Thrombosis Research 207 (2021) 40–49

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Full Length Article

Resistance to thrombomodulin correlates with liver stiffness in chronic liver disease a prospective single-center cohort study

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ARTICLE INFO

Keywords:
Chronic liver disease
Blood coagulation
Thrombin generation
Thrombomodulin resistance
Liver stiffness

ABSTRACT

Introduction: Chronic liver disease (CLD) is characterized by changes in haemostasis, embracing both hypo- and hypercoagulability. Global hemostatic tests such as thrombin generation assays evaluate the hemostatic balance, to better assess bleeding and thrombotic risks. In addition, procoagulant state in patients with CLD has been demonstrated using modified thrombin generation assays with thrombomodulin, a cofactor for protein C activation. In this study, we prospectively determined thrombin generation and thrombomodulin resistance in patients with CLD staged with liver stiffness measurement (LSM), using both the fully automated analyzer ST Genesia® Thrombin Generation System (STG) and the calibrated automated thrombogram assay (CAT).

Materials and methods: Demographic, clinical and laboratory characteristics, and blood samples were collected from 65 patients with CLD. Liver stiffness was measured by transient elastography, and thrombin generation and thrombomodulin resistance, by STG and CAT.

Results: Patients were separated based on LSM of <21 and ≥21 kilopascals (kPa). The propagation rate of thrombin generation was higher in patients with LSM ≥21 kPa and the thrombin generation rate increased as LSM increased. In addition, thrombomodulin resistance assessed by STG and CAT was higher in patients with LSM ≥21 kPa. However, ETP inhibition by activated protein C was comparable in patients with LSM <21 and ≥21 kPa. Finally, LSM correlated with most thrombin generation parameters.

Conclusion: The STG automated system may have value in the assessment of patients with chronic liver disease in the routine coagulation laboratory. LSM ≥21 kPa identify a procoagulant phenotype in these patients, including thrombomodulin resistance.

1. Introduction

Chronic liver disease (CLD) is defined as a progressive destruction of the liver parenchyma over a period greater than 6 months leading to fibrosis and cirrhosis. This condition affects both pro- and anticoagulant forces of the hemostatic system [1]. Therefore, the hemostatic balance in CLD is in a fragile equilibrium and may easily shift toward an increased or decreased thrombin generation (TG) that eventually may result in life-threatening bleeding or thrombotic complications [2–4]. CLD has been associated to an increased incidence of thromboembolism [5–7] and portal vein thrombosis occurs more frequently in patients with CLD [8–10]. Moreover, coagulation factors have been reported to promote

Abbreviations: AIH, autoimmune hepatitis; ALD, alcoholic liver disease; ALP, alkaline phosphatase; APC, activated protein C; AST, aspartate transaminase alanine transaminase; CAT, calibrated automated assay; CLD, chronic liver disease; ETP, endogenous thrombin potential; HBV, hepatitis B virus; HCV, hepatitis C virus; HFE, hereditary hemochromatosis; INR, international normalized ratio; LSM, liver stiffness measurement; NAFL, non-alcoholic fatty liver; PBC, primary biliary cholangitis; PFP, platelets frozen plasma; PPP, platelets poor plasma; PT, prothrombin time; STG, ST Genesia® Thrombin generation system; STG-BLS, ST Genesia® Thrombin generation system BleedScreen; STG-TS, ST Genesia® Thrombin generation system ThromboScreen; TF, tissue factor; TG, thrombin generation; TM, thrombomodulin.

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https://doi.org/10.1016/j.thromres.2021.09.007
Received 28 June 2021; Received in revised form 19 August 2021; Accepted 10 September 2021
Available online 14 September 2021

fibrosis progression. Several mechanisms have been proposed such as thrombin-mediated activation of stellate cells via protease activated-receptor-1, as well as tissue ischemia and parenchymal extinction, reviewed by Anstee et al. [11].

Consequently, anticoagulation might be considered for both fibrosis and thrombosis prevention in patients with CLD. Notably, anticoagulation with the low molecular weight heparin enoxaparin reduces the intrahepatic vascular resistance and improves portal pressure in a murine model of cirrhosis [12] and has been shown to delay the occurrence of decompensation and to improve survival in patients with CLD [13].

Coagulation alterations in patients with CLD comprise a concomitant decrease of pro-and anticoagulant proteins, except for factor VIII, which is increased. Therefore, the hypercoagulability of plasma in these patients results mainly from increased factor VIII and decreased protein C levels [3]. The elevated factor VIII/protein C ratio appears to be responsible for the resistance to the anticoagulant effect of thrombomodulin (TM), an endothelial receptor playing a key role in the inhibitory process of TG [1,14]. Using a modified TG assay that adds soluble TM, it has been shown that the coagulation potential observed in patients with liver disease is preserved indicating that the deficiency of anticoagulant proteins compensates for the deficiency of procoagulant proteins [15]. Even in CLD patients with prolonged coagulation times, TM-modified TG is normal or even increased compared to healthy individuals [16,17].Global hemostatic tests such as TG assays provide currently the most accurate representation of the coagulation phenotype at the individual level [18] and have been used for investigating coagulopathy in CLD in several studies due to their ability to taking into account the protein C pathway [9,15,19–23]. Using this methodologic approach, La Mura et al. have shown that TM resistance is associated with portal vein thrombosis and might predict an adverse outcome in patients with CLD [24].

Measurement of liver stiffness by transient elastography is a reliable method to detect and stage liver fibrosis, and to stratify the prognosis. A recent meta-analysis demonstrated for diagnosis clinically significant portal hypertension (CSPH) in patients with CLD, that the most sensitive validated cut-off is 13 kPa and the most specific cut-off is 21 kPa [25–27]. Recently, Dillon et al. reported that liver stiffness measurements (LSM) identify differences in parameters of TG measured by the calibrated automated thrombogram (CAT) assay in patients with cirrhosis before changes in coagulation times occur [22].

In the present study, both the new fully automated analyzer ST Genesia® Thrombin Generation System (STG) and the CAT assay were evaluated in a cohort of patients with CLD. The aim was to prospectively determine TG and resistance to TM in CLD patients staged with LSM, using both STG and CAT.

2. Methods

2.1. Study design

Patients affected by CLD were recruited prospectively at a single center over a 2-year period (2017–2019).

Patients presenting with CLD of different etiologies and all stage of liver cirrhosis with or without a previous history of clinical decompensation (esophagogastric variceal bleeding, ascites, hepatic encephalopathy, spontaneous bacterial peritonitis or hepatorenal syndrome) were eligible for this study. Inclusion criteria were: CLD defined as a progressive destruction of the liver parenchyma over a period greater than 6 months leading to fibrosis and cirrhosis, age 18–79 years. Exclusion criteria were: age <18 and ≥ 80 years [28], malignancy, ongoing anticoagulation therapy, previous history of venous thromboembolism, active bacterial/parasitic infection, liver cyst, pancreatitis, inherited bleeding disorder, orthotopic liver transplantation, Rendu-Osler-Weber disease. The ethic committee at our institution approved the study (general consent: 01.02.2016 and retrospective/prospective study 2018-00487: 28.03.2018), and written informed consent was obtained from all participants. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

2.2. Clinical data collection

For all enrolled patients, baseline demographic information (age and sex, etiology of the CLD, body mass index (BMI), LSM, spleen size, history of previous decompensation, smoking status, alcohol use, history of diabetes, hematocrit and hemoglobin levels, white blood cell and platelet counts, prothrombin time (PT), international normalized ratio (INR), creatinine, serum albumin, total bilirubin and liver function tests such as aspartate transaminase (AST), alanine transaminase and alkaline phosphatase (ALP)) were collected.

2.3. Transient elastographic analysis

LSM was performed using transient elastography (Fibroscan 502; Echosens, Paris, France) as previously reported [29]. Briefly, patients in a fasted state were lying in the dorsal decubitus position with the right arm in maximal abduction. LSM were realized on the right lobe of the liver through intercostal spaces. Using the appropriate probe according to the skin-to-capsule distance (M probe or XL probe), an experienced operator located a liver portion of at least 6-cm thick and free of large vascular structures. At least 10 successful measurements were performed on each patient. Success rate was calculated as the ratio of the number of successful measurements over the total number of acquisitions. Only LSM with a success rate of at least 60% and an interquartile range/median value below 30% were considered reliable. LSM were expressed in kPa and median value was used as representative of LSM.

2.4. Blood collection and processing

Venous blood was obtained through antecubital venipuncture and collected into 3.2% sodium citrate (2×4.3 mL, S-Monovette, Sarstedt, Nümbrecht, Germany). Samples were then processed by double centrifugation according to the recommendation of the subcommittee of the Scientific and Standardization Committee of the ISTH [28,30]. Briefly, whole blood samples (2×4.3 mL) were centrifuged at 2500*g* for 15 min at room temperature and 3 mL of platelet-poor plasma (PPP) was collected in the middle of the tube, thus avoiding the platelets at the top surface and those at the buffy coat. The collected plasma was recentrifuged at 2500*g* for 15 min at room temperature and 2.6 mL of supernatant (platelet-free plasma, PFP) was collected. The resulting PFP was stored in aliquots at -80 °C. Measurements were all completed within 2 months after blood collection.

2.5. TG assays

TG measurements were performed with the STG assay (Stago, Asnières-sur-Seine, France) and with the CAT assay (Stago, Asnières-sur-Seine, France).

For the STG assay, two types of reagents were used: the STG-BleedScreen (STG-BLS) and the STG-ThromboScreen (STG-TS). TG with the STG-BLS assay was triggered by a mixture of procoagulant phospholipids and low picomolar level of human tissue factor (TF), balanced for sensitivity to procoagulant factor deficiencies while minimizing contact activation. The assay contained two quality controls for low and normal TG potential, respectively, and a reference plasma for parameters normalization. In the STG-TS assay, TG was initiated by a mixture of procoagulant phospholipids and medium picomolar level of human TF in the presence or absence of TM, balanced for sensitivity to deficiencies in natural anticoagulants, without interfering contact activation. The assay contained three quality controls for low, normal and high TM resistance and a reference plasma for parameter normalization.

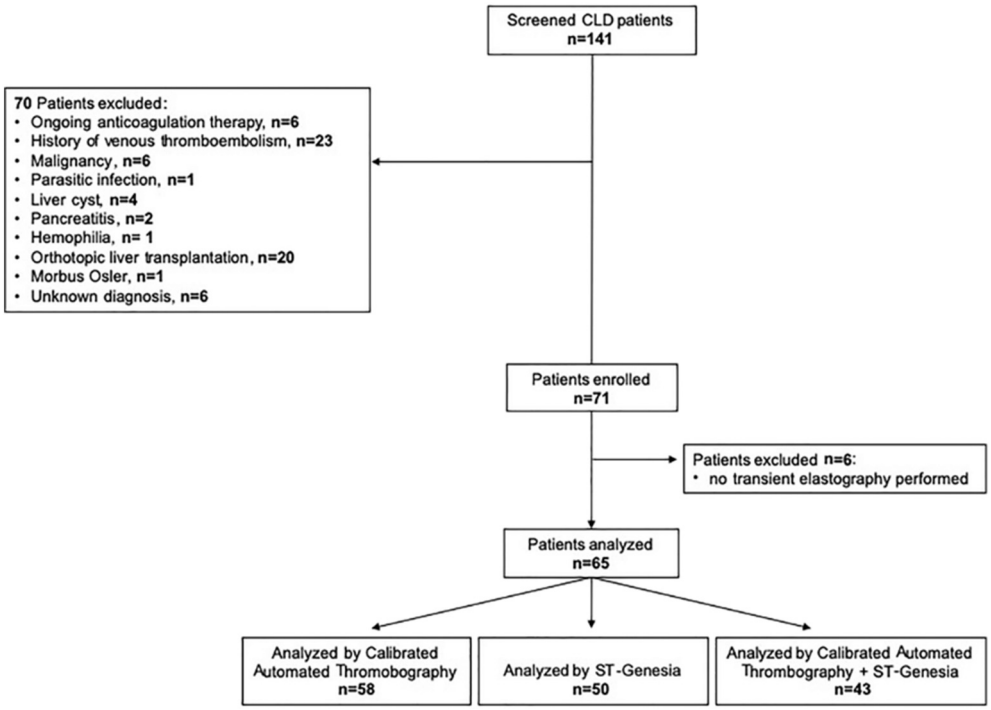


Fig. 1. Flow diagram of patients included in the study. CLD, advanced chronic liver disease.

In the presence of both reagents, TG was triggered by dispensing a fluorogenic substrate and $CaCl_2$. The following parameters were measured: lag time, peak height, time to peak, endogenous thrombin potential (ETP), ETP inhibition, and velocity index. Lag time measures the time from test initiation until thrombin is detected (min), peak is the highest amount of thrombin generated (nM) and time to peak measures the time from test initiation until peak (min). ETP represents the area under the curve (nM.min). Velocity index is the curve slope until peak (nM/min); it represents the rate of thrombin propagation. The ETP inhibition evaluates the ETP reduction in presence of TM. For the data analysis, only the normalized values of each TG parameters were taken into account. Data normalization was performed in each assay in relation to an internal reference control plasma (STG-Reference Plasma, Stago). Normal reference ranges, expressed as interquartile range (25th–75th percentile), were recalculated based on data recently published [28] excluding donors under oral contraceptive and hormonal replacement therapy ($n = 110$).

For the CAT assay, two experimental settings were used.

In the first setting (referred later on as $CAT_{low\ TF}$), 74 μL PFP was added to 20 μL of a mixture of 1 pmol/L TF and 4 μmol/L phospholipids (PPP reagent LOW, Stago), and of recombinant human TM (Sekisui, Alveo AG, Switzerland) or 6 μL of HN-buffer (Hepes 20 mM, NaCl 140 mM, pH 7.4 + 5 mg/mL BSA), in a 96-well round bottom microtiter plate (Immulon2HB, Thermo Fischer Scientific, Reinach, Switzerland). The concentration of TM of 2 nM was tested in a preliminary assay and selected by the ability to decrease by 50% the peak of thrombin.

For the second setting (referred later on as $CAT_{high\ TF}$), 74 μL PFP was added to 20 μL of a mixture of TF and phospholipids (7:3 mixture PPP reagent HIGH and MP reagent, Stago, Asnières-sur-Seine, France) and 6 μL of recombinant human activated protein C (APC) (Enzyme Research, Swansea, United Kingdom) or HN-buffer, in a 96-well round bottom microtiter plate. The concentration of APC of 16 nM was tested in a preliminary assay and selected by the ability to decrease by 90% the ETP [31].

The reaction was initiated with 20 μL of a mixture of fluorogenic substrate and $CaCl_2$ (Fluobuffer, Stago, Asnières-sur-Seine, France) and fluorescence measured using a fluorescence plate reader (Fluoroskan Ascent, Thermo Labsystems, Helsinki, Finland). All experiments were carried out in duplicate at 37 °C for each assay. In addition, the same normal control plasma (Cryocheck Reference Control Normal, PrecisionBiologic, Dartmouth, Canada) was tested in all experiments in order to correct day-to-day variations. TG curves were generated using the Thrombinoscope software version 5.0.0.742 (Thrombinoscope BV, Maastricht, The Netherlands). Lag time, peak height, time to peak, ETP and the ETP ratio obtained in presence/absence of TM or APC was calculated. Results from the control plasma were used to calculate the normalized ETP ratio as follow [32,33]: (Patient ETP_{+TM}/Patient ETP_{-TM})/(Control ETP_{+TM}/Control ETP_{-TM}) and (Patient ETP_{+APC}/Patient ETP_{-APC})/(Control ETP_{+APC}/Control ETP_{-APC}).

Based on the measurement on 111 normal plasma samples, we determined that normalized ETP ratio in presence/absence of TM above 1 and normalized ETP ratio in presence/absence APC above 2 are normal.

2.6. Statistical analysis

Continuous variables were expressed as median and interquartile range (25th–75th percentile) or as mean and standard deviation (SD). Groups were compared by using the Mann–Whitney *U* test for independent samples and Tukey's multiple comparisons test. The Spearman correlation was calculated to verify the association between different parameters. Contingency tables were analyzed by Fisher's test. Statistical analysis was performed using Graphpad Prism 7 software (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Characteristics of the patients cohort

Of 141 screened patients with CLD, we excluded 70 at the time of the TG analyses because of ineligibility and 6 after enrollment due to no LSM, leaving a study sample of 65 patients (Fig. 1). Due to volume limitation of the plasma samples, 58 samples were assessed by CAT, 50 samples by STG and 43 by both STG and CAT (Fig. 1). Characteristics at

Table 1
Demografic, clinical and laboratory characteristics of the studied population according to liver stiffness measurements and thrombin generation analysis. LSM, liver stiffness measurement; BMI, body mass index; INR, international normalized ratio; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; AIH, autoimmune hepatitis; ALD, alcoholic liver diseases; HBV, hepatitis B virus; HCV, hepatitis C virus; HFE, hereditary hemochromatosis, NAFLD, non-alcoholic fatty liver disease; PBC, primary biliary cholangitis.

Characteristic	All	ST Genesia		P	Calibrated automated thrombography		P
		<21 kPa	≥21 kPa		<21 kPa	≥21 kPa	
n	65	38	12	–	43	15	–
Sex (% men)	60	50	83	ns	54	80	*
Age (years)	58 (48–67)	55 (43–64)	60 (56–68)	ns	55 (43–64)	67 (57–70)	*
Etiology	AIH (n = 6) ALD (n = 16) HBV (n = 8) HFE (n = 4) HCV (n = 16) NAFLD (n = 5) Unknown (n = 5) PBC (n = 4) drug-induced (n = 1)	AIH (n = 5) ALD (n = 2) HBV (n = 8) HFE (n = 2) HCV (n = 10) NAFLD (n = 4) Unknown (n = 3) PBC (n = 4)	ALD (n = 8) HCV (n = 2) Unknown (n = 1) drug-induced (n = 1)	–	AIH (n = 6) ALD(n = 2) HBV (n = 8) HFE (n = 3) HCV (n = 12) NAFLD (n = 3) Unknown (n = 4) PBC (n = 4)	ALD (n = 11) HCV (n = 2) Unknown (n = 1) drug-induced (n = 1)	–
BMI (kg/cm^2)	26.5 (22.9–30.4)	25.4 (22–28.8)	29.8 (27.8–34.4)	**	24.4 (22.1–28.8)	28.4 (26.7–33.5)	**
LSM (kPa)	9 (5.0–26.6)	5.9 (4.7–9.3)	34.3 (27.3–61.6)	****	5.9 (4.7–10.9)	36.9 (28.1–53.3)	****
Spleen size (cm)	12.6 (11.0–14.1)	11.6 (10.7–13.7)	13.1 (10.8–16.2)	ns	11.3 (10.5–14.1)	13.4 (12.5–16.0)	ns
Previous decompensation (%)	8	3	33	**	0	29	**
Smoking status (%)	45	42	58	ns	40	47	ns
Alcohol use (%)	49	48	33	ns	54	47	ns
Diabetes (%)	8	8	17	ns	5.0	7	ns
Hematocrit (L/L)	0.40 (0.37–0.42)	0.41 (0.37–0.42)	0.36 (0.33–0.41)	ns	0.41 (0.38–0.42)	0.37 (0.34–0.41)	*
Hemoglobin (g/L)	139 (126–150)	139 (130–150)	126 (117–139)	ns	140 (133–151)	127 (118–140)	*
White cell count (G/L)	6.4 (5.1–8.3)	6.7 (5.1–8.3)	5.4 (4.5–6.8)	ns	6.8 (5.3–8.4)	5.6 (4.6–7.7)	ns
Platelets count (G/L)	196 (116–241)	214 (166–270)	112 (79–134)	***	210 (167–270)	99 (76–127)	****
Prothrombin time (s)	11 (10.6–12.0)	10.7 (10.3–11.0)	12.6 (11–14.4)	****	10.7 (10.5–11.1)	12.5 (10.9–15.5)	****
INR (ratio)	1.03 (1.0–1.1)	1.01 (1.0–1.03)	1.2 (1.05–1.4)	***	1.01 (1–1.03)	1.2 (1.05–1.5)	****
Creatinine (μmol/L)	71 (61–84)	70 (62–88)	77 (60–81)	ns	70 (62.5–88)	77 (57.8–82.5)	ns
Albumin (g/L)	38 (34–40)	39 (37–40.3)	29 (27–34)	****	38 (36.5–40)	27 (25–33)	****
Bilirubin (μmol/L)	11 (7–24)	8.5 (6–16)	25 (11.8–48)	**	9 (7–18.5)	28 (12.5–48)	**
AST (U/L)	30 (23–50)	26.5 (22.8–41.5)	49.5 (31–75.2)	**	28.5 (23–40)	44.5 (31–72.3)	*
ALT (U/L)	31 (21–44.5)	30 (21–43.8)	32 (19.8–45)	ns	31 (23–45)	31 (18–39.5)	ns
AST:ALT (ratio)	1.1 (0.9–1.4)	1 (0.85–1.19)	1.9 (1.1–2.2)	****	0.97 (0.8–1.2)	1.8 (112–2.0)	****
ALP (U/L)	82 (65–117.5)	74.5 (64.7–88.5)	119.5 (99.5–145.5)	***	76 (62.5–91.5)	116 (96.5–132.5)	**

Groups were compared using the Mann-Whitney U test. Data are expressed by median and interquartile range (25th–75th percentiles). ns, non-significant.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.
**** $P < 0.0001$.

the time of the TG analyses are shown in Table 1.

Overall, 39 patients (60%) were men with a median age of 58 years (interquartile range [IQR] 48–67 years). CLD etiology was identified in 92% of patients. Disease severity was staged based on LSM according to the EASL-ALEH guidelines [29]. A cut-off of 21 kPa was used to identify patients with a high likelihood of bearing clinically significant portal hypertension [22,26,34,35].

Patients with elevated LSM (≥21 kPa) at the time of the TG measurement were slightly older in the group assessed by CAT than in the group assessed by STG (median age of 67 versus 60 years, $P < 0.05$). Patients with elevated LSM had a higher BMI and showed higher prevalence of previous decompensation. Only patients with elevated LSM belonging to the CAT group had a slightly lower hematocrit and hemoglobin levels. Patients with elevated LSM had lower platelet counts and albumin concentration. They had also more prolonged PT as well as higher INR, bilirubin, AST, AST:ALT ratio and ALP.

3.2. TG parameters and liver stiffness measurements

Both the lag time to TG and peak TG assessed either by STG or CAT were comparable in the group with LSM <21 kPa and in the group with LSM ≥21 kPa (Figs. 2-3).

The time to peak TG was shorter in the group with LSM <21 kPa when measured either by STG-TS (medium picomolar TF concentration)

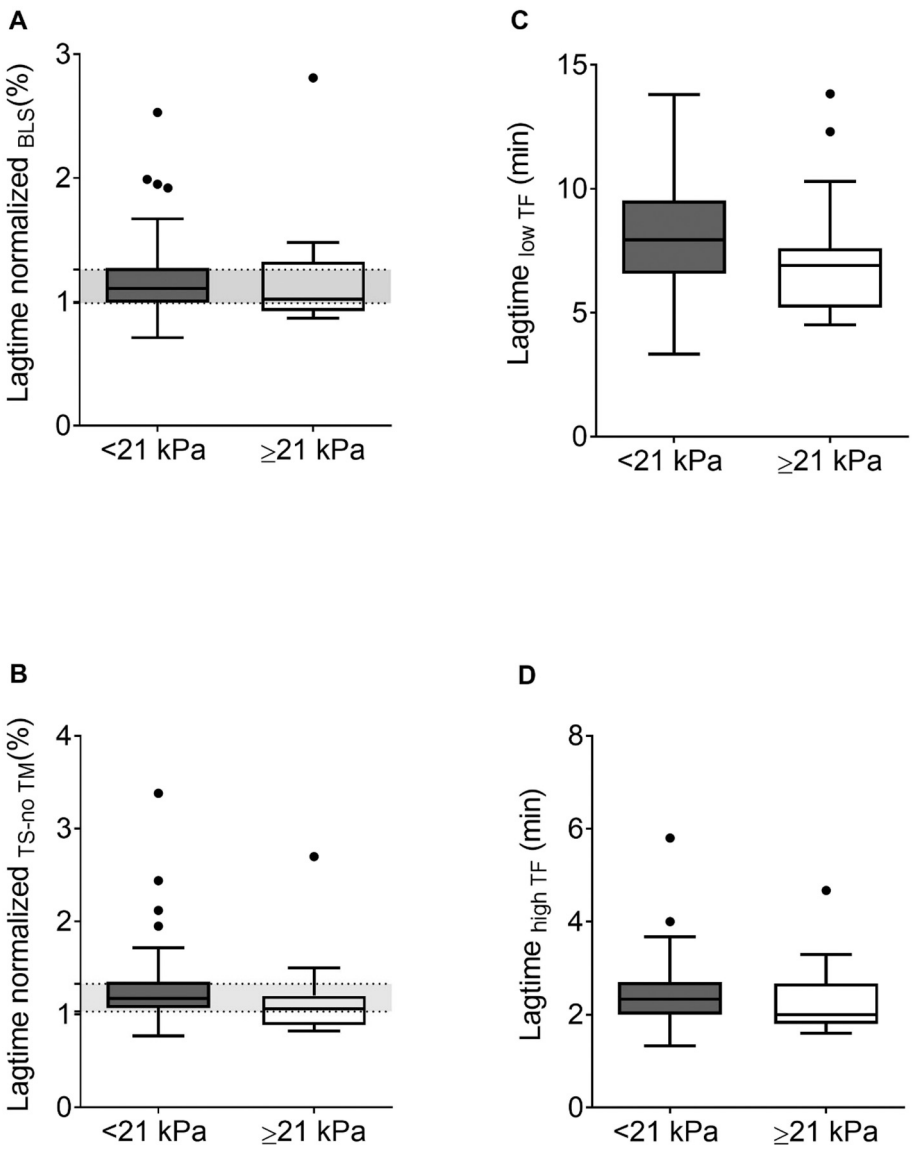


Fig. 2. Lag time to thrombin generation (TG) measured by ST Genesia (STG) and CAT assay in a cohort of patients with CLD separated based on liver stiffness measurement (LSM). (A) Lag time to TG normalized measured by STG-BleedScreen (BLS) assay. (B) Lag time to TG normalized measured by STG-ThromboScreen (TS) assay (without thrombomodulin, TM). (C) Lag time to TG measured by CAT assay with low tissue factor (TF) concentration. (D) Lag time to TG measured by CAT assay with high TF concentration. Median, 25th to 75th percentiles, minimal and maximal values are indicated. The region filled in gray indicates the reference range (25th–75th percentiles) in a healthy adult population [28]. Groups were compared using the Mann-Whitney *U* test.

or CAT assay with high TF concentration (LSM <21 kPa: 1.31 ± 0.33; LSM ≥21 kPa; 1.08 ± 0.38; mean ± SD; $P = 0.0058$), but not when measured either with STG or CAT in presence of a low TF concentration (Fig. 4A-D). The ETP measured either by STG or CAT assay was comparable in the group with LSM <21 kPa and in the group with LSM ≥21 kPa (Supplementary Fig. 1).

ETP inhibition assessed by STG was higher in the group with LSM <21 than in the group with LSM ≥21 kPa (LSM <21 kPa: 57.5 ± 20.7; LSM ≥21 kPa; 33.4 ± 24.4, mean ± SD, $P = 0.0057$; Fig. 5A). Notably, ETP inhibition in the LSM group ≥21 kPa was remarkably decreased when compared with normal reference interval [28] (gray area in Fig. 5A). Similarly, ETP ratio normalized values with or without TM measured by CAT were significantly different in the two LSM groups (LSM <21 kPa: 1.09 ± 0.50; LSM ≥21 kPa: 2.23 ± 1.52; $P = 0.007$, respectively; Fig. 5B). Since the not normalized ratio is currently used in the literature, it is worth noting that the ETP ratio not normalized was also increased in the LSM group ≥21 kPa (LSM <21 kPa: 0.33 ± 0.19; LSM ≥21 kPa: 0.52 ± 0.29; mean ± SD; $P = 0.0196$; Supplementary Fig. 2A). Importantly, ETP inhibition (measured by STG-TS) negatively correlated with the not normalized ETP ratio (measured by CAT) (Supplementary Fig. 2B). All together, these data suggest that patients with LSM ≥21 kPa displayed a hypercoagulability due to a resistance to TM.

Since reagents and settings are different in STG and CAT, the relationship between ETP inhibition evaluated by the two systems was analyzed. STG-ETP inhibition correlated strongly with CAT-ETP ratio normalized ($r = -0.74$, $p < 0.0001$; Fig. 6A).

Resistance to the anticoagulant activity of TM can result from an impaired protein C, protein S, factor V and factor VIII synthesis or activity. In order to define the role of protein C in the observed resistance to TM, we analyzed TG by CAT in presence or absence of exogenous activated PC. Activated protein C-modified TG was comparable in the LSM group <21 kPa and the LSM group ≥21 kPa (Fig. 5C). Finally, there was no correlation between activated protein C and TM sensitivities (assessed by CAT or STG, Fig. 6A-C).

The velocity index (or rate of propagation), only when measured by STG-TS (medium picomolar TF concentration), was higher in the LSM group ≥21 kPa than in the LSM group <21 kPa (Fig. 7A-D).

3.3. Correlation between TG and liver stiffness measurements

Since the evaluation of the liver stiffness is a reliable method for the staging of CLD [29] and is associated with clinically significant portal hypertension [36], we calculated the correlation (coefficients) of the

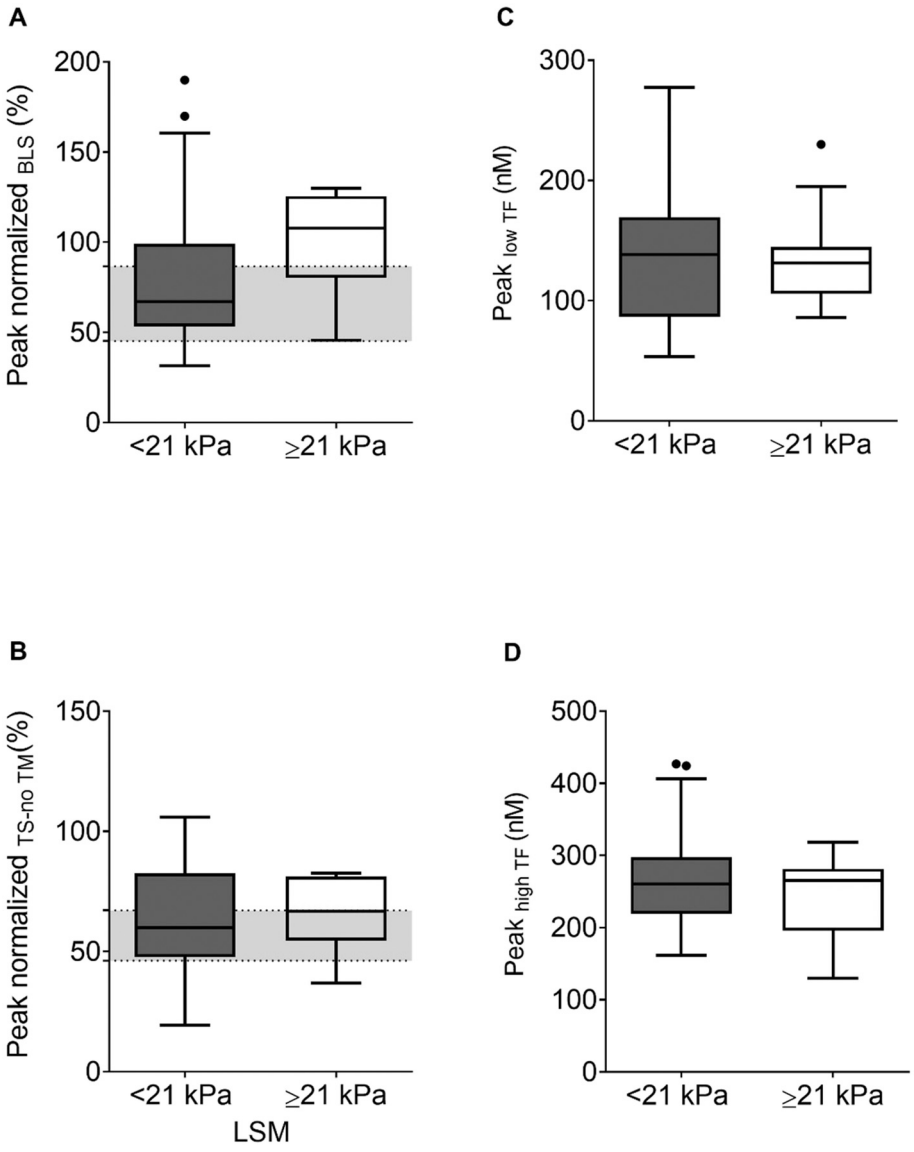


Fig. 3. Peak thrombin generation (TG) measured by ST Genesia (STG) and CAT assay in a cohort of patients with CLD separated based on liver stiffness measurement (LSM). (A) Peak TG normalized measured by STG-BleedScreen (BLS) assay. (B) Peak TG normalized measured by STG-ThromboScreen (TS) assay (without thrombomodulin, TM). (C) Peak TG measured by CAT assay with low TF concentration. (D) Peak TG measured by CAT assay with high TF concentration. Median, 25th to 75th percentiles, minimal and maximal values are indicated. The region filled in gray indicates the reference range (25th–75th percentiles) in a healthy adult population [28]. Groups were compared using the Mann-Whitney *U* test.

linear regression model (*r*) and *P* values between TG parameters and LSM in the study group (Supplemental Table 1).

In STG-BLS (low picomolar TF concentration), normalized values of peak height, ETP and velocity index positively correlated with LSM, while normalized values of time to peak negatively correlated with LSM. In STG-TS (medium picomolar TF concentration), normalized values of peak height and velocity index correlated positively with LSM, while normalized values of time to peak and ETP inhibition correlated negatively with LSM. In contrast, ETP did not correlate with LSM.

When using CAT assay, both peak height and ETP did not correlate with LSM. When using CAT assay with low picomolar TF concentration, lag time and time to peak correlated negatively with LSM, while normalized ETP ratio with or without TM positively correlated with LSM. When using CAT assay with high picomolar TF concentration, lag time and time to peak correlated negatively with LSM.

Finally, normalized ETP ratio with or without APC did not correlate with LSM.

4. Discussion

In this study, we report TG data from the newly automated analyzer ST Genesia® (STG) and the CAT assay in a prospective cohort of heterogeneous patients affected by CLD and provide a comparison. Since the measurement of the liver stiffness is a reliable method for the staging of CLD [29,36], LSM was used to stage the patients included in the CLD cohort. We used a cut-off of 21 kPa to subgroup the patients' sample, as a LSM ≥21 kPa is currently considered the best non-invasive surrogate marker of clinically significant portal hypertension [22].

STG is the first fully automated TG analyzer that could be suitable for the accurate measurement of TG parameters in patients. Reference intervals have been previously determined in a single study involving a small population of healthy adults and need further validation [28]. The main finding of the current study is that resistance to TM assessed by STG and CAT assays was higher in the group of CLD patients with clinically significant portal hypertension as identified by LSM ≥21 kPa. However, ETP inhibition by APC was comparable in patients with LSM of <21 and ≥21 kPa. Consequently, resistance to TM determined in this study seems to be related to an impaired protein C synthesis/activity more than to an increased factor VIII and/or factor V synthesis/activity, in line with a previous report [37].

We also observed that the time to peak TG (or the time to reach the highest thrombin concentration) measured by STG and CAT in presence of high TF was shorter in patients with LSM ≥21 kPa. For the group with LSM ≥21 kPa, the time to peak was shorter compared with normal

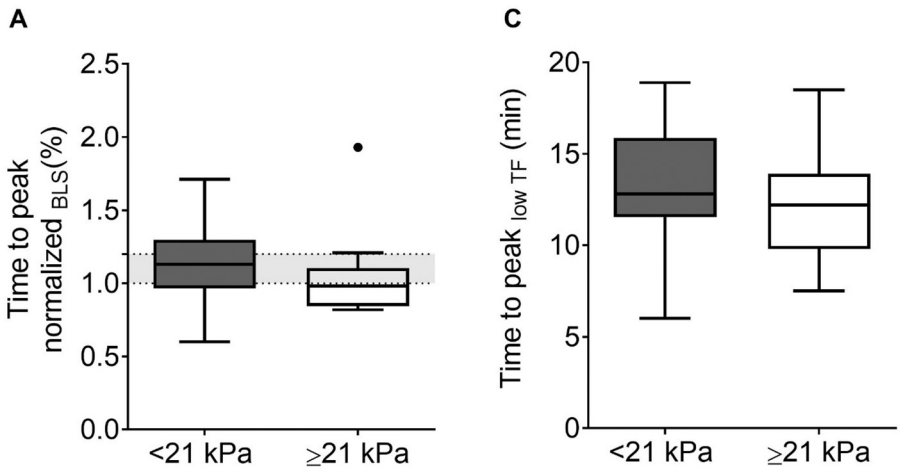
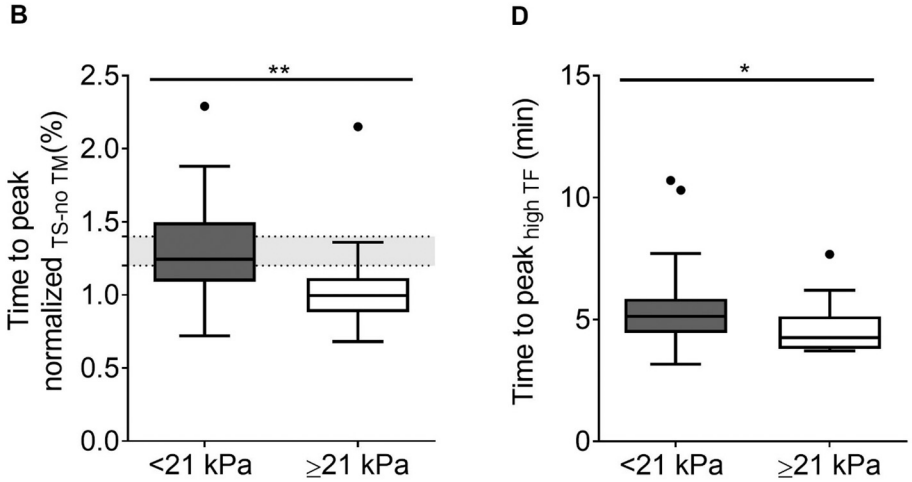


Fig. 4. Time to peak thrombin generation (TG) measured by ST Genesia (STG) and CAT assay in a cohort of patients with CLD separated based on liver stiffness measurement (LSM). (A) Time to peak TG normalized measured by STG-BleedScreen (BLS) assay. (B) Time to Peak TG normalized measured by STG-ThromboScreen (TS) assay (without thrombomodulin, TM). (C) Time to peak TG measured by CAT assay with low TF concentration. (D) Time to peak TG measured by CAT assay with high TF concentration. Median, 25th to 75th percentiles, minimal and maximal values are indicated. The region filled in gray indicates the reference range (25th–75th percentiles) in a healthy adult population [28]. Groups were compared using the Mann-Whitney *U* test. $^{*}P < 0.05$, $^{**}P < 0.01$.



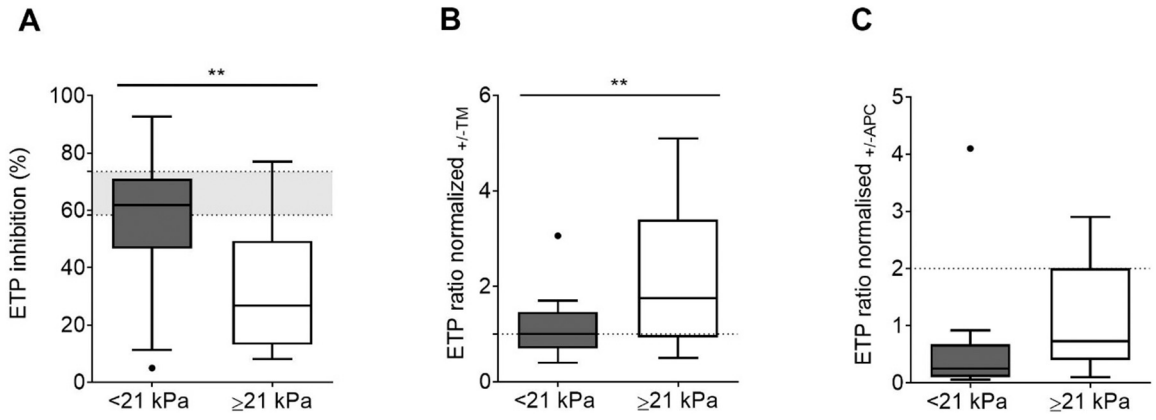


Fig. 5. Resistance to thrombomodulin (TM) measured by ST Genesia (STG) and CAT assay in a cohort of patients with CLD separated based on liver stiffness measurement (LSM). (A) Endogenous thrombin potential (ETP) inhibition measured by STG-ThromboScreen (TS) assay. The region filled in gray indicates the reference range (25th–75th percentiles) in a healthy adult population [28]. (B) Normalized ETP ratio with and without TM measured by CAT assay. Values above the dot line at 1 are considered normal. (C) Normalized ETP ratio with and without activated protein C (APC) measured by CAT assay was used as a control. Values above the dot line at 2 are considered normal. $^{**}P < 0.01$.

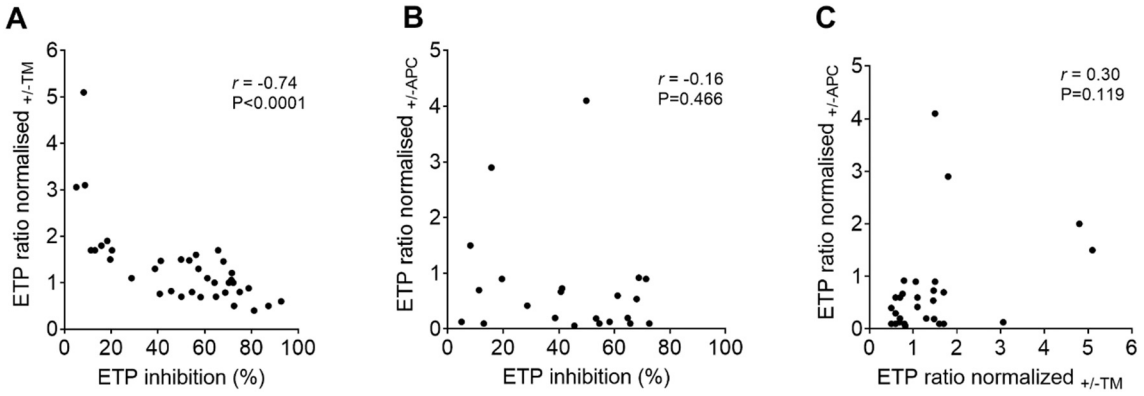


Fig. 6. Correlations between ETP ratio normalized and ETP inhibition with and without thrombomodulin (TM) and with an without APC assessed by ST Genesia and Calibrated automated thrombography in a cohort of patients with CLD (A) Correlation between the ETP ratio normalized with and without TM (measured by CAT assay) and the ETP inhibition (measured by STG-TS) in a subgroup of the cohort ($n = 43$). (B) Correlation between the ETP inhibition (measured by STG-TS) and the ETP ratio with and without APC (measured by CAT) in a subgroup of the cohort ($n = 22$). (C) Correlation between the normalized ETP ratio with and without TM and with and without APC measured by CAT assay, in a subgroup of the cohort ($n = 29$), 25th to 75th percentiles, minimal and maximal values are indicated.

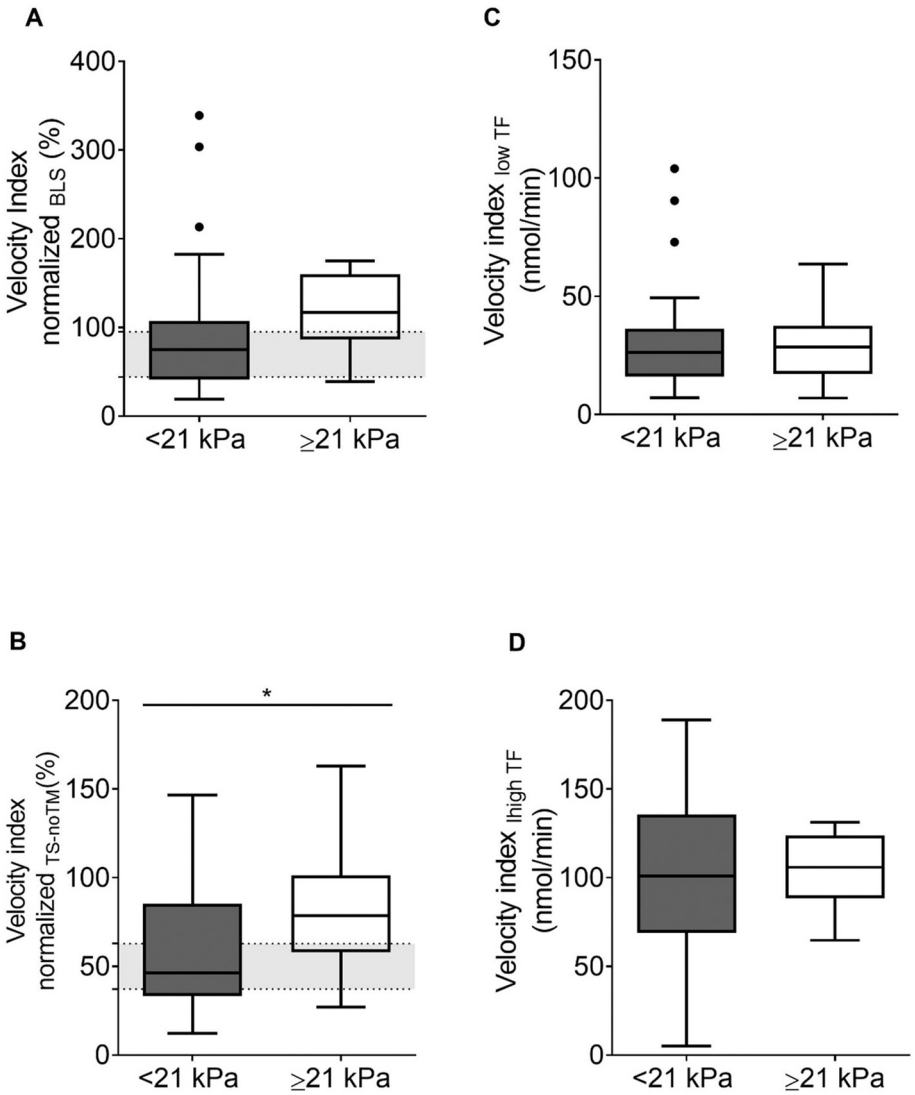


Fig. 7. Velocity index (or rate of propagation) measured by ST Genesia (STG) and CAT assay in a cohort of patients with CLD separated based on liver stiffness measurement (LSM). (A) Velocity index measured by STG-BleedScreen (BLS) assay. (B) Velocity index measured by STG-ThromboScreen (TS) assay (without thrombomodulin, TM). (C) Velocity index measured by CAT with low TF concentration (TF). (D) Velocity index evaluated by CAT with high TF concentration. Median, 25th to 75th percentiles, minimal and maximal values are indicated. The region filled in gray indicates the reference ranges (25th–75th percentiles) in a healthy adult population [28]. Groups were compared using the Mann-Whitney U test. $^*P < 0.05$.

reference ranges [28] as depicted by the gray area in Fig. 4B.

Liver stiffness shows correlation with both STG and CAT, although the STG assay may be better as more of its parameters correlate with LSM in the studied cohort. In particular, liver stiffness showed a moderate correlation with the velocity index (or TG rate) measured with STG. Moreover, when samples were grouped according to LSM cutoff,

there was a significant difference in the velocity index measured by STG only in presence of high TF. For the group of patients with LSM ≥21 kPa, the velocity index was increased compared with normal reference ranges [28] as depicted by the gray area in Fig. 7B. However, the increased TG rate in the group with LSM ≥21 kPa was not observed in CAT data (Fig. 7C-D). This difference between the two instruments could be due to differences in reagents composition and to the distinctive algorithms used for the evaluation of TG rate (Stago, Asnières-sur-Seine, France, personal communication). Taken together these data suggest that the propagation phase of TG is impaired with liver disease worsening (defined by LSM increase).

Conventional coagulation tests do not reflect the hemostatic balance in patients with CLD [1]. In patients with CLD, TG and TM-modified TG are currently considered a more accurate measure of the in vivo coagulation, because it can assess both pro- and anticoagulant forces [15,19–22,38–40]. There is evidence of a procoagulant imbalance in patients with CLD. Similarly to Gatt et al., we found that patients with CLD and clinically significant portal hypertension (identified by LSM) displayed an increased propagation rate compared to healthy controls [19]. In addition, in line with Dillon et al., we were able to show that LSM measurements identify differences in TG parameters in patients with CLD [22]. However, the study of Dillon et al. focused on patients with well-compensated cirrhosis and observed a reduced TG predominantly in patients with LSM >35 kPa and did not report on TM resistance. Another publication [24] has shown the association between TM resistance and de novo portal vein thrombosis as well as low survival in patients with cirrhosis.

Our study has some limitations. First, the study included only 65 patients with CLD from a single center. Second, only a small number of patients with LSM ≥21 kPa (12 analyzed by STG and 15, by CAT) have been included in the study. Thus, confirmation on a larger cohort comprising a higher number of patients with LSM ≥21 kPa would be required, and a multicenter study would certainly be the most appropriate design. Third, the absence of follow-up of the patients precluded an assessment of bleeding and thrombotic events. Fourth, due to volume limitation of the plasma samples, not all of the samples were assessed by both STG and CAT. Fifth, about 10 patients LSM <21 kPa displayed a resistance to TM, indicating that a possible procoagulant state has been detected at an early stage of the disease. A prolonged follow-up of these patients would have been necessary to figure out the impact of this finding. Sixth, patients with LSM ≥21 kPa had a higher BMI. This is a potential confounding factor because high BMI can increase TG [41]. Seventh, we did not measure protein C and S and coagulation factors. All these limitations will be taken into account for the design of a larger study aiming at confirming our current findings.

In conclusion, the STG automated system with BleedScreen and ThromboScreen assays may be a suitable routine coagulation laboratory instrument for the measurement of TG parameters in patients with CLD. Since LSM is broadly used to assess CLD progression, its use in combination with TG changes and the development of a TM resistance may constitute a new approach for the follow up of CLD patients that has to be investigated in larger, multicenter, cohort studies. In particular, these future studies will be necessary to determine whether resistance to TM and LSM in patients with CLD could help to predict decompensation or identify patients at higher risk of portal vein thrombosis at an early stage on. These findings could give more insight to the treated hepatologist to decide whenever an anticoagulation therapy should be started in CLD patients.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank Mrs. Sara Bohni for her help in patient recruitment and blood sample collection.

Funding

This work was supported by the Swiss National Science Foundation (grant number 314730_173127).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.thromres.2021.09.007.

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