


# More Pronounced Hypercoagulable State and Hypofibrinolysis in Patients With Cirrhosis With Versus Without HCC

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In patients with cirrhosis, particularly those with hepatocellular carcinoma (HCC), hypercoagulability may be associated with purported increased risks of portal vein thrombosis and cirrhosis progression. In this study, we extensively investigated hemostatic alterations potentially responsible for the thrombotic tendency in HCC, and evaluated whether such alterations were predictive of hepatic decompensation. Patients with cirrhosis at all stages were prospectively recruited and underwent an extensive hemostatic assessment, including all procoagulant factors and inhibitors, thrombin generation with and without thrombomodulin (TG), profibrinolytic and antifibrinolytic factors, and plasmin-antiplasmin complex. In study part 1 (case control), we compared alterations of coagulation and fibrinolysis in patients with cirrhosis with versus without HCC. In study part 2 (prospective), the subgroup of patients with decompensated cirrhosis was followed for development of further decompensation, and predictors of outcome were assessed by multivariate analysis. One-hundred patients were recruited (50 each with and without HCC). Severity of cirrhosis was comparable between groups. Median HCC volume was 9 cm<sup>3</sup> (range: 5-16). Compared with controls, patients with HCC demonstrated a significantly more prothrombotic hemostatic profile due to increased TG and reduced activation of fibrinolysis, independent of cirrhosis stage. During a median follow-up of 175 days, 20 patients with decompensated cirrhosis developed further episodes of decompensation that were predicted by low FVII and high plasminogen activator inhibitor-1 levels, independent of Model for End-Stage Liver Disease score. **Conclusion:** Patients with cirrhosis with HCC have profound hyper-coagulable changes that can account for their increased thrombotic tendency. In contrast, hypercoagulability in patients with decompensated cirrhosis is more likely a consequence of chronic liver disease rather than a driver for cirrhosis progression. (*Hepatology Communications* 2021;5:1987-2000).

Patients with cirrhosis have complex alterations of hemostasis that include low platelet count and increased levels of von Willebrand factor, reduced hepatic synthesis of both procoagulant factors and inhibitors, and altered fibrinolysis.<sup>(1)</sup>

Current theory posits that these alterations result in a *rebalanced* but precarious hemostatic system that may easily tilt toward either hypocoagulability (increased bleeding tendency) or hypercoagulability (increased clotting tendency).<sup>(2-4)</sup>

*Abbreviations:* ACLF, acute-on-chronic liver failure; AFP, alpha-fetoprotein; AT, antithrombin; CI, confidence interval; ETP, endogenous thrombin potential; FII, factor II; FIX, factor IX; FV, factor V; FVII, factor VII; FVIII, factor VIII; FX, factor X; FXI, factor XI; FXII, factor XII; FXIII, factor XIII; HCC, hepatocellular carcinoma; INR, international normalized ratio; MELD, Model for End-Stage Liver Disease; OR, odds ratio; PAI-1, plasminogen activator inhibitor-1; PC, protein C; PS, protein S; PVT, portal vein thrombosis; TAFI a/ai, activated/inactivated thrombin-activatable fibrinolysis inhibitor; TFPI, tissue factor pathway inhibitor; TGA, thrombin generation assay; TM, thrombomodulin; t-PA, tissue plasminogen activator antigen; VTE, venous thromboembolism.

Received May 4, 2021; accepted June 17, 2021.

Additional Supporting Information may be found at [onlinelibrary.wiley.com/doi/10.1002/hep4.1781/supinfo](https://onlinelibrary.wiley.com/doi/10.1002/hep4.1781/supinfo).

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Supported by a research grant from the Italian Ministry of Education, University and Research (to P.S.). The funders had no role in study design, data collection and analysis, or preparation of the manuscript.

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In patients with cirrhosis, however, it has yet to be demonstrated whether alterations of hemostasis are truly responsible for the development of thrombotic complications, including portal vein thrombosis (PVT).<sup>(5)</sup> In a recent study including 310 patients with mostly compensated cirrhosis followed prospectively for a median of 48 months, Turon et al. found that portal vein flow <15 cm/s, but not plasmatic hypercoagulability, was predictive of PVT.<sup>(6)</sup>

On the other hand, the pathogenesis of PVT in cirrhosis is multifactorial and likely results from alterations in one or more components of the Virchow's triad (i.e., decreased portal vein inflow, hypercoagulability, and local damage of the portal vein wall).<sup>(5)</sup> Therefore, it is plausible that a more profound hypercoagulable state, at least in some patients, could contribute to the development of this thrombotic complication.<sup>(5)</sup>

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer occurring in patients with cirrhosis,<sup>(7)</sup> and is associated with a purported increased risk of thrombosis, particularly PVT.<sup>(8-11)</sup>

Cancer is a well-known risk factor for venous thromboembolism (VTE).<sup>(12)</sup> Development of VTE in patients with cancer is related to plasmatic hypercoagulability triggered by neoplastic cells with procoagulant activity, host response to cancer, and additional factors such as chemotherapy, immobilization, and infections.<sup>(13)</sup>

Our group previously demonstrated that HCC is associated with increased platelet number and function, higher concentration of plasmatic fibrinogen, and

elevated levels of plasma circulating microvesicles,<sup>(11,14,15)</sup> which all are potential risk factors for VTE.<sup>(16-18)</sup>

On the other hand, the effect of HCC on coagulation and fibrinolysis, which may also be implicated in cancer-associated thrombosis,<sup>(19-21)</sup> has not been investigated yet.

Understanding the factors responsible for the purported increased thrombotic tendency in patients with cirrhosis and HCC may have significant implications for the prevention and treatment of these potentially life-threatening complications.<sup>(22,23)</sup>

Therefore, our first objective in this study was to extensively assess alterations of coagulation and fibrinolysis in patients with cirrhosis and HCC.

Hypercoagulability in cirrhosis, however, may be implicated not only in macrovascular venous thrombosis,<sup>(24)</sup> but also in sinusoidal micro-thrombosis and parenchymal extinction.<sup>(25)</sup> In fact, in a seminal randomized control trial including patients with *decompensated* cirrhosis, anticoagulant treatment with enoxaparin was associated not only with reduced incidence of PVT, but also with lower risks of disease progression and liver decompensation.<sup>(26)</sup> To our knowledge, however, no study has yet investigated the correlation between markers of hypercoagulability and risk of liver decompensation.

Therefore, our secondary objective was to determine, in the subgroup of patients with *decompensated* cirrhosis, whether alterations of hemostasis, particularly markers of hypercoagulability as assessed by thrombin generation, were predictive of further decompensation.

DOI 10.1002/hep4.1781

Potential conflict of interest: Nothing to report.

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## Materials and Methods

### PATIENT SELECTION

Adult (>18 years) patients with cirrhosis at all stages who attended or were referred to the liver clinics of Multivisceral Transplant Unit, Gastroenterology, and Hepatobiliary Surgery and Liver Transplantation Center of Padova University Hospital from July 1, 2020, to October 30, 2020, were prospectively screened to determine eligibility to participate in the study.

The diagnosis of cirrhosis was confirmed with available data including histology, radiology, laboratory, and clinical assessment. Decompensation was defined by the presence or history of clinically evident decompensating events (ascites, variceal hemorrhage, and hepatic encephalopathy).<sup>(27)</sup> Diagnosis of HCC was based on the European Association for the Study of the Liver clinical practice guidelines.<sup>(7)</sup>

Patients with history of HCC but no evidence of active tumor at the last available imaging, patients with HCC but with a treatment-free interval <60 days at time of screening, and patients with history of mixed HCC-cholangiocarcinoma were not eligible.

At screening, patient's medical records, past medical history, and laboratory data were reviewed for the following exclusion criteria: history of major bleeding or portal hypertensive-related bleeding and/or bacterial infections in the 30 days before evaluation<sup>(28)</sup>; acute kidney injury or chronic kidney dysfunction<sup>(29)</sup>; presence or history of extrahepatic tumors or known hematologic diseases; recent (within 30 days) major surgery; human immunodeficiency virus (HIV) infection, history of any organ transplantation; antiplatelets and/or anticoagulant therapy; treatment with selective serotonin reuptake inhibitors; and recent (1-week) transfusion of any blood product.

Upon screening and having determined eligibility to participate, patients with cirrhosis were categorized into cases (with HCC) and controls (without HCC) and underwent an extensive hemostatic assessment.

A third group of age-matched and sex-matched healthy subjects were recruited as controls for hemostatic assessment. This group constituted of 53 healthy controls with no history of acute or chronic disease. None of these controls were taking antithrombotic, anticoagulant, antibiotic, or hormonal therapy.

### STUDY DESIGN AND OBJECTIVES

This was a two-part study. In study part 1 (case control), we compared alterations of coagulation and fibrinolysis in patients with cirrhosis with HCC (cases) versus patients with cirrhosis without HCC (controls). This constituted the main part of this study and our primary objective. In study part 2 (prospective), the subgroup of patients with decompensated cirrhosis were followed prospectively for development of further decompensation, and predictors of outcome were assessed by multivariate analysis (secondary objective). Further decompensation was defined as development of recurrent variceal hemorrhage, refractory ascites, sepsis, hepatorenal syndrome, recurrent hepatic encephalopathy, acute-on-chronic liver failure (ACLF), or a combination thereof.<sup>(27,30)</sup>

The study was approved by the Padova University Hospital Ethical Committee (protocol #0034435-08/06/20) and was conducted in compliance with the Declaration of Helsinki. All patients gave written, informed consent before enrollment.

### SAMPLE COLLECTION AND HEMOSTASIS ASSESSMENT

#### Blood Sampling

At inclusion, peripheral blood was collected through venipuncture in citrate-containing vacutainer tubes with 0.109 M (3.2%) sodium citrate (9:1 blood to anticoagulant ratio). The first few milliliters were discarded. Platelet-poor plasma was prepared within 1 hour by double centrifugation ( $2 \times 10$  minutes at 1,500g) at room temperature. Aliquots (1 mL) were immediately frozen and then stored at  $-80^{\circ}\text{C}$  until use.

#### Hemostasis Assessment

Hemostasis assessment included coagulation and fibrinolysis. All tests were performed at the Coagulation Lab of the Thrombotic and Hemorrhagic Diseases Unit of Padova University Hospital.

### EVALUATION OF COAGULATION

#### Procoagulant Factors and Inhibitors

The following procoagulant factors and inhibitors were determined: fibrinogen (n.v. 150–450 mg/dL), factor II (FII, n.v. 80%–120%), factor V (FV, n.v.

80%-120%), factor VII (FVII, n.v. 80%-120%), factor VIII (FVIII, n.v. 60%-160%), factor IX (FIX, n.v. 80%-120%), factor X (FX, n.v. 80%-120%), factor XI (FXI, n.v. 80%-120%), factor XII (FXII, n.v. 80%-120%), protein C coagulometric (PC coag, n.v. 80%-120%), protein C chromogenic (PC chromo, n.v. 70%-130%), protein S coagulometric (PS, n.v. 70%-130%), antithrombin (AT, n.v. 80%-120%), and tissue-factor pathway inhibitor (TFPI, n.v. 51.2-110.6 ng/mL), as previously described.<sup>(31-34)</sup> See the Supporting Information for more information.

### Thrombin Generation Assay With and Without Thrombomodulin

Thrombin generation assay (TGA) was determined in platelet-poor plasma with the calibrated automated thrombogram method (Thrombinoscope BV, Maastricht, the Netherlands), as previously described.<sup>(4)</sup> Briefly, 80  $\mu$ L of plasma was dispensed into the wells of a 96-well microtiter plate, and coagulation was triggered with 20  $\mu$ L of platelet-poor plasma (Reagent Low; Thrombinoscope BV), a mixture of tissue factor (1 pmol/L final concentration) and synthetic phospholipids (4  $\mu$ mol/L final concentration). The reaction was initiated by adding 20  $\mu$ L of a mixture consisting of a thrombin fluorogenic substrate and CaCl<sub>2</sub> (FluCa-Kit; Thrombinoscope BV). Thrombin Calibrator (Thrombinoscope BV) was used to correct each curve for inner filter effects and substrate consumption. Fluorescence was read in a Fluoroskan Ascent reader (Thermo LabSystems, Helsinki, Finland), and thrombin generation curves were calculated using the Thrombinoscope Software version 5.0.0.742 (Thrombinoscope BV). Thrombin generation curves were described in terms of lag time, time to peak, peak thrombin, velocity index (slope), and area under the curve, known also as endogenous thrombin potential (ETP) (Supporting Fig. S1).

TGA was run both with and without thrombomodulin (TM), with concentration of 1.5 nmol/L (rabbit TM; Sekisui Diagnostics, Stamford, CT). TM is the main cofactor in the thrombin-induced activation of the natural anticoagulant PC, and its addition brings in the effect of the PC in the TGA. In normal plasma, the addition of TM leads to a reduction in the generation of thrombin (reduction of ETP). In this study, the concentration of TM (1.5 nmol/L) was chosen to reduce the ETP by 54%

in normal pool plasma (resulting in an ETP ratio of 0.46).

Plasma from 53 normal healthy subjects was also tested to evaluate the effect of TM on ETP and acted as control group for TGA. This group consisted of 23 males and 30 females without history of cardiovascular, autoimmune and acute diseases, and not taking antithrombotic, antibiotic, and hormonal therapy. All tests were performed in duplicate.

The ETP ratio was calculated as follows: ETP with TM/ETP without TM, reflecting the "resistance" to the anticoagulant effect of PC. The lower the ETP ratio, the better preserved the level and the function of PC. Conversely, higher ETP ratio means more severe PC deficiency/PC resistance and a potentially greater susceptibility for thrombosis.

### EVALUATION OF FIBRINOLYSIS

The following fibrinolytic factors were determined: plasminogen (n.v. 75%-140%), alfa-2 antiplasmin (n.v. 80%-120%), factor XIII (FXIII, n.v. 70%-140%), plasminogen activator inhibitor-1 antigen (PAI-1; n.v. 1-25 ng/mL), tissue plasminogen activator antigen (t-PA, n.v. <10 ng/mL), and activated inactivated thrombin-activatable fibrinolysis inhibitor (TAFI a/ai; n.v. 8.5-22.1 ng/mL), as previously described.<sup>(4,35)</sup> Plasmin-antiplasmin complexes were determined as marker of fibrinolysis activation, as previously described.<sup>(4,35)</sup> See the Supporting Information for more information.

### DATA COLLECTION

Data collected from the medical record included patient demographics, etiology and severity of cirrhosis, laboratory data (including conventional coagulation parameter international normalized ratio [INR]), and development of further decompensation.

Model for End-Stage Liver Disease (MELD) score and Child class were calculated based on biochemical values and clinical characteristics from the day of enrollment.

In patients with HCC, the following variables were also collected: number of nodules, size of each nodule, presence of extrahepatic spread, history of previous treatments for the HCC, and HCC stage according to the Barcelona Clinic Liver Cancer staging.<sup>(7)</sup>

Total tumor volume was calculated as the sum of the volumes of all tumors according to the following



formula:  $(4/3) \pi r^3$ , where  $r$  is the maximum radius of each nodule of HCC.

## DATA ANALYSIS

### Sample Size Determination

Sample size was calculated for primary objective. We previously demonstrated that HCC in cirrhosis was associated with a significantly higher platelet count ( $122 \times 10^9/L$  vs.  $87 \times 10^9/L$  in HCC vs. non-HCC, respectively).<sup>(11)</sup> Because when designing this protocol we did not find any study regarding thrombin generation in patients with cirrhosis with HCC, we based our sample size on that difference. Sample size was calculated assuming a continuous endpoint compared between two independent groups, two-sided type I error of 0.05, and statistical power of 0.90. The required per-group sample size was 48.

### Statistical Analysis

Qualitative data are described using frequency and percentage. Quantitative data are described using median with 25% and 75% quartile ranges. Comparison between independent groups was performed using the Mann-Whitney U test and t-test for continuous variables, and chi-square test of Fisher's exact test for categorical variables. Univariate logistic regression analyses were performed to evaluate parameters significantly associated with development of further decompensation (study part 2). Only variables highly significant associated ( $P \leq 0.01$ ) were included in multivariate analysis. Because of the relatively small sample size and the low number of clinical outcomes, only two variables at time were entered in the multivariate analysis. Odds ratios (ORs) with 95% confidence intervals (CIs) for association were calculated. Statistical significance was set at  $P \leq 0.05$ . All analyses were completed using SPSS version 26.

## Results

### DEMOGRAPHICS

Of the 142 patients with cirrhosis screened for recruitment, 100 were included (50 each with and without HCC). Reason for exclusion were as follows:

history of mixed HCC-cholangiocarcinoma ( $n = 2$ ), no active HCC ( $n = 4$ ), recent treatments for HCC ( $n = 6$ ), chronic kidney disease ( $n = 4$ ), recent bacterial infection ( $n = 3$ ), recent variceal bleeding ( $n = 2$ ), previous transplantation ( $n = 3$ ), extrahepatic tumors ( $n = 4$ ), hematological disease ( $n = 1$ ), recent surgery ( $n = 2$ ), HIV ( $n = 1$ ), antiplatelet therapy ( $n = 6$ ), recent transfusions of blood product ( $n = 2$ ), and refusal to participate ( $n = 2$ ).

Baseline characteristics are presented in Table 1. Demographics, severity of cirrhosis (MELD score and Child class), and standard lab tests assessing hemostasis (platelet count and INR) were comparable between patients with and without HCC.

In contrast, alpha-fetoprotein (AFP) was significantly higher in patients with HCC (9 ng/mL [range: 4-47] vs. 3 ng/mL [range: 2-4] vs. non-HCC). Among patients with HCC, 20% had AFP  $\geq 100$  ng/mL and 8% had AFP  $\geq 400$  ng/mL, respectively.

Median total tumor volume was 9 cm<sup>3</sup> (range: 5-16). According to the Barcelona Clinic Liver Cancer staging, 29% patients had stage 0/A, 57% had stage B, and 14% had stage C/D HCC.

Fifty percent of patients with HCC were treatment-experienced, with transarterial chemo-embolization and laparoscopic radiofrequency ablation being the most common treatments. In treated patients, median time from last treatment to recruitment was 105 days (75-152 days).

### COAGULATION: PATIENTS WITH HCC HAVE A MORE SEVERE HYPERCOAGULABLE STATE COMPARED WITH CONTROLS WITHOUT HCC, INDEPENDENT OF CIRRHOSIS STAGE

Levels of procoagulant factors and inhibitors were comparable between patients with cirrhosis and HCC and patients with cirrhosis without HCC (Table 2).

At TGA, endogenous thrombin potential (ETP) was comparable among patients with cirrhosis with HCC, patients with cirrhosis without HCC, and healthy controls (Table 3). The addition of TM significantly reduced the ETP in healthy controls but not in patients with cirrhosis (Table 3). Indeed, the ETP ratio was significantly higher in patients with cirrhosis than in healthy subjects (Fig. 1).

**TABLE 1. BASELINE CHARACTERISTICS IN PATIENTS WITH CIRRHOSIS**

	HCC (n = 50)	No HCC (n = 50)
Age, years	65 (58-69)	61 (55-71)
Male gender, %	80	66
Etiology of cirrhosis, %		
Alcohol	46	36
HCV	26	42
NASH	10	8
HBV ± HDV	16	12
Other	2	2
Child class A/B/C, %	46/36/18	62/22/16
MELD score	11 (8-16)	10 (8-14)
History of decompensation, %		
Ascites	46	28
Variceal hemorrhage	22	22
Hepatic encephalopathy	6	20
Hemoglobin, g/dL	12 (11-14)	12 (10-14)
Platelet count, 10 <sup>9</sup> /L	95 (64-114)	108 (67-140)
Total bilirubin, mg/dL	1.2 (0.9-2.7)	1.2 (0.8-3.5)
INR	1.3 (1.2-1.5)	1.2 (1.1-1.6)
Creatinine, mg/dL	0.8 (0.7-0.9)	0.8 (0.7-0.9)
Albumin, g/dL	31 (29-36)	34 (29-38)
AFP, ng/mL	9 (4-47)	3 (2-4)
Multinodular, %	68	–
Number of nodules	3 (2-7)	–
TTV, cm <sup>3</sup>	9 (5-16)	–
TTV > 10 cm <sup>3</sup> , %	45	–
History of previous treatment, %		–
Yes/No	45	
TACE*	39 in 19	
RF*	31 in 20	
Resection*	5 in 5	
PEI	2 in 2	
Capecitabine	1	

Note: Median values reported with 25th and 75th percentile values in parentheses.

\* Number of procedures in n patients.

Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; HDV, hepatitis D virus; NASH, nonalcoholic steatohepatitis; PEI, percutaneous ethanol injection; RF, radiofrequency; TACE, transarterial chemoembolization; TTV, total tumor volume.

In patients with cirrhosis, those with HCC had higher ETP with TM and higher ETP ratio compared with those without HCC ( $P = 0.04$  and  $P < 0.001$ , respectively) (Fig. 1). Among other parameters of TGA, peak and velocity index were higher, whereas time to peak was lower, respectively, in patients with HCC versus patients without HCC (Table 3).

Because levels of procoagulant factors and inhibitors as well as TGA are directly affected by severity of chronic liver disease, to better ascertain the effect of HCC on secondary hemostasis, we analyzed Child A and Child B/C patients separately (with vs. without HCC).

As indicated in Supporting Table S1, demographics and severity of liver dysfunction were comparable between patients with and without HCC in Child class A and Child class B/C analyzed separately. Regarding hemostatic alterations, in Child class A, patients with HCC had lower FII, FV, FVII, FIX, FXI, FXII, AT, PC coagulometric, and PS compared to those without HCC. Conversely, fibrinogen, FVIII, and TFPI were comparable between the groups (Supporting Table S2). At TGA, ETP without TM was comparable between patients with and without HCC, whereas ETP with TM and ETP ratio were higher in patients with versus without HCC (Fig. 2).

In Child class B/C, patients with HCC had higher fibrinogen, FII, FV, FVII, FIX, FXI, AT, and PC chromogenic than patients without HCC. Conversely, FX and FXII were comparable between the groups (Supporting Table S2). At TGA, ETP without TM was comparable between patients with and without HCC, whereas ETP with thrombomodulin and ETP ratio were both higher in those with HCC (Fig. 2). The trends of other parameters of TGA in patients with versus without HCC according to Child class were comparable with that of the overall analysis noted previously (Supporting Table S3).

## FIBRINOLYSIS: PATIENTS WITH HCC HAVE A SIGNIFICANTLY REDUCED ACTIVATION OF FIBRINOLYSIS COMPARED WITH CONTROLS WITHOUT HCC

Levels of FXIII, plasminogen, alfa2-antiplasmin, t-PA, and PAI-1 were comparable between patients with and without HCC (Table 2). Conversely, level of PAP was lower in patients with HCC versus patients without HCC (45 ng/mL [range: 39-50] vs. 50 ng/mL [range: 47-56];  $P = 0.01$ ) (Fig. 3).

Compared with healthy subjects, patients with HCC demonstrated lower levels of PAP (45 ng/mL [range: 39-50] vs. 49 ng/mL [range: 52-62];  $P = 0.01$ ), whereas no difference was found between healthy subjects and patients with cirrhosis without HCC (Fig. 3).

**TABLE 2. LEVELS OF COAGULATION AND FIBRINOLYSIS FACTORS IN PATIENTS WITH CIRRHOSIS WITH VERSUS WITHOUT HCC**

	HCC (n = 50)	No HCC (n = 50)	PValues
<i>Secondary hemostasis (coagulation)</i>			
Fibrinogen, mg/dL (n.v.: 150-450)	263 (224-424)	260 (192-328)	0.2
FII, % (n.v.: 80-120)	64 (40-86)	74 (47-92)	0.3
FV, % (n.v.: 80-120)	90 (62-112)	91 (67-120)	0.7
FVII, % (n.v.: 80-120)	66 (41-95)	77 (47-103)	0.2
FVIII, % (n.v.: 60-160)	175 (144-221)	178 (140-201)	0.5
FIX, % (n.v.: 80-120)	74 (56-91)	88 (55-102)	0.1
FX, % (n.v.: 80-120)	77 (59-97)	93 (69-109)	0.1
FXI, % (n.v.: 80-120)	59 (44-80)	74 (48-94)	0.07
FXII, % (n.v.: 80-120)	74 (58-93)	91 (57-101)	0.2
Antithrombin, % (n.v.: 80-120)	65 (43-85)	75 (48-89)	0.3
PC coag, % (n.v.: 80-120)	54 (33-80)	68 (32-104)	0.2
PC chromo, % (n.v.: 70-130)	62 (39-86)	74 (37-95)	0.4
PS activity, % (n.v.: 70-130)	67 (54-87)	72 (54-90)	0.8
TFPI, ng/mL (n.v.: 51.2-110.6 ng/mL)	61 (44-83)	60 (45-77)	0.5
<i>Tertiary hemostasis (fibrinolysis)</i>			
FXIII, % (n.v.: 70-140)	96 (71-123)	105 (82-127)	0.3
Plasminogen, % (n.v.: 75-140)	68 (59-89)	85 (60-102)	0.1
Alfa-2 antiplasmin, % (n.v.: 80-120)	72 (60-90)	74 (47-90)	0.8
t-PA, ng/mL (n.v.: <10)	12 (8-18)	12 (8-17)	0.6
PAI-1, ng/mL (n.v.: 1-25)	20 (10-32)	18 (11-30)	0.9
TAFIa/ai, ng/mL (n.v.: 8.5-22.1 ng/mL)	18 (15-24)	21 (16-28)	0.1

Note: Median values are reported with 25th and 75th percentile values in parentheses.

Figure 4 shows trends of profibrinolytic and anti-fibrinolytic factors in patients with cirrhosis with and without HCC according to Child class. FXIII, plasminogen, and alfa2-antiplasmin significantly decreased, whereas t-PA significantly increased, respectively, from Child A to Child C class. Levels of PAI-1 and PAP were comparable among Child A, B, and C patients (Fig. 4).

## CORRELATION OF HEMOSTATIC ALTERATIONS WITH RISK OF FURTHER DECOMPENSATION

Fifty-seven patients had decompensated cirrhosis at inclusion and were considered for study part 2. Ascites was the most common reason for decompensation, followed by variceal hemorrhage and hepatic encephalopathy (Table 1). Of these 57 patients, 1 underwent liver transplantation 3 days after enrollment and was excluded from the analysis. Hence, 56 patients (33 with HCC and 23 without HCC) were finally

included and prospectively followed for a median of 175 days (range: 101-205). Median MELD score in these patients was 16 (range: 13-22). Twenty patients (33%) developed further decompensation, specifically 3 recurrent variceal hemorrhage, 1 refractory ascites, 2 sepsis, 4 hepatorenal syndrome (2 with spontaneous bacterial peritonitis), 1 recurrent hepatic encephalopathy, and 9 ACLF (3 alcohol-related, 2 postprocedural, 1 due to sepsis, and 3 with no clear precipitating factor). Two of the patients who developed ACLF were on prophylactic dose of anticoagulation due to partial PVT before decompensation.

Univariate analysis showed that ETP ratio, FV, FVII, FXII, PAI-1, PC coagulometric, TAFI a/ai, and MELD score were significantly associated with the outcome (Supporting Table S4).

In a two-variables at time multivariate analysis, levels of FVII (OR: 0.97; CI 95%: 0.95-0.99;  $P = 0.03$ ) and PAI-1 (OR: 1.05; CI 95%: 1.01-1.10;  $P = 0.01$ ) were the only predictors of further decompensation, independent of MELD (Supporting Table S5).

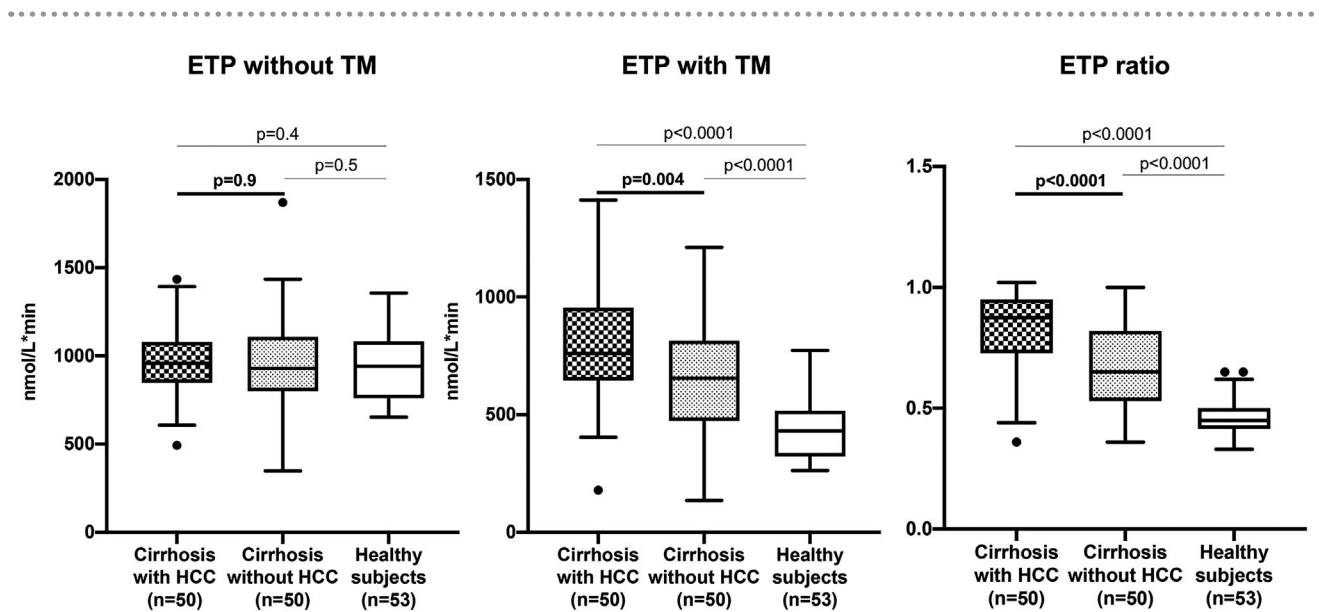
**TABLE 3. THROMBIN GENERATION ASSAY IN PATIENTS WITH CIRRHOSIS WITH VERSUS WITHOUT HCC AND IN HEALTHY SUBJECTS**

	HCC (n = 50)	No HCC (= 50)	Healthy Controls (n = 53)	PValues HCC vs. No HCC*	PValues Group Comparison†
<i>TGA without TM</i>					
Lag time, minutes	4.3 (3.7-5.3)	4.6 (3.8-5.7)	5.2 (4.7-5.7)	0.4	0.001
Peak height, nM	143 (112-185)	129 (96-152)	81.7 (66-105)	0.01	<0.0001
Time to peak, minutes	7.9 (6.7-9.8)	9.3 (7.2-10.8)	11.3 (10.4-11.7)	0.03	<0.0001
Velocity index, nM/min	43 (27-68)	33 (20-43)	13.8 (11-18)	0.004	<0.0001
Start tail, minutes	26 (24-28)	27 (26-31)	34 (31-36)	0.003	<0.0001
ETP, nmol/L*min	958 (848-1,079)	929 (800-1,108)	942.3 (761-1,083)	0.9	0.6
<i>TGA with TM</i>					
Lag time, minutes	4.4 (3.9-5.5)	4.6 (4.0-5.7)	5 (4.8-5.6)	0.7	0.01
Peak height, nM	136 (103-177)	109 (78-136)	60 (450-80)	0.002	<0.0001
Time to peak, minutes	8 (6.8-8.9)	8.2 (7.1-9.5)	9.2 (8.7-9.7)	0.2	<0.0001
Velocity index, nM/min	42 (27-67)	32 (21-40)	15 (13-20)	0.002	<0.0001
Start tail, minutes	25 (23-26)	24 (23-27)	26 (25-27)	0.6	0.01
ETP, nmol/L*min	760 (646-955)	655 (474-815)	431 (323-516)	0.004	<0.0001
ETP ratio	0.87 (0.72-0.95)	0.65 (0.53-0.82)	0.45 (0.41-0.50)	<0.0001	<0.0001

Note: Median values reported with 25th and 75th percentile values in parentheses.

\*Wilcoxon rank-sum test.

†Kruskal-Wallis test.



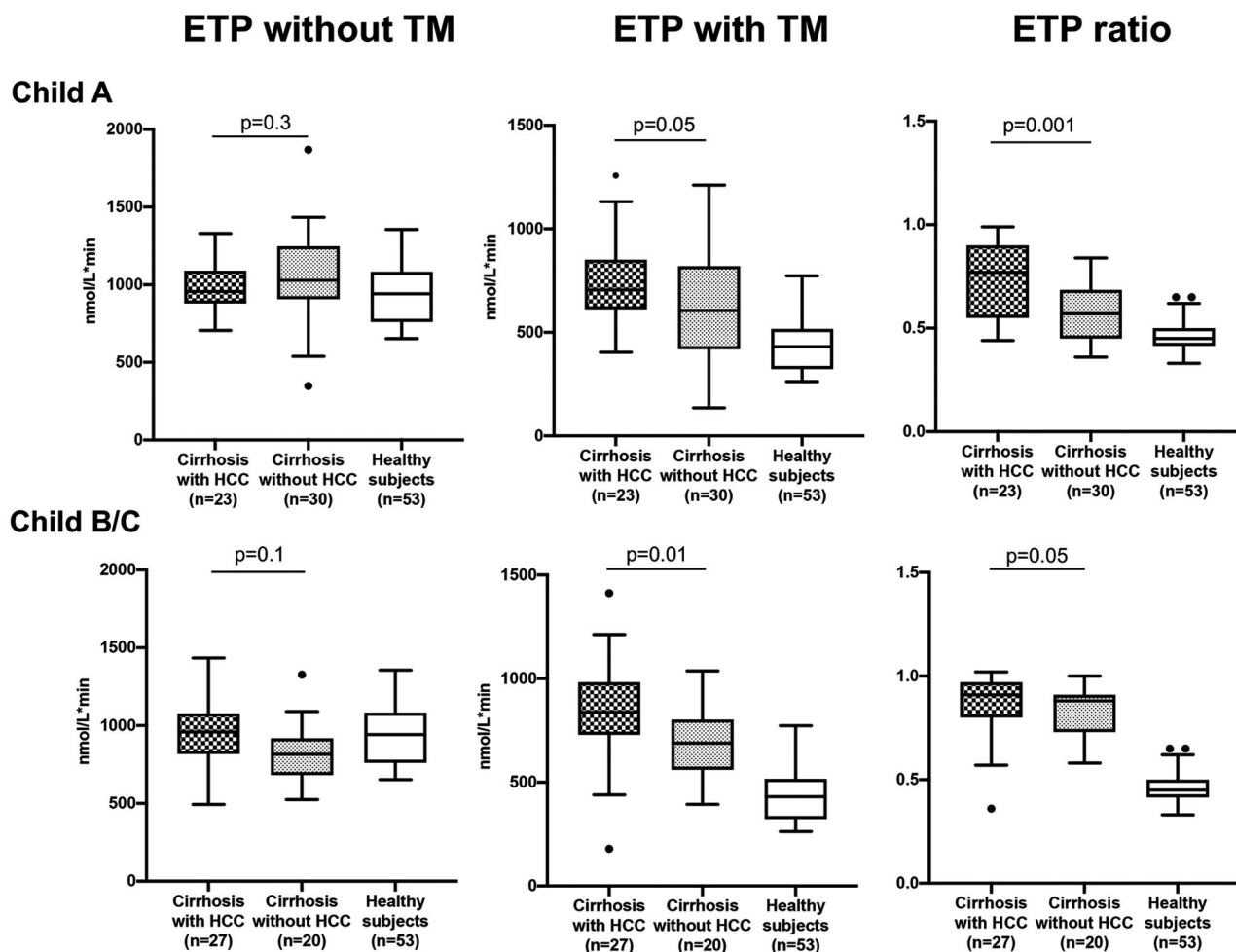
**FIG. 1.** Coagulation, as evidence by higher ETP with TM and ETP ratio, is significantly more in altered (more pro-thrombotic) in patients with cirrhosis with HCC than in patients with cirrhosis without HCC and healthy subjects.

## Discussion

Patients with cirrhosis and HCC may be at increased risk of thrombosis, particularly PVT,<sup>(8-11)</sup> but the hemostatic alterations that contribute to this

purported increased thrombotic tendency have not been thoroughly investigated.<sup>(21)</sup> In patients without liver disease, increased activation of coagulation is one of the main factors responsible for the increased thrombotic tendency in patients with cancers.<sup>(20,36)</sup>





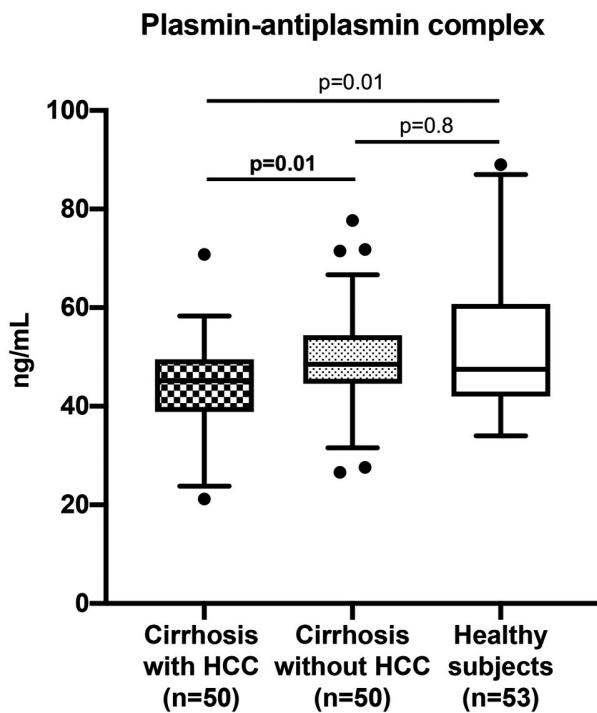
**FIG. 2.** HCC is associated with a more pronounced hypercoagulable state, independent of cirrhosis stage. For numerical values, see Supporting Table S1.

While awaiting large prospective trials to establish whether alterations of hemostasis are truly implicated in the pathogenesis of PVT in patients with cirrhosis,<sup>(5,6)</sup> and to further confirm the association between HCC and increased thrombotic tendency in these patients, we performed a case-control study to investigate alterations of coagulation in this patient population. Because hemostasis in cirrhosis is complex,<sup>(1,3)</sup> and altered fibrinolysis may also be responsible for the thrombotic tendency in digestive cancers,<sup>(19,37)</sup> we included determination not only of coagulation but also of fibrinolysis.

This study demonstrates that, compared to controls without HCC and similar severity of liver dysfunction, patients with cirrhosis and HCC have a more profound hypercoagulable profile due to significantly

increased thrombin generation and significantly reduced activation of fibrinolysis. These hypercoagulable hemostatic changes, together with the HCC-driven increased platelet function<sup>(15)</sup> and higher levels of circulating microvesicles,<sup>(14)</sup> may tilt the precarious hemostatic balance of patients with cirrhosis towards hypercoagulability, thus likely explaining the association between HCC and increased risk of PVT in cirrhosis (Fig. 5).

However, as the differences between patients with and without HCC were relatively modest and perhaps somewhat influenced by a nonidentical disease severity between the two groups, this hypothesis needs confirmation by further studies. Additionally, further prospective studies with development of PVT as clinical endpoint are still required to assess whether

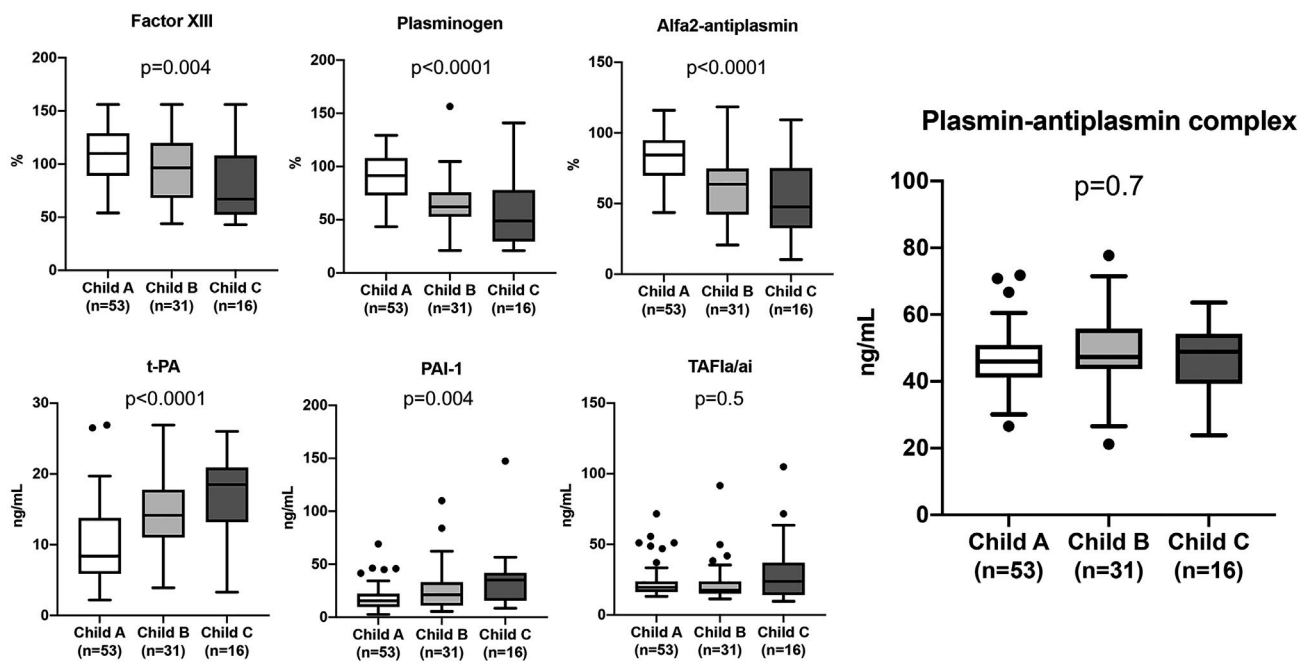


**FIG. 3.** HCC is associated with reduced activation of fibrinolysis, as evidenced by lower levels of PAP. Conversely, levels of PAP were comparable between patients with cirrhosis without HCC and healthy subjects.

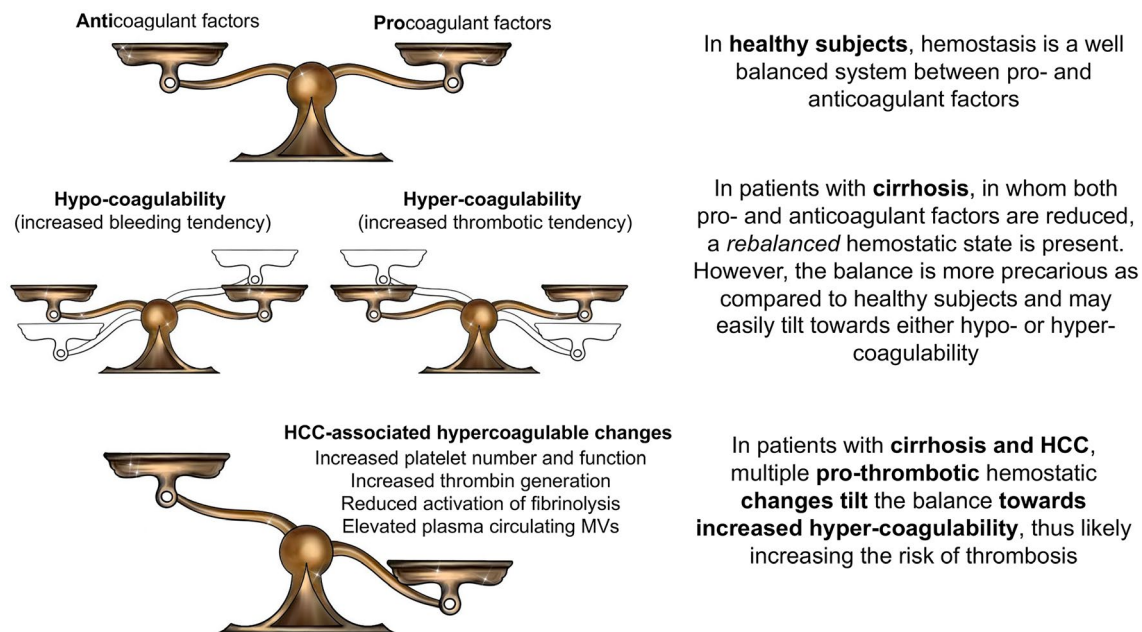
prothrombotic alterations of hemostasis, as detected in our study, are implicated in the pathogenesis of PVT in patients with cirrhosis,<sup>(5,6)</sup> including those with HCC.

The thrombomodulin-modified thrombin generation assay that we used to assess coagulation is a well-recognized research tool for the evaluation of clotting alterations in cirrhosis.<sup>(38,39)</sup> Seminal works showed that cirrhosis is associated with normal thrombin generation (i.e., clotting capacity) despite low levels of procoagulant factors, as reflected by a high ETP.<sup>(40)</sup> Current theory posits that decreased levels of procoagulant factors is *rebalanced* by the increased levels of procoagulant factors synthesized outside the liver (FVIII) and by the decreased hepatic synthesis of anticoagulant factors (PC, PS, and AT).<sup>(3)</sup> In line with previous findings, we show that thrombin generation is preserved in patients with cirrhosis and, contrary to healthy subjects, is not significantly reduced by the addition of thrombomodulin (a PC activator). Interestingly, when comparing patients with cirrhosis with versus without HCC, we found that ETP with TM and ETP ratio were both significantly increased in those with HCC, indicative of a more pronounced hypercoagulable state.<sup>(41)</sup>

The determinants of such hypercoagulability in HCC were complex and rather different in patients



**FIG. 4.** Levels of profibrinolytic and antifibrinolytic factors either increased or decreased in parallel with severity of cirrhosis. In contrast, levels of plasmin-antiplasmin complex, a marker for fibrinolysis activation, remained unchanged between Child A, B, and C patients.



**FIG. 5.** HCC-driven hypercoagulable changes tilt the feeble hemostatic balance of patients with cirrhosis toward a more severe hypercoagulable state, thus likely explaining their increased thrombotic tendency. Abbreviation: MVs: microvesicles.

with compensated versus decompensated cirrhosis. In compensated cirrhosis (Child class A), patients with HCC had lower levels of hepatic-dependent procoagulant factors and inhibitors, possibly indicative of a deleterious effect of HCC on liver synthetic function. In contrast, levels of procoagulant FVIII, which is primarily synthesized by sinusoidal cells and not by hepatocytes, were comparable between patients with and without HCC. As the reduction in anticoagulant factors, particularly PC, was more profound compared with that in procoagulant factors; however, the coagulation balance was ultimately tipped toward hypercoagulability. On the other hand, in patients with decompensated cirrhosis (Child class B and C), HCC was associated with higher levels of fibrinogen and other hepatic-dependent procoagulant and anticoagulant factors, which may potentially reflect an acute phase reaction to HCC.<sup>(42)</sup> The fact that the increase in procoagulant factors was higher than in anticoagulant factors, although ultimately pushed again the balance toward hypercoagulability (Fig. 5).

As hypercoagulability is a risk factor for venous thrombosis in patients with cancers,<sup>(20,36)</sup> these results support the association between HCC and increased thrombotic tendency in patients with cirrhosis.<sup>(8-11)</sup> However, further prospective studies are required to

determine whether hypercoagulability in cirrhosis with HCC, as detected in our study, is a true risk factor for PVT in these patients.

For fibrinolysis analysis, our data demonstrate that levels of profibrinolytic (FXIII and plasminogen) and antifibrinolytic (alfa2-antiplasmin, PAI-1, and TAFI a/ai) factors were comparable between patients with and without HCC. Yet, the reduced levels of PAP, a marker for fibrinolysis activation, in patients with cirrhosis with versus without HCC would suggest that the overall balance of fibrinolysis converged toward a relatively hypofibrinolytic status in HCC. This appears particularly striking because one might expect the HCC-driven activation of coagulation (secondary hemostasis) to be followed by a hyperfibrinolytic (tertiary hemostasis) compensatory response.

However, as PAP is a breakdown product of fibrinolysis that in normal condition is cleared by the liver, it is likely that an altered hepatic clearance in patients with cirrhosis may somewhat interfere with its assessment.<sup>(43)</sup> Furthermore, the difference in PAP levels between patients with and without HCC in our cohort was relatively modest, and our analysis of single protein levels entirely overlooked regulatory interactions and cellular contributions. For all of these reasons, our finding that patients with HCC had a

lower fibrinolysis compared to controls without HCC needs confirmation by further studies, including more global fibrinolytic assays.

Hypofibrinolysis may also be responsible for the thrombotic tendency in patients with cancer,<sup>(19,37)</sup> thus supporting a potential contribution for reduced fibrinolysis to the development of PVT in cirrhosis and HCC.<sup>(19,37)</sup> However, as per our secondary hemostasis findings, clinical studies are required to validate this hypothesis.

There is an ongoing debate on fibrinolysis in patients with cirrhosis with most data converging toward hyperfibrinolysis,<sup>(1)</sup> and one recent study observed a hypofibrinolytic potential in patients with compensated cirrhosis.<sup>(44)</sup> In this study including patients with cirrhosis at all stages, we demonstrate that hepatic-dependent fibrinolytic factors (plasminogen and alfa2-antiplasmin) significantly decreased from Child A to Child C class, whereas non-hepatic-dependent factors (t-PA and TAFI a/ai) significantly increased in parallel with cirrhosis severity. Indeed, levels of PAP were comparable among different Child classes and between patients with cirrhosis without HCC and healthy subjects. This adds to the current literature regarding fibrinolysis in cirrhosis and indicates that (1) fibrinolysis is not hyperactivated in decompensated versus compensated patients, and (2) that degree of fibrinolysis activation is comparable between patients with cirrhosis and healthy subjects.

Whether altered hemostasis and hypercoagulability in decompensated cirrhosis are just a consequence of chronic liver disease or are also involved in disease progression and development of hepatic decompensation is still unclear.<sup>(25,26)</sup> With the hypothesis that patients with decompensated cirrhosis with more severe hypercoagulability could be at higher risk of cirrhosis progression, in the second part of our study, we prospectively evaluated whether hemostatic alterations, particularly markers of hypercoagulability as assessed by TM-modified TGA, were predictive of further decompensation.

We found low levels of FVII and high levels of PAI-1, respectively, to be associated with development of further decompensation, independent of MELD score. The association between low levels of FVII and increased risk of decompensation is in line with previous data by Violi et al., who showed that a level of FVII < 34% could identify patients with cirrhosis at higher risk for death.<sup>(45)</sup> With regard to PAI-1, the

association with increased risk of decompensation is a new finding and may perhaps reflect a potential role of PAI-1 in progression of chronic liver injury.<sup>(46)</sup>

On the other hand, ETP ratio and other markers of hypercoagulability were not independent predictors of further decompensation, which are at odds with the hypothesis that plasmatic hypercoagulability is a driver for cirrhosis progression and a potential therapeutic target.<sup>(25,26)</sup> However, as our analysis was limited due to small number of outcomes and inclusion of an heterogenous cohort of patients with and without HCC and different severity of decompensation, larger studies are required to further look at the complex relationship between hypercoagulability and outcomes in cirrhosis.<sup>(47,48)</sup> Another potential explanation for the lack of association between hypercoagulability and risk of further decompensation is that we assessed coagulation in peripheral and not in portal blood, in which alterations of coagulations may be different.<sup>(49)</sup>

Despite the comprehensive hemostatic analysis, our study has some additional limitations. First, as in any study regarding hemostasis, the lack of interaction between blood components and vessel walls is unavoidable. Second, drugs might have interfered with hemostasis assessment, although robust inclusion criteria were considered and potential cofounders such as other cancers, recent treatments for HCC, kidney dysfunction, sepsis, and bleeding were excluded. Finally, other factors such as plasma-circulating microvesicles that could also contribute to increased hypercoagulability in cancers,<sup>(50)</sup> including HCC,<sup>(14)</sup> were not included in this study.

In conclusion, our extensive hemostatic profiling of coagulation and fibrinolysis in a group of patients with cirrhosis with and without HCC demonstrates that those with HCC have a more pronounced hypercoagulable profile due to increased hypercoagulability and reduced activation of fibrinolysis. These hypercoagulable changes provide explanation for the increased risk of PVT in patients with cirrhosis and HCC and further reinforce the need for studies on anticoagulation for prevention and treatment of thrombotic complications in these patients. We also demonstrate that, in patients with decompensated cirrhosis, low levels of FVII and high levels of PAI-1 are predictors of further decompensation, independent of MELD. In contrast, the lack of association between plasmatic markers of hypercoagulability and risk of hepatic further decompensation suggests that hypercoagulability



in decompensated cirrhosis is more likely a consequence of chronic liver disease rather than a driver for cirrhosis progression.

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