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Characterization of a prothrombotic phenotype using thrombin generation and thrombin activity in cirrhosis and portal hypertension

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ABSTRACT

Background: Patients with advanced chronic liver disease (ACLD) may develop a prothrombotic phenotype that seems to be more pronounced with more severe liver dysfunction. An imbalance of endogenous pro- and anti-coagulants is not fully captured by routine coagulation assays.
Methods: In a cohort of ACLD patients undergoing hepatic venous pressure gradient (HVPG) measurement, we assessed thrombin generation (TGA) using two commercially available assays (Technothrombin and Thrombinoscope) with and without addition of soluble thrombomodulin (TM), as well as thrombin activity, alongside a panel of coagulation parameters.
Results: The cohort encompassed 37 patients (median age 55.3 years, mean HVPG 16 ± 5 mm Hg). In the TM-modified Thrombinoscope TGA, the endogenous thrombin generation potential (ETP) was significantly increased in Child-Pugh-Score (CPS) B/C patients (N = 23, 62 %) compared to CPS A patients (N = 14, 38 %) (ETP: 546 nM*min (443–696) vs. 404 nM*min (289–573), p = 0.028). Using the Technothrombin TGA without TM, patients with CPS B/C had decreased ETP compared to CPS A patients (ETP: 2792 ± 1336 nM*min vs. 5040 ± 816 nM*min, p < 0.001) and with addition of TM (final concentration: 5 nM; ETP: 2545 ± 1327 nM*min vs. 4824 ± 929 nM*min, p < 0.001). Thrombin activity levels were 0.6pM in median (0.2–1.6pM) and above the level of detectability (0.10pM) in 94.6 % of patients but were not correlated to severity of cirrhosis (CPS A 0.7pM vs CPS B/C 0.4pM, p = 0.377) nor to parameters of TGA.
Conclusion: Thrombin plasma levels are elevated in liver disease patients without apparent correlation to TGA or severity of cirrhosis. TGAs can be modified with TM to enable protein C-dependent anticoagulation, but result in differences with regard to severity of liver disease.

1. Introduction

Advanced chronic liver disease (ACLD) is associated with both increased risk of thrombosis and bleeding [1–3]. The risk of bleeding is considered to be largely mediated by increased portal pressure predisposing to portal hypertensive bleedings, as well as intercurrent bacterial

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infections and renal dysfunction [2,4,5]. The risk of thrombosis, which is manifested in splanchnic vein thrombosis but also systemic venous thromboembolism, is considered to originate from the imbalance of pro- and anticoagulant, as well as pro- and antifibrinolytic proteins [6].

With increasing severity of ACLD, the hepatic synthetic capacity decreases as evidenced by increasing INR. However less apparent, protein C (PC), an endogenous anticoagulant is also synthesized in the liver and has a shorter half-life than other serine proteases involved in hemostasis. The ratio of coagulation factor VIII to PC (FVIII/PC) has been suggested to reflect the imbalance in plasmatic coagulation and the prothrombotic nature of advanced liver disease [6] and was correlated with portal vein thrombosis development (PVT) [7] as well as with liver disease progression [7]. However, our group could not confirm the association between FVIII/PC and thrombotic, hypercoagulability, or bleeding events [8]. Indeed, several factors may cause thrombosis development in ACLD patients [9] and not all thrombotic events in liver disease are associated with increased FVIII/PC [10].

Thrombin generation potential measured in-vitro is investigated as a biomarker for the risk of thromboembolism in patient populations at high risk of thromboembolism, but is limited in its capacity to reflect the endogenous anticoagulation pathway by PC, because complexes of thrombin and thrombomodulin, an endothelial cell-bound transmembrane protein, are required for efficient PC activation [11]. Unlike in other patient samples e.g. samples from patients using anticoagulant drugs [12], in cirrhosis patients thrombin generation potential increases relative to healthy individuals in the presence of thrombomodulin, giving rise to the concept of thrombomodulin resistance in cirrhosis [13,14].

Thrombin is a coagulation protease with rapid inactivation and degradation in circulation. In healthy subjects, circulating levels are low [15]. After activation of coagulation under conditions like surgery, thrombosis, or malignancy, systemic thrombin levels are increased [15,16]. Plasma thrombin activity levels can be measured with a highly sensitive oligonucleotide aptamer-based enzyme capture assay [17].

Aim of the current investigation was the characterization of a prothrombotic phenotype using thrombin plasma levels and two different thrombin generations assays with and without addition of soluble thrombomodulin in a cohort of patients with varying degrees of ACLD undergoing extensive clinical, laboratory and hemodynamic characterization.

2. Methods

2.1. Study population

This study includes 37 patients undergoing hepatic-venous pressure gradient measurement (HVPG) within the prospective Vienna Cirrhosis Study (VICIS) at the Hepatic Hemodynamic Laboratory of the Medical University of Vienna. All patients were diagnosed with advanced chronic liver disease (ACLD) and portal hypertension (HVPG $\geq$6 mm Hg) [5]. Inclusion criteria were age $\geq$ 18 years, diagnosis of ACLD (liver stiffness $\geq$10 kPa, HVPG $>$ 5 mm Hg, or histologic F3/F4 fibrosis) and written informed consent. Exclusion criteria were missing/inconclusive HVPG measurements, anticoagulant or antiplatelet therapy, presence of acute decompensation, severe alcoholic hepatitis, acute-on-chronic liver failure, hepatocellular carcinoma, liver metastasis, congestive heart failure, previous liver transplantation or transjugular intrahepatic portosystemic shunt. Patients underwent extensive routine clinical, laboratory and hemodynamic characterization at the time of HVPG-measurement. Additionally, two citrate plasma vials (Vacuette, Greiner-Bio One, Kremsmünster, Austria; containing 1/10 volume sodium citrate at 0.129 mmol$\times$L^{-1}) as well as one argatroban-containing vial (Thrombin blood collection tubes, LOXO GmBH, Dossenheim, Germany) were collected via a central venous line at the end of HVPG-measurement, centrifuged at 2500*g* for 15 min at room temperature and frozen at −20 °C until further processing. Furthermore, we also included a control group of 35 patients without liver disease.

2.2. Hepatic venous pressure gradient (HVPG) measurement

HVPG was measured as according to a standardized operating procedure using a balloon catheter under fluoroscopic control [18,19]. Potential concomitant treatment with non-selective β-blockers was interrupted for at least 3 days prior to HVPG measurement. Subclinical portal hypertension was defined by an HVPG of 6–9 mm Hg and clinically significant portal hypertension (CSPH) by HVPG values $\geq$10 mm Hg [5,20].

2.3. Thrombin activity assay

The thrombin activity assay measures the plasma levels of circulating thrombin. Sample aliquots were thawed in a water bath for 15 min at 37 °C. Thrombin plasma levels were measured using a commercially available fluorogenic enzyme-capture assay (OLIGOBIND Thrombin activity assay, LOXO GmBH, Dossenheim, Germany) according to manufacturer instructions. Standards and patient samples were pipetted in duplicate into separate wells of the aptamer-coated microtiter plate and incubated at room temperature for 1 h in the dark, before washing four times with wash buffer. Addition of a thrombin substrate and a fluorogenic substrate to the aptamer-bound thrombin started the cleavage of the fluorogenic substrate. We measured the increase in fluorescence at room temperature with extinction at 360 nm and emission at 460 nm over 30 min at 5-min intervals inside a fully automated computer-controlled microplate reader (BioTek ®, FLX800). The rate of change in fluorescence was converted into thrombin plasma levels (pM) using a 6-point standard curve. The manufacturer-specified limit of detection is 0.10 pM thrombin, and the lower limit of quantification is 0.35 pM thrombin.

2.4. Thrombin generation assay (Technothrombin)

Both thrombin generation assays measure the potential of plasma to generate thrombin upon stimulation with an activation mix. Thrombin generation was measured using a commercially available fluorogenic assay (Technothrombin TGA, Technoclone, Vienna, Austria) according to manufacturer instructions. Coagulation of the thawed platelet-poor plasma samples was initiated with addition of the TGA RC low reagent, containing a final concentration of 5 pM recombinant human tissue factor lipidated in 3.2 μmol/L phospholipid micelles (phosphatidylcholine [2.56 μmol/L] and phosphatidylserine [0.64 μmol/L]). The generated thrombin cleaves the fluorogenic substrate Z-Gly-Gly-Arg-AMC (1 mM, + 15 mM $CaCl_2$) (Technoclone), while fluorescence was measured at 360 nm extinction and 460 nm emission on the same microplate reader over a period of 120 min in 1-minute intervals. The parameters lag time, peak thrombin, time to peak thrombin, velocity index (VI), and area under the curve (endogenous thrombin generation potential) were recorded for analysis. In parallel to the standard TGA measurements, recombinant soluble thrombomodulin (Sekisui Diagnostics, Pfungstadt, Germany) was added to reagents at a final concentration of 5 nM and the respective parameters were recorded. Experiments were repeated after a second centrifugation at 2500*g* for 10 min as described previously [21].

2.5. Thrombin generation assay (Thrombinoscope)

Blood samples for Thrombinoscope TGA measurements were processed as outlined above and then transferred to the Surgical Research Laboratory, University Medical Center Groningen, The Netherlands. Samples were centrifuged again at 10,000*g* for 10 min and then the Thrombinoscope assay (Stago, Maastricht, The Netherlands) was performed based on protocols of Thrombinoscope BV (Maastricht, the Netherlands) as described previously [16]. Coagulation was initiated by

Table 1
Patient characteristics and test results by Child-Pugh Stage.

Patient characteristics	All patients	Child-Pugh Stage (CPS)		p-Value
	N = 37	CPS A (n = 23)	CPS B/C (n = 14)	
Age, years	55.3 ± 13.2	54.7 ± 13.9	56.2 ± 12.5	0.754
Sex (M/F, %M)	23/14 (62 %)	14/9 (61 %)	9/5 (64 %)	0.835
Etiology				
ALD/NAFLD	22 (59 %)	12 (52 %)	10 (71 %)	0.507
Viral	8 (22 %)	6 (26 %)	2 (14 %)	
Other	7 (19 %)	5 (22 %)	2 (14 %)	
HVPG, mm Hg	16 ± 5	15 ± 5	18 ± 4	**0.040**
MELD, points	12 ± 5	9 ± 2	17 ± 5	**<0.001**
Albumin, $g \times L^{-1}$	36.9 ± 5.1	39.6 ± 3.6	32.4 ± 3.9	**<0.001**
ALT (U/l)	36 (22–48.5)	41 (24–51)	33 (20–43)	0.267
AST (U/l)	47.5 (33.3–53.5)	46 (29–54)	49 (46–52)	0.360
Bilirubin, $mg \times dL^{-1}$	1.2 (0.7–2.2)	0.9 (0.7–1.2)	2.4 (1.2–3.1)	**<0.001**
INR	1.3 (1.1–1.6)	1.2 (1.1–1.3)	1.7 (1.5–2.0)	**<0.001**
Prothrombin time (%)	64 (53–75)	69 (59–77)	52 (41–59)	**0.004**
aPTT (s)	40.3 (36.3–48.3)	37.3 (34.3–40.3)	48.3 (42.5–53.8)	**<0.001**
Fibrinogen ($mg \times dl^{-1}$)	283 (240–358)	317 (260–360)	252 (211–294)	0.077
D-dimer (μg/mL)	0.6 (0.3–1.4)	0.53 (0.32–0.85)	0.79 (0.41–2.68)	0.082
Creatinine, $mg \times dL^{-1}$	0.8 (0.6–0.9)	0.8 (0.6–1.0)	0.6 (0.4–0.8)	**0.024**
Sodium, $mmol \times L^{-1}$	138 ± 4	139 ± 3	135 ± 5	**0.003**
FVIII/prot. C-ratio	2.0 (1.3–3.1)	1.8 (1.2–2.3)	4.2 (2.0–8.8)	**0.001**
Advanced coagulation assays				
Thrombin activity, pM	0.6 (0.3–1.5)	0.7 (0.3–1.8)	0.4 (0.2–0.8)	0.377
Technothrombin TGA ETP, nM*min	4228 ± 1493	5040 ± 816	2792 ± 1336	**<0.001**
Technothrombin TGA ETP +5 nM TM, nM*min	4001 ± 1542	4824 ± 929	2545 ± 1327	**<0.001**
Technothrombin TGA peak thrombin, nM	225 (156–356)	273 (205–375)	123 (64–179)	**<0.001**
Technothrombin TGA peak thrombin +5 nM TM, nM	217 (132–343)	296 (196–356)	121 (71–170)	**0.001**
Thrombinoscope TGA ETP, nM*min	433 (381–600)	404 (289–573)	546 (433–696)	**0.028**
Thrombinoscope TGA peak thrombin, nM	88 (67–109)	77 (61–105)	92 (87–111)	0.179
Technothrombin TGA results after second centrifugation				
ETP, nM*min	2131 ± 1145	2295 ± 1222	1729 ± 860	0.216
ETP +5 nM TM, nM*min	1899 ± 1103	2019 ± 1176	1606 ± 893	0.353
Peak thrombin, nM	89 (59–126)	94 (61–151)	89 (55–117)	0.654
Peak thrombin +5 nM TM, nM	88 (52–148)	88 (59–151)	88 (52–93)	0.716

P-Values printed in bold denote statistical significance ($p<0.05$).

5 pM tissue factor (Innovin, Siemens Healthineers, Den Haag, The Netherlands) in the presence of 4 μmol/L phospholipid vesicles (PS/PC/PE, 20/40/40, Avanti Polar Lipids, Alabaster, Al, USA), and soluble thrombomodulin (10 nM, Synapse B.V., Maastricht, The Netherlands). The thrombin calibrator and fluorogenic substrate were from Stago (Asnieres, France).

2.6. Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics 26 (IBM, Armonk, NY, USA) and GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA). Continuous variables are reported as mean ± standard deviation or median (interquartile range), and categorical variables are shown as numbers (n) and proportions (%) of patients. Comparisons of continuous variables were performed using Student's *t*-test or Mann-Whitney *U* test, as applicable.

p-Values ≤0.05 were considered statistically significant.

2.7. Ethics

Liver disease patients were recruited within the ethics committee-approved (EC 1262/2017) prospective Vienna Cirrhosis Study (VICIS, NCT03267615) and all patients gave written informed consent. Subjects without liver disease were recruited with approval of the ethics committee (EC 1711/2014).

3. Results

3.1. Study population

In total, 37 patients undergoing HVPG measurement at the Vienna Hepatic Hemodynamic Laboratory of the Medical University of Vienna between 09/2017 and 05/2019 were included in this study. While thrombin generation assays were technically successful in 36 patients, thrombin plasma levels were valid in all 37 patients. Additionally, we included a control cohort of 35 patients without liver disease in whom the Technothrombin TGA was performed.

3.2. Patient characteristics (Table 1)

The majority (62 %) of patients was male with a mean age of 55.3 ± 13.2 years. The predominant etiology of ACLD was fatty liver disease (alcoholic/non-alcoholic, 59 %), while 8 patients had virus hepatitis (22 %) and 7 (19 %) patients were diagnosed with other underlying liver diseases. The vast majority of patients (n = 34, 92 %) was diagnosed with clinically significant portal hypertension (CSPH) with a mean HVPG of 16 ± 5 mm Hg. While 23 patients (62 %) had CPS A cirrhosis, 14 (38 %) patients were diagnosed with advanced cirrhosis (CPS B/C). As expected, patients with more advanced cirrhosis (CPS B/C) had a higher HVPG (18 ± 4 vs. 15 ± 5 mm Hg, $p = 0.040$), a significantly higher UNOS MELD score (17 ± 5 vs. 9 ± 2, $p < 0.001$), as well as a lower serum sodium (135 ± 5 vs. 139 ± 3 $mmol \times L^{-1}$, $p = 0.003$). Baseline characteristics of the control group are displayed in Supplemental Table 1.

Table 2
Patient characteristics and test results by hepatic venous pressure gradient (HVPG).

Patient characteristics	Level of HVPG		p-Value
	≤15 mm Hg n = 15	>15 mm Hg n = 22	
Age, years	57.1 ± 11.1	54.0 ± 14.6	0.495
Sex (M/F, %M)	7/8 (47 %)	16/6 (73 %)	0.109
Etiology			
ALD/NAFLD	9 (60 %)	13 (59 %)	0.165
Viral	5 (33 %)	3 (14 %)	
Other	1 (7 %)	6 (27 %)	
MELD, points	11 ± 5	12 ± 5	0.653
Albumin, g×L^{-1}	38 ± 5	36 ± 5	0.301
ALT (U/l)	43 (20–59)	35 (25–44)	0.253
AST (U/l)	48 (35–76)	47 (33–52)	0.595
Bilirubin, mg×dL^{-1}	0.9 (0.7–1.7)	1.2 (1.0–2.4)	0.290
INR	1.3 (1.1–1.3)	1.3 (1.1–1.7)	0.572
Prothrombin time (%)	68 (59–71)	63 (50–77)	0.693
aPTT (s)	38.7 (34.3–44.0)	42.1 (38.8–50.4)	0.071
Fibrinogen (mg×dL^{-1})	300 (219–429)	278 (246–354)	0.614
D-dimer (μg/mL)	0.6 (0–3-0.8)	0.62 (0.33–1.59)	0.531
Creatinine, mg×dL^{-1}	0.8 (0.6–0.9)	0.7 (0.5–0.8)	0.366
Sodium, mmol×L^{-1}	138 ± 5	137 ± 4	0.505
FVIII/prot. C-ratio	2.1 (1.6–2.9)	1.9 (1.2–3.1)	0.382
Advanced coagulation assays			
Thrombin activity, pM	0.7 (0.5–1.5)	0.4 (0.2–1.7)	0.161
Technothrombin TGA ETP, nM*min	4833 ± 1014	3797 ± 1647	**0.026**
Technothrombin TGA ETP +5 nM TM, nM*min	4637 ± 1103	3547 ± 1671	**0.035**
Technothrombin TGA ETP ratio (5 nM TM/0 nM TM)	1.0 ± 0.1	0.9 ± 0.1	0.256
Technothrombin TGA peak thrombin, nM	272 (202–370)	205 (116–329)	0.096
Technothrombin TGA peak thrombin +5 nM TM, nM	254 (195–368)	181 (97–316)	0.053
Technothrombin TGA peak ratio (5 nM TM/0 nM TM)	1.0 ± 0.1	0.9 ± 0.1	0.302
Thrombinoscope TGA ETP, nM*min	440 (378–703)	411 (383–548)	0.446
Thrombinoscope TGA peak thrombin, nM	89 (54–143)	87 (70–95)	0.680

P-Values printed in bold denote statistical significance (p<0.05).

3.3. Comparison of coagulation tests within different CPS stages (Table 1)

As expected, INR was significantly higher in patients with CPS B/C cirrhosis when compared to CPS A (1.7 (1.5–2.0) vs. 1.2 (1.1–1.3), p < 0.001). Additionally, FVIII/PC–ratio was significantly increased in patients with more advanced liver disease (4.2 (2.0–8.8) vs. 1.8 (1.2–2.3), p = 0.001).

In line with these findings, endogenous thrombin potential (ETP) measured with the Technothrombin TGA was significantly higher in liver disease patients compared to liver-healthy subjects (4228 ± 1493 vs. 3451 ± 805 nM*min, p = 0.001), while peak thrombin generation was lower in liver disease patients (225 [156–356] vs. 322 nM [375–237], p = 0.037).

Next, results of the Technothrombin TGA assay were compared between different CPS stages. Interestingly, patients with more advanced liver disease (CPS B/C) showed decreased in-vitro potential to generate thrombin without activation of the anticoagulant PC-pathway by thrombomodulin (ETP: 2792 ± 1336 vs. 5040 ± 816 nM*min, p < 0.001; peak height: 123 (64–179) vs. 273 nM (205–375), p < 0.001). These results were confirmed even after addition of thrombomodulin at a final concentration of 5 nM (ETP: 2545 ± 1327 vs. 4824 ± 929 nM*min, p < 0.001; peak height: 121 (71–170) vs. 296 nM (196–356), p = 0.001). In contrast, the ETP was significantly increased in CPS B/C patients in the Thrombinoscope TGA assay performed with the addition of thrombomodulin (ETP: 546 (443–696) vs. 404 (289–573) nM*min, p = 0.028) while peak thrombin was numerically, but not statistically significantly higher (peak thrombin: 92 (87–111) vs. 77 (61–105) nM, p = 0.179).

The thrombin activity plasma levels had a median of 0.6 pM (25th to 75th percentile 0.3–1.5 pM) and ranged from 0.0 to 6.3 pM in the full cohort, but only two sample had undetectable thrombin activity. A decrease in thrombin activity in CPS B/C patients compared to CPS A patients (0.4 [0.2–0.8] pM vs. 0.7 [0.3–1.8] pM, p = 0.377) did not reach statistical significance.

3.4. Comparison of coagulation tests within different severity of portal hypertension (Table 2)

In order to evaluate the effects of portal hypertension on coagulation studies, patients were classified according to the presence or absence of severe portal hypertension (HVPG ≥16 mm Hg; n = 22 [59 %]). Baseline characteristics were well balanced between the two groups. Again, in the Technothrombin TGA assay in-vitro thrombin potential was significantly reduced in patients with more pronounced portal hypertension without (ETP: 3797 ± 1647 vs. 4833 ± 1014 nM*min, p = 0.026; peak height: 205 (116–329) vs. 272 (202–370) nM, p = 0.096) and with thrombomodulin (ETP: 3547 ± 1671 vs. 4637 ± 1103 nM*min, p = 0.035; peak height: 181 (97–316) vs. 254 (195–368) nM, p = 0.053). In the Thrombinoscope TGA assay there was no significant difference between less or more advanced portal hypertension (ETP: 411 (383–548)

Table 3
Comparison of patient characteristics with high vs. low in-vitro ETP-AUC in the (A) Technothrombin and (B) Thrombinoscope thrombomodulin-modified TGA assays.

Patient characteristics	(A) Technothrombin TGA + 5 nM TM ETP (stratified by median)		p-Value	(B) Thrombinoscope TGA + 5 nM TM ETP (stratified by median)		p-Value
	<4077 (n = 18)	≥4077 (n = 18)		<433 (n = 17)	≥433 (n = 19)	
Age, years	55.1 ± 12.0	55.6 ± 15.0	0.901	56 ± 15	56 ± 11	0.900
Sex (M/F, %M)	9/9 (50 %)	13/5 (72 %)	0.171	10/7 (59 %)	12/7 (63 %)	0.790
Etiology						
ALD/NAFLD	10 (56 %)	11 (61 %)	0.189	11 (65 %)	11 (58 %)	0.823
Viral	6 (33 %)	2 (11 %)		3 (18 %)	5 (26 %)	
Other	2 (11 %)	5 (28 %)		3 (18 %)	3 (16 %)	
HVPG, mm Hg	17 ± 5	16 ± 5	0.358	17 ± 5	16 ± 5	0.482
CPS	7.9 ± 2.3	5.4 ± 0.6	**<0.001**	5.9 ± 2.4	7.3 ± 2.4	**0.034**
Albumin, g×L^{-1}	34.7 ± 5.6	39.2 ± 3.6	**0.009**	38.1 ± 4.7	35.9 ± 5.5	0.203
Bilirubin, mg×dL^{-1}	2.3 (1.2–3.0)	0.8 (0.7–1.2)	**<0.001**	0.8 (0.7–1.3)	1.2 (1.0–2.8)	**0.038**
INR	1.6 (1.2–1.9)	1.2 (1.1–1.3)	**0.002**	1.2 (1.1–1.3)	1.3 (1.1–1.7)	0.156
Creatinine, mg×dL^{-1}	0.7 (0.4–0.8)	0.8 (0.7–1.0)	**0.014**	0.8 (0.7–1.2)	0.8 (0.5–0.8)	0.165
Sodium, mmol×L^{-1}	137 ± 5	139 ± 4	0.221	139 ± 3	137 ± 5	0.205
FVIII/prot. C-ratio	3.1 (1.5–7.3)	2.0 (1.3–2.7)	**0.039**	1.3 (1.1–2.0)	2.9 (1.9–4.9)	**0.002**
Thrombin activity, pM	0.6 (0.2–1.5)	0.5 (0.3–1.2)	0.839	0.6 (0.2–2.5)	0.6 (0.3–0.8)	0.802

P-Values printed in bold denote statistical significance (p<0.05).

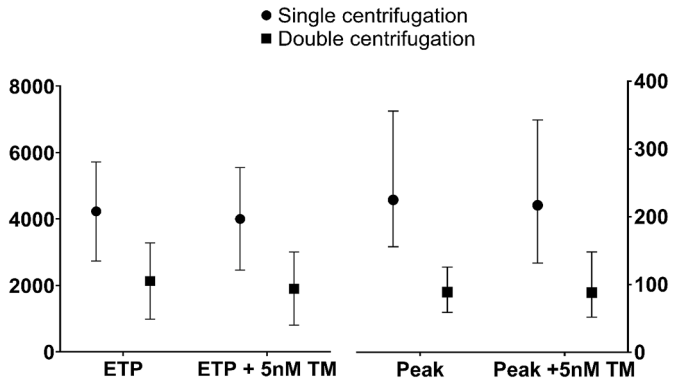

Fig. 1. Technothrombin TGA results after single and double centrifugation. Abbreviations: ETP endogenous thrombin potential, TM thrombomodulin, Peak peak thrombin generation.

vs. 440 (378–703) nM*min, p = 0.446; peak height: 87 (70–95) vs. 89 (54–143) nM p = 0.680). Similarly, the thrombin plasma levels were non-significantly decreased in patients with higher HVPG (0.4 (0.2–1.7) pM vs. 0.7 (0.5–1.5) pM, p = 0.161).

3.5. Comparison of patient characteristics according to in-vitro thrombin generation potential as measured by the Technothrombin TM-modified TGA assay (Table 3A) and the Thrombinoscope TM-modified TGA assay (Table 3B)

Next, we aimed to compare patient characteristics according to the in-vitro thrombin potential as measured by the Technothrombin TM-modified TGA assay. Therefore, patients were stratified by the median thrombin generation potential (ETP: 4077 nM*min, peak thrombin generation: 217 nM). On the Technothrombin TGA assays, patients with higher ETP had less severe liver disease as indicated by lower CPS scores (ETP: 7.9 ± 2.3 nM*min vs. 5.4 ± 0.6 nM*min, p < 0.001), higher serum albumin (39.2 ± 3.6 vs. 34.7 ± 5.6 $g \times L^{-1}$, p = 0.009), and lower bilirubin (0.8 vs. 2.3 $mg \times dL^{-1}$, p < 0.001). There was no significant difference between thrombin plasma levels between the samples with high and low ETP, but lower FVIII/PC ratio in samples with high ETP (2.0 (1.3–2.7) vs. 3.1 (1.5–7.3), p = 0.039).

On the Thrombinoscope TGA assays, patients with higher ETP showed higher CPS scores (7.3 ± 2.4 vs. 5.9 ± 2.4, p = 0.034) and higher bilirubin levels (1.2 (1.0–2.8) vs. 0.8 (0.7–1.3) $mg \times dL^{-1}$, p = 0.038). Additionally, FVIII/PC-ratio was statistically significantly higher (ETP: 2.9 nM*min (1.9–4.9) vs. 1.3 nM*min (1.1–2.0), p = 0.002) in the group of patients with higher ETP. There was no difference in thrombin plasma levels between patients with high or low Thrombinoscope ETP (0.6 pM (0.3–0.8) vs. 0.6 pM (0.2–2.5), p = 0.802).

3.6. Re-performance of Technothrombin TGA assay after second centrifugation (Table 1, Fig. 1)

As results of the Technothrombin and the Thrombinoscope TGA assays were significantly different, we re-performed Technothrombin TGA assay after second centrifugation. Importantly, no thrombin generation could be achieved in 5 (14 %) patients after second centrifugation and overall thrombin generation was significantly lower compared to results after single centrifugation (after re-centrifugation: ETP: 2131 ± 1145; peak thrombin: 89 (59–126); ETP with 5 nM TM: 1899 ± 1103; peak thrombin with 5 nM TM: 88 (52–148)). Even after second centrifugation, patients with CPS A cirrhosis had higher thrombin generation without (ETP: 2295 ± 1222 nM*min; peak thrombin generation: 94 nM (61–151)) and with TM (ETP: 2019 ± 1176 nM*min; peak thrombin: 88 (59–151) nM) compared to CPS B/C patients without (ETP: 1729 ± 860 nM*min; peak thrombin generation: 89 nM (55–117)) and with TM (ETP: 1606 ± 893 nM*min; peak thrombin generation: 88 nM (52–93)).

Table 4
Comparison of patient characteristics with low vs. high in-vitro enzymatic thrombin activity levels.

Patient characteristics	Thrombin activity (<≥median)		p-Value
	<0.6 pM (n = 18)	≥0.6 pM (n = 19)	
Age, years	57 ± 12	54 ± 14	0.583
Sex (M/F, %M)	12/6 (%)	11/8 (%)	0.582
Etiology			
ALD/NAFLD	12 (%)	10 (%)	0.317
Viral	2 (%)	6 (%)	
Other	4 (%)	3 (%)	
HVPG, mm Hg	18 ± 5	15 ± 5	0.111
CPS	6.8 ± 1.8	6.5 ± 2.3	0.652
MELD, points	13 ± 6	11 ± 4	0.150
Albumin, $g \times L^{-1}$	36.5 ± 4.7	37.4 ± 5.6	0.603
Bilirubin, $mg \times dL^{-1}$	1.1 (0.7–2.2)	1.2 (0.8–2.4)	0.558
INR	1.3 (1.2–1.6)	1.2 (1.1–1.7)	0.258
Creatinine, $mg \times dL^{-1}$	0.7 (0.5–0.8)	0.8 (0.6–0.9)	0.391
Sodium, $mmol \times L^{-1}$	135 ± 4	140 ± 3	**<0.001**
FVIII/prot. C-ratio	2.7 (1.3–3.2)	2.0 (1.4–2.8)	0.782
Technothrombin TGA ETP +5 nM TM, nM*min	3860 ± 1623	4142 ± 1491	0.591
Technothrombin TGA peak thrombin +5 nM TM, nM	221 (141–368)	217 (123–334)	0.888
Thrombinoscope TGA ETP, nM*min	595 ± 425	444 ± 160	0.168
Thrombinoscope TGA peak thrombin, nM	88 (65–134)	88 (70–102)	0.606
Bile acids, µmol/L	25 (11–92)	17 (6–39)	0.226
CRP $mg \times dL^{-1}$	0.3 (0.2–1.6)	0.3 (0.1–0.7)	0.284
IL6, pg/mL	10.0 (7.0–18)	11 (3.5–15.6)	0.425
LBP, µg/mL	7.9 (6.5–9.8)	6.5 (4.5–7.7)	**0.027**
PCT, ng/mL	0.1 (0.1–0.2)	0.1 (0.1–0.2)	0.799
Transferrin, $mg \times dL^{-1}$	269 (202–287)	272 (198–298)	0.988
Fibrinogen, $mg \times dL^{-1}$	289 (246–392)	273 (217–354)	0.707
VWF:Ag, %	310 (193–381)	228 (192–331)	0.594

P-Values printed in bold denote statistical significance (p<0.05).

3.7. Comparison of patient characteristics between patients with high vs. low thrombin activity levels (Table 4)

The median thrombin activity level found in the full cohort was 0.6 pM. Given thrombin levels are not detectable in patients without ongoing coagulation, we were surprised to find >90 % of the samples above the limit of detection of the assay at 0.1 pM. We investigated differences in patient characteristics between patients with low and high enzymatic thrombin activity. Therefore, patients were stratified by the median value (0.6 mU/mL). Interestingly, patients with low enzymatic thrombin activity had lower serum sodium levels (135 ± 4 vs. 140 ± 3 $mmol \times L^{-1}$, p < 0.001) indicating a more pronounced systemic hyperdynamic circulation and higher levels of lipopolysaccharide binding protein (LBP) (7.9 (6.4–9.8) vs. 6.5 (4.5 ± 7.7) $mmol \times L^{-1}$, p = 0.027) indicating bacterial translocation-induced inflammation. Importantly, the ETP both on the Technothrombin and the Thrombinoscope TGA were similar in patients with low vs. high in-vitro enzymatic thrombin activity.

4. Discussion

In our analysis of thrombin generation in patients with chronic liver disease, we found an increase in ETP in CPS B/C patients compared to CPS A patients using the TM-modified Thrombinoscope TGA alongside an increase in FVIII/PC ratio. The TM-modified Technothrombin TGA did not reproduce the results of the Thrombinoscope TGA, but correlated with markers of liver disease including CPS, albumin, bilirubin, INR, and

FVIII/PC ratio. We found elevated levels of circulating thrombin plasma levels in the cohort, which did not correlate with thrombin generation, CPS or HVPG.

Opposed to thrombin generation, which measures the potential to generate thrombin upon in-vitro activation with exogenous triggers, the thrombin activity assay measures the level of thrombin in circulation using argatroban to capture and stabilize thrombin [22]. In healthy subjects, thrombin plasma levels are below the limit of detection and only in patients with ongoing coagulation, thrombin plasma levels are increased [15]. In the current model of coagulation in liver disease, a precariously rebalanced coagulation system is postulated with reduced hepatic derived clotting factors, reduced protein C and antithrombin, but elevated FVIII [23]. Elevated thrombin plasma levels may reflect a tipping of this balance that warrants prospective investigation regarding the risk of decompensated liver disease and occurrence of thromboembolism.

The TM-modified thrombin generation assay can be used to demonstrate the rebalanced coagulation system in liver disease [6,13]. As a global coagulation assay, thrombin generation captures the coagulation capacity collectively taking decreased and increased coagulation factor levels into account opposed to for example INR and FVIII/PC ratio that describe isolated imbalances in coagulation [7,8]. The increasing prothrombotic phenotype with advanced stages of cirrhosis is, however, not apparent on thrombin generation assays, without modifying the thrombin generation assay with TM [24]. Addition of thrombomodulin to the thrombin generation assay results in binding of generated thrombin to thrombomodulin which allows activation of PC [12]. Nevertheless, despite addition of thrombomodulin, the ETP consistently decreased with increasing CPS when using the Technothrombin TGA assay in our experiments but increased when using the Thrombinoscope assay. Even after adjusting and replicating pre-analytic methods, the assays maintained the difference in their results. In general, lack of standardization between pre-analytic methods and concentration of reagents makes comparison of different TGA assays difficult and differences in results remain unexplained [24]. The premise of TGA manipulations in cirrhosis patients is to reduce the thrombin burst as much as possible by twice centrifugation, low TF concentrations and low phospholipid concentrations in order to amplify the effects of activated PC. Under these conditions, TGA reveals the prothrombotic phenotype of patients with advanced cirrhosis [25]. The Technothrombin TGA did not verify these previous findings [26], because the thrombin generation potential was not reduced enough by means of second centrifugation and thrombomodulin addition for the anticoagulation potential of activated PC to take effect.

A strength of this study is that patients are not only characterized by liver disease severity (CPS) but also by severity of portal hypertension as measured by HVPG, which translates to the pressure gradient between wedged and free hepatic venous pressures [19]. Importantly, there might be a link between intrasinusoidal thrombosis and liver disease/portal hypertension severity. Wanless and co-workers postulated the theory of parenchymal extinction caused by intrasinusoidal microthrombosis and recently extended their theory by the description of a ‘congestive escalator’ following vascular damage ultimately resulting in liver disease progression [27,28].

When divided into two groups of patients with low or high HVPG at 16 mm Hg, patients with higher HVPG generally had lower ETP in both assays with and without addition of thrombomodulin compared to low HVPG patients.

Limiting aspects in our study are lack of prospective observational data to support the risk of thromboembolism. Further, we observed difficulties in obtaining stable thrombin generation curves in the Technothrombin assay when applying equal pre-analytic conditions. After twice centrifugation, the concentration of subcellular structures is so low in plasma, that thrombin generation could not be sustained in some samples. Decreasing the ETP of thrombin generation to 50 % of the pre-TM runs was in most cases not possible, because the thrombin generation curves collapsed without rendering reproducible results. Thrombin generation assays are not standardized and substantial differences in the reagent concentrations exist between manufacturers. While thrombin generation assays may be a useful tool to determine a potential procoagulant phenotype in patients with ACLD or to monitor anticoagulant therapy [29] in these complex patients, further studies are required to determine the optimal pre-analytical conditions as well as the optimal TF and TM concentrations [24].

5. Conclusions

In our method comparison of different thrombin generation assays in a cohort of patients with cirrhosis, we found an increased thrombin generation potential compared to control persons as an expression of a prothrombotic phenotype using the Technothrombin TGA. With increasing severity of liver disease characterized by CPS, we found a decrease of ETP using the Technothrombin assay, but an increase in FVIII/PC ratio and ETP using the Thrombinoscope TGA modified with the addition of TM. Further, we found elevated levels of circulating thrombin using an enzyme-capture assay in the majority of ACLD patients, but confirmed that thrombin plasma levels are unrelated to thrombin generation potential, because there was no correlation to thrombin generation or markers of liver disease. The relevance of this thrombotic marker for the characterization of prothrombotic phenotype in ACLD patients remains to be elucidated.

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Competing interest

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