.27)	**<0.001**
Factor VIIa, ng/ml	3.4 ± 1.8	2.4 ± 1.5	1.29 (1.11–1.51)	**0.001**
Factor XIIa, ng/ml	3.8 ± 1.7	3.3 ± 1.6	1.17 (0.96–1.43)	0.11
D-dimer, ng/ml	466.8 ± 225.8	460.9 ± 221.2	1 (0.99–1)	0.95
PAP, µg/ml	1024.1 ± 268.4	949.6 ± 338.46	1(1–1)	0.21
Microparticles	24.3 ± 10.4	18.0 ± 9.1	1.05 (1.02–1.08)	**<0.001**
Global functional tests				
ETP (without TM), nM IIa*min	322.9 ± 27.7	349.8 ± 60.6	0.98 (0.97–0.99)	**<0.001**
ETP (with TM), nM IIa*min	268.6 ± 24.2	282.9 ± 44.3	0.98 (0.97–0.99)	**0.009**
Clot lysis time, min	82 ± 40	71 ± 25	1.01 (0.99–1.02)	0.072
Permeability, Ks	$4.2 \times 10^{-9} \pm 1.9 \times 10^{-9}$	$4.7 \times 10^{9} \pm 8 \times 10^{-9}$	0.99 (0.95–1.03)	0.61
Clot weight, mg	55 ± 10	60 ± 9	0.95 (0.90–0.99)	**0.042**
Ratios				
Von Willebrand ratio (VWF co-factor/Ag)	0.96 ± 0.33	0.98 ± 0.27	0.76 (0.11–5.01)	0.78
Ratio FVIII/Protein C	2.34 ± 0.98	1.89 ± 0.75	1.58 (1.17–2.14)	**0.0028**
ETP ratio (with/without TM)	0.83 ± 0.08	0.81 ± 0.06	1.70 (0.77–3.72)	0.20
Inflammatory markers				
Cell-free DNA, ug/ml	0.89 ± 0.16	0.89 ± 0.22	0.97 (0.22–4.27)	0.97
MPO-DNA (AU)	0.21 ± 0.29	0.29 ± 0.46	0.68 (0.28–1.67)	0.40
IL-6, pg/ml	7.7 ± 7.9	8.4 ± 12.5	0.99 (0.97–1.02)	0.70
TNF-α,pg/ml	12.4 ± 5.1	11.6 ± 10.5	0.01 (0.99–1.03)	0.32
CRP, ng/ml	5315 ± 8044	3584 ± 6631	1 (1–1)	0.24

CRP, C-reactive protein; ETP, endogenous thrombin potential; IL-6, interleukin-6; MPO-DNA, complexes of myeloperoxidase and DNA; PAI-1, plasminogen activator inhibitor-1; PAP, plasmin/antiplasmin complex; sTF, soluble tissue factor; TM, thrombomodulin; TNF, tumor necrosis factor; VWF, von Willebrand factor.
Values in bold denote statistical significance.
Predictors were estimated by regression modeling of subdistribution functions in competing risks scenarios. The statistical test used to define these variable associations is the Wald test, which under the null hypothesis follows an asymptotic $\chi 2$-distribution with 1 degree of freedom.

8.3% compares with the previously study from Nery *et al.*, especially when considering the 6-month ultrasound screening strategy (8%).[3] We are confident that this low incidence is real and not due to false negative ultrasound studies. Indeed, this is a prospective study specifically aimed at ruling out PVT and US-Doppler was always performed by the same 5 US experts.

Our study shows that the severity of portal hypertension, estimated by history of variceal bleeding and low platelet count, together with low PBFV are the main factors associated with PVT development. Different studies have already described previous decompensation and low platelet count as potential risk factors for PVT.[4,6,10] Controversies were raised about the reproducibility of PBFV measurements and therefore their potential role as a determinant factor for PVT development.[7] Our study clearly shows that accurate measurement of PBFV, by an experienced professional, is a useful predictive tool for PVT development. Indeed, our study confirms the previously described threshold of 15 cm/sec[4,9] as an independent risk factor for PVT.

Patients with cirrhosis have been shown to have a fragile rebalanced hemostatic system with a decrease in both pro and anticoagulant factors.[15] It has been suggested that, in different situations, this balance can be lost and a hypo or hypercoagulable state develops. Some studies have evaluated the potential role of the hemostatic state of patients with cirrhosis in promoting PVT development. Indeed, FVIII,[18] low ADAMTS13,[19] thrombomodulin resistance in a thrombin generation test[17] and an increased FVIII/protein C ratio[16] have been proposed as risk factors for PVT. However, previous studies have not comprehensively analyzed a full hemostatic profile or considered its potential independent role of other relevant variables such as severity of liver disease or PBFV in relation to PVT development. Our study confirms that patients with cirrhosis developing PVT had significantly reduced levels of several anticoagulant factors as well as a significant increase in the FVIII/protein C ratio, suggesting that a hypercoagulable state may also play a role in PVT development. When these results were adjusted, in different statistical models, by clinical and ultrasonographic variables, low levels of the liver synthesized procoagulant factor X were the only coagulation parameter that independently predicted PVT. Therefore, these data suggest that alterations of most of these acquired defects are a consequence of the more advanced liver disease state.

A well-known relationship exists between inflammation and coagulation.[38] Indeed, increased IL-6 levels have been reported to be associated with the presence of PVT in cirrhosis[39] and NETs have previously been implicated in thrombotic disease, such as deep vein thrombosis or myocardial infarction,[23] but their specific role in PVT development in the setting of cirrhosis has never been evaluated. In addition, NETs have been implicated in progression of various liver diseases[40] and have been shown to be associated with activation of coagulation during liver transplant surgery.[30] With this background, we decided to evaluate the potential role of several inflammatory markers in predicting PVT development. Our study did not find any association between the inflammatory markers evaluated, including IL-6 or NETs markers (MPO-DNA or cfDNA), and the risk of PVT development. Thus, our results do not support that cirrhosis-associated inflammation or NETs predict PVT development in cirrhosis. However, it cannot be excluded that an acute inflammatory response or an acute increase in the generation of NETs rather than baseline inflammatory status, or even a local increase of circulating inflammatory markers in the portal vein,[41] may drive PVT development.

In our broad evaluation of factors promoting blood hypercoagulability, we also evaluated the potential role of inherited coagulation disorders. Previous studies, including a recent meta-analysis, suggested that inherited thrombophilic factors such as FVLeiden and prothrombin G20210A mutation might play a role in PVT development in patients with cirrhosis.[20] Nevertheless, this has not been confirmed in other studies.[3,21] Most of these studies were retrospective and with a high probability of selection bias, therefore the potential role of the inherited prothrombotic factors was uncertain. In the current study, none of the 23 patients developing PVT of the 266 patients in whom inherited disorders were tested were carriers of FVLeiden or the prothrombin G20210A variant. The results of our study therefore argue against a role for these inherited disorders in PVT development in patients with cirrhosis.

Endothelial dysfunction/injury, reduction in blood flow and hypercoagulability are the 3 main pathophysiological mechanisms leading to venous thrombosis. Although we did not specifically evaluate the severity of endothelial dysfunction/injury, several clinical and experimental studies have shown a clear relationship between endothelial dysfunction and the severity of portal hypertension in cirrhosis.[42,43] History of variceal bleeding and low platelets are highly likely a reflection of a more severe degree of portal hypertension and of endothelial dysfunction. It is important to remark that in the subgroup of patients in whom HVPG was available, it was significantly higher in those patients who developed PVT. All patients developing PVT had an HVPG ≥10 mmHg and the risk seems to be much increased when HVPG is >20 mmHg. Additionally, it is clear that in the portal venous system there is a close relationship between endothelial dysfunction and portal hypertension, since endothelial dysfunction is one of the main mechanisms leading to an increased hepatic resistance to portal blood flow.[44] Increased portal pressure promotes the development of porto-collateral circulation (resulting in clinically relevant esophageal varices) diverting part of the portal blood flow to the systemic circulation, bypassing the liver and therefore reducing PBFV in the portal vein. Therefore, these 2 components, although independent, synergize with each other. Therefore, the results of our study suggest that PVT in patients with cirrhosis is mainly related to changes in portal blood flow and to endothelial dysfunction/injury while the role of hypercoagulability, either acquired or inherited, seems to be minor.

To shed more light on the controversy surrounding the use of NSBBs, we evaluated the role of NSBBs on PVT risk in a time-dependent analysis. Taking into consideration NSBB use, presence or development of esophageal varices and variceal bleeding, no association was found between NSBBs and PVT development. This observation goes against that from a recently published meta-analysis that reported a 4.6-fold increase in the risk of PVT with NSBB use.[14] However, it included 9 heterogeneous studies, mainly retrospective, with limited follow-up and without considering dynamic changes in NSBB use, variceal bleeding and variceal size. Thus, according to our results, the presence of risk factors for PVT development should not preclude the use of NSBBs if indicated.

Other factors previously suggested as potential risk factors for PVT such as obesity,[45] large esophageal varices, previous variceal endoscopic treatment[8,13,14] or etiology of liver disease, especially

metabolic dysfunction-associated fatty liver disease (MAFLD)[8,13,46,47] were not confirmed in our prospective study. However, only 13 patients in our cohort had MAFLD, so no firm conclusions can be drawn.

Combining the 3 variables with independent predictive value we were able to build a PVT risk score that identified 2 different populations (Fig. S1). Using our score, for every 4 high-risk patients that develop PVT after 4 years of follow-up, 1 low-risk patient will develop PVT. However, this model should be validated in future prospective cohorts.

We have to acknowledge some limitations of the study. First, the low number of *de novo* cases of PVT during follow-up reduces the number of parameters that can be included in the multivariate analysis and therefore some additional parameters related to PVT development may be missed. Although our study shows that the severity of portal hypertension is one of the strongest parameters related to PVT development, most patients included in the study were Child-Pugh A. Whether the risk factors for PVT are the same in Child-Pugh B and C patients is not known. Additionally, this is a single-center study and, although a multicenter study would have allowed for a larger sample, the design of our study had the advantage of maximizing the homogeneity of the data. Secondly, blood samples (to determine hemostatic and inflammatory status) were taken at inclusion rather than immediately before PVT development. Thus, while these factors are not predictive of PVT development according to our results, it cannot be excluded that they may play a pathophysiological role in a given situation (*i.e.* acute increase of inflammatory markers). Third, patients who achieve SVR after HCV antiviral treatment were censored due to its potential impact on the natural history of cirrhosis and PVT. This was inevitable since it would have been unethical to delay or withhold effective treatment for HCV patients after it became broadly available. However, this only occurred in 100 patients and their follow-up (45 ± 14 months) did not differ significantly from the follow-up of the whole cohort; thus, we think this is likely to have had minimal impact on our results.

In conclusion, factors related to more severe portal hypertension, including PBFV <15 cm/sec, low platelet count and history of variceal bleeding are independently associated with a higher risk of developing PVT in the setting of cirrhosis. Our results do not support that acquired hypercoagulability alterations observed in patients with cirrhosis, inherited disorders of coagulation or inflammatory status were predictive of PVT development. In addition, NSBB use was not independently associated with PVT risk.

Abbreviations

cfDNA, cell-free DNA; CLT, clot lysis time; Doppler-US, Doppler ultrasound; EBL, endoscopic band ligation; ETP, endogenous thrombin potential; FVIII, Factor VIII; FVLeiden, Factor V Leiden; HCC, hepatocellular carcinoma; HVPG, hepatic venous pressure gradient; IL-6, interleukin 6; MAFLD, metabolic dysfunction-associated fatty liver disease; MELD, model for end-stage iver disease; MPO-DNA,complexes of myeloperoxidase and DNA; NETs, neutrophil extracellular traps; NSBBs, non-selective beta-blockers; OLT, orthotopic liver transplantation; PBFV, portal blood flow velocity; PI, pulsatility index; PVT, portal vein thrombosis; RI, resistance index; sHR, subdistribution hazard ratio; sTF, soluble tissue factor; SVR, sustained virological response; TIPS, transjugular intrahepatic portosystemic shunt; TM, thrombomodulin; VWF, von Willebrand factor.

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Conflict of interest

Prof JB is a consultant for Gilead Science, Surrozen, Actelion, BMS, Biovie and BLB. Prof JCGP is a consultant for GORE and research grants from NOVARTIS. AGC receives speaker fees from BTG and Terumo.

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

Authors’ contributions

Fanny Turon: study concept and design, acquisition of data, analysis and interpretation of data, statistical analysis, drafting of the manuscript. Ellen G. Driever: Performed laboratory tests, analysis and interpretation of data, critical revision of the manuscript for important intellectual content. Anna Baiges: acquisition of data, drafting of the manuscript. Eira Cerda: acquisition of data, drafting of the manuscript. Ángeles Garcia-Criado: ultrasound acquisition, drafting of the manuscript. Rosa Gilabert: ultrasound acquisition, drafting of the manuscript. Concepció Bru: ultrasound acquisition, drafting of the manuscript. Annalisa Berzigotti: ultrasound acquisition, drafting of the manuscript. Isabel Nuñez: ultrasound acquisition, drafting of the manuscript. Lara Orts: acquisition of data, obtaining blood samples for biobank, monitoring patients. Juan Carlos Reverter: Performed and supervised laboratory tests, analysis and interpretation of data, critical revision of the manuscript for important intellectual content. Marta Magaz: acquisition of data, drafting of the manuscript. Genis Camprecios: acquisition of data, drafting of the manuscript. Pol Olivas: acquisition of data, drafting of the manuscript. Fanian Betancourt-Sanchez: acquisition of data, drafting of the manuscript. Valeria Perez-Campuzano: acquisition of data, drafting of the manuscript. Annabel Blasi: analysis and interpretation of data, drafting of the manuscript. Susana Seijo: acquisition of data, drafting of the manuscript. Enric Reverter: acquisition of data, drafting of the manuscript. Jaume Bosch: obtained funding, critical revision of the manuscript for important intellectual content. Roger Borràs: Analysis and interpretation of data, drafting of the manuscript. Virginia Hernandez-Gea: critical revision of the manuscript for important intellectual content. Ton Lisman: Supervised laboratory tests, analysis and interpretation of data, obtained funding, critical revision of the manuscript for important intellectual content. Juan Carlos Garcia-Pagan: study concept and design, analysis and interpretation of data, statistical analysis, drafting of the manuscript, critical revision of the manuscript for important intellectual content, obtained funding and study supervision.

All the authors read and approved the final version of the paper.

Data availability statement

The data used in the manuscript are available in case of need.

Supplementary data

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Author names in bold designate shared co-first authorship

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