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ORIGINAL ARTICLE

In vivo generation of thrombin in patients with liver disease without apparent evidence of activation of the intrinsic or extrinsic pathway of coagulation

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Abstract

Background: Patients with liver diseases are in a hypercoagulable state, as evidenced by enhanced *in vitro* thrombin generating capacity and elevated plasma levels of markers of *in vivo* thrombin generation. However, it is unknown by which mechanism *in vivo* activation of coagulation occurs.

Objectives: We aimed to clarify the mechanisms underlying enhanced *in vivo* thrombin generation to provide a rationale for targeted anticoagulant therapy.

Patients/Methods: Overall, 191 patients diagnosed with stable or acutely decompensated cirrhosis, acute liver failure or injury, acute-on-chronic liver failure, or sepsis without underlying chronic liver disease were recruited from King's College Hospital, London, from 2017 to 2021 and compared with reference values of 41 healthy controls. We measured levels of markers of *in vivo* activation of coagulation and activation of the intrinsic and extrinsic pathways, their respective zymogens, and natural anticoagulants.

Results: Thrombin-antithrombin complexes, prothrombin fragment 1+2 (F1+2), and D-dimer levels were increased in acute and chronic liver disease, proportional to disease severity. Plasma levels of free activated factor XII (FXIIa), C1-esterase-inhibitor (C1inh)-FXIIa, C1inh-factor XI, C1inh-plasma kallikrein, factor-VIIa-antithrombin-complexes, and activated FVII were reduced in acute and chronic liver disease, even after adjusting for zymogen levels, which were also substantially reduced. Natural anticoagulants antithrombin and protein C were profoundly reduced in liver patients.

Conclusions: This study provides evidence of enhanced thrombin generation in liver disease without detectable activation of the intrinsic or extrinsic pathway. We propose that defective anticoagulant mechanisms highly amplify the low-grade activation of coagulation by either pathway.

KEYWORDS
acute, blood coagulation, cirrhosis, hemostasis, liver failure, thrombosis

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1 | INTRODUCTION

Liver disease is characterized by a rebalanced hemostatic state, which is instable and can easily change into hypo- or hypercoagulable profile [1]. Indeed, multiple hypercoagulable features such as increased levels of von Willebrand factor and factor VIII, low levels of ADAMTS-13, low levels of anticoagulant proteins, increased *ex vivo* thrombin generating capacity, elevated levels of procoagulant microvesicles, and increased neutrophil extracellular traps are present in patients with liver disease [2–7]. Multiple studies have shown increased *in vivo* activation of coagulation in liver disease as evidenced by elevated plasma levels of prothrombin fragment 1+2 (F1+2), thrombin-antithrombin complexes (TAT), or D-dimers [8–11]. This hypercoagulable state may contribute to thrombotic risk in liver disease, as patients with liver disease are at an increased risk for development of venous thromboembolism [12,13].

Although there is laboratory and clinical evidence for a hypercoagulable state in patients with chronic and acute liver diseases [14], it is currently unclear what triggers thrombin generation in these patients. Animal studies have suggested a role of the extrinsic pathway of coagulation in initiating thrombin generation. It has been demonstrated that healthy livers express tissue factor, the initiator of extrinsic coagulation, in an inactive or "encrypted" state. Upon liver injury, however, decryption of tissue factor occurs, which contributes to coagulation activation in models of acute and chronic liver injury [15,16]. Alternatively, activation of coagulation in liver disease may be driven by the intrinsic system, which may be activated in patients with liver diseases via various mechanisms [17]. For example, contact system activation is induced by collagen that is highly expressed in the cirrhotic liver, platelet-derived polyphosphate that may be released following platelet activation, and circulating extracellular DNA that may be derived from dying liver cells or from neutrophil extracellular traps [18–22]. As anticoagulant therapy may reduce the progression of liver disease and the risk of portal vein thrombosis [23], and as thromboprophylaxis is indicated for patients with liver disease at risk of venous thrombosis [24], an understanding of the route by which thrombin generation occurs is vital for targeted anticoagulant therapy.

Here, we investigated *in vivo* activation of coagulation in liver disease, specifically focusing on markers of *in vivo* activation of the intrinsic or extrinsic pathway of coagulation in patients with chronic liver disease at various clinical stages of their disease, patients with acute liver failure, and patients with sepsis without underlying liver disease.

Essentials

- Mechanisms of enhanced thrombin generation and thrombotic risks in liver disease are unclear.
- Plasma samples of 191 patients with chronic and acute liver diseases were studied.
- We found enhanced thrombin generation *in vivo* without enhanced intrinsic or extrinsic activation.
- Defective anticoagulant mechanisms may amplify the activation of coagulation by either pathway.

2 | MATERIALS AND METHODS

2.1 | Study population

The present study analyzed plasma samples of 191 patients diagnosed with chronic liver diseases, acute liver failure, and sepsis without underlying liver disease, which were sampled at King's College Hospital in London, United Kingdom, between June 2017 and August 2021. Ethical approval was granted by the National Research Ethics Service Committee London – Westminster (Study Number 12/LO/1417) in accordance with the Declarations of Helsinki and Istanbul. Informed consent was obtained in writing by participants or, in case of mental incapacity, by their personal consultees.

The main inclusion criteria were stable cirrhosis, decompensated cirrhosis, acute liver injury (ALI), acute liver failure (ALF), acute-on-chronic liver failure (ACLF), and sepsis with organ support, but without underlying chronic liver disease. Cirrhosis was defined as 2 or more of (1) histologic evidence on liver biopsy consistent with cirrhosis, (2) laboratory abnormalities consistent with cirrhosis, or (3) radiological findings consistent with cirrhosis and portal hypertension. Decompensated disease was characterized by development of one or more of ascites, jaundice, variceal hemorrhage, or hepatic encephalopathy. ACLF marked the transition from stable or decompensated cirrhosis, defined and assessed by the number of organ failures and 28-day mortality rate according to the CANONIC study [25]. ALI was defined as an acute impairment in hepatic function with impaired coagulation (international normalized ratio > 1.5), no previous history of chronic liver disease, and >24 weeks duration of illness. In the additional presence of encephalopathy, patients were categorized as ALF [26]. Because of limited patient numbers, we grouped ALI and ALF patients in the analysis. Sepsis without underlying chronic liver disease was defined according to the Sepsis-3 criteria [27]. Exclusion criteria were age below 18 years old or above 75 years old, evidence of a pregnancy, congenital coagulation disorder, human immunodeficiency virus positivity, serologic hepatitis B or C, extrahepatic malignancy, or hepatocellular carcinoma.

To establish reference values, blood samples were obtained from 41 healthy volunteers aged >18 years at King's College Hospital. Similar exclusion criteria were applied. Additionally, healthy volunteers with pre-existing liver disease, untreated medical conditions, or current use of anticoagulants, antiplatelet medications or oral contraceptives were excluded.

2.2 | Data collection

Data were collected on patient demographics, disease etiologies, comorbidities, hematology, biochemistry, ascites grade, hepatic encephalopathy grade, illness severity scores, and outcome (death or

liver transplant). We calculated Child–Pugh scores, Model of End-Stage Liver Disease (MELD) scores, Chronic Liver Failure - Consortium (CLF-C) Organ Failure scores (CLF-C-OF), CLF-C Acute decompensation (CLF-C-AD), and CLF-C Acute-on-chronic Liver Failure scores (CLF-C-ACLF).

2.3 | Blood sampling

Patient blood samples were drawn by venipuncture or from a non-heparinized central venous catheter or arterial line within 2 days after admission into 3.2% sodium citrate tubes. Collection was done prior to the administration of blood products, anticoagulants, or antiplatelet medications. Blood from healthy volunteers was obtained by venipuncture. Within 2 hours, all samples were processed to plasma by double centrifugation at 2000 g and 10 000 g for 10 minutes and then stored at −80 °C for subsequent analysis.

2.4 | Coagulation assays

Activation of coagulation in patient plasma was estimated by measuring TAT complexes and F1+2 with commercially available ELISAs (Siemens, Berlin, Germany), and D-dimer levels were measured on the StaCompact 3 (Stago) using reagents and protocols from the manufacturer. Activation of coagulation via the extrinsic pathway of coagulation was assessed via levels of FVIIa-AT complexes and FVIIa by a commercially available clotting test (Stago) assessed on a StaCompact 3 (Stago). We used nanobody-based assays to assess various markers of activation of the intrinsic pathway as described previously [28–30]. Specifically, we measured free FXIIa, and C1Inh-FXIa, C1Inh-FXIIa, and C1Inh-PKa complexes. Briefly, FXII is converted to its active enzyme (FXIIa) by PKa or by auto-activation. To control for PKa-mediated activation, we measured PKa bound by its regulating enzyme, the C1-inhibitor, which is then bound to our nanobodies and detected. Small amounts of FXIIa are free in plasma, and the remaining amounts are also bound by the C1-inhibitor enzyme. We measured C1Inh-XIa, as FXIa becomes activated by FXIIa to further activate the contact system. Zymogen levels of FVII, FXI, FXII, AT, protein C (PC), and free protein S (PS) were assessed on a StaCompact 3 (Stago) using reagents and instructions from the manufacturer. Additionally, prekallikrein (PK) and high-molecular-weight-kininogen (HK) were quantified by commercially available paired antibody sets from Affinity Biologicals and Abcam, respectively. Antithrombin concentrate was purchased from Shire. Purified human protein C was a generous gift from Dr J.C.M. Meijers, Sanquin, Amsterdam, The Netherlands. Thrombomodulin-modified thrombin generation tests were performed as described previously described [9] https://pubmed.ncbi.nlm.nih.gov/33006808/.

2.5 | Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8 for Windows (GraphPad Software) and IBM SPSS Statistics version 28. Continuous variables were expressed as mean ± SD or medians with minimum and maximum, as appropriate. Categorical variables are reported as count with percentage. Normality was assessed by the Shapiro-Wilk Test and D'Agostino and Pearson test. To compare the plasma levels of each analyte of each patient group with reference values measured in healthy individuals and between subgroups of patients, nonparametric one-way analysis of variance (Kruskal–Wallis test) with Dunn's post hoc test or one-way analysis of variance with Dunnett's post hoc test were used, as appropriate. We used linear regression with Spearman's correlation coefficients to assess correlations. A p value of ≤ 0.05 was considered to be statistically significant.

3 | RESULTS

3.1 | Patient characteristics

This study included a total of 168 patients with liver disease clustered in stage and syndrome stable cirrhosis (n = 39), decompensated cirrhosis (n = 63), ACLF (n = 50), and ALI, or ALF (n = 16). In addition, we included patients with sepsis without underlying liver disease (n = 23) to dissect the effects of liver disease and infection in critically ill cirrhosis patients as described previously [9]. Patient values were compared with 41 healthy controls. Demographic, clinical, and laboratory characteristics of patients and controls are summarized in the Table. Of 191 patients, 138 (72%) were male, 45 (24%) were transplanted, 35 (15%) died within 3 months after sampling, 48 (21%) within 1 year, and 64 (28%) within 5 years.

3.2 | Evidence of activation of coagulation in liver disease

We analyzed the plasma of patients and controls for activation of coagulation and subsequent lysis by measuring plasma levels of TAT complexes, F1+2, and D-dimers. As shown in Figure 1A, levels of TAT were significantly higher in patients with ACLF, ALI/ALF, and sepsis compared with healthy controls. Similarly, we observed a mild increase in median plasma levels of F1+2 in patients with ACLF, ALI/ALF, and sepsis (Figure 1B). D-dimers were elevated in AD, ACLF, ALI/ALF, and Sepsis (Figure 1C). Plasma levels of all 3 markers of activation of coagulation increased proportional to the severity and acuteness of the disease.

TABLE Demographic, clinical, and laboratory data of patients and controls.

Variable	Control (*n* = 41)	Stable cirrhosis (*n* = 39)	Decompensated cirrhosis (*n* = 63)	ACLF (*n* = 50)	ALI/ALF (*n* = 16)	Sepsis (*n* = 23)
Age (y)	32.9 ± 7.8	57.1 ± 9.3	54.2 ± 11.9	50.2 ± 8.8	39.9 ± 13.2	59.4 ± 12.1
Male	14 (34)	29 (74)	45 (71)	39 (78)	10 (63)	15 (65)
Etiology	–	–	–	–	–	–
Autoimmune hepatitis	–	0 (0.0)	0 (0.0)	1 (2)	3 (19)	–
Biliary	–	6 (15)	9 (14)	1 (2)	1 (6)	–
Budd–Chiari syndrome	–	0 (0.0)	0 (0.0)	1 (2)	0 (0.0)	–
Cryptogenic	–	1 (3)	4 (6)	1 (2)	0 (0.0)	–
Drugs and toxins	–	17 (44)	38 (60)	37 (74)	3 (19)	–
Infection	–	8 (21)	2 (3)	1 (2)	0 (0.0)	–
NAFLD/NASH	–	5 (13)	9 (14)	8 (16)	1 (6)	–
Sepsis without Liver disease	–	–	–	–	–	23 (100)
Others[a]	–	0 (0.0)	0 (0.0)	0 (0.0)	8 (50)	–
Wilsons's disease or hemochromatosis	–	2 (5)	1 (2)	0 (0.0)	0 (0.0)	–
Examination	–	–	–	–	–	
BMI (kg/m^2)	22.5 ± 2.9	26.3 ± 4.0	27.2 ± 5.2	28.0 ± 5.4	27.1 ± 7.1	29.5 ± 8.1
Mean arterial pressure (calculated)	N/A	91.2 ± 11.2	81.8 ± 9.7	77.1 ± 11.2	83.4 ± 14.8	77.8 ± 10.9
Ascites grade 1 or 2 (mild to severe)	N/A	3 (8)	39 (62)	37 (74)	2 (13)	–
Hepatic encephalopathy grade 3–4	N/A	0	0	13 (27)	3 (21)	–
Child class (%)	–	–	–	–	–	
A	N/A	39 (100)	1 (2)	4 (8)	–	–
B	N/A	0	31 (49)	4 (8)	–	–
C	N/A	0	31 (49)	42 (84)	–	–
Child–Pugh scores	N/A	5.4 ± 0.5	9.4 ± 1.7	10.6 ± 2.1	–	–
MELD scores	N/A	8.8 ± 1.6	20.2 ± 4.6	31.8 ± 7.3	–	–
CLIF-C AD scores	N/A	42.7 ± 5.7	50.7 ± 7.2	59.7 ± 10.3	–	–
CLIF-C ACLF scores	N/A	–	–	53.1 ± 11.1	–	–
CLIF-OF scores	N/A	6.0 ± 0.2	6.8 ± 0.8	11.8 ± 2.8	–	–
Hematology and biochemistry on admission	–	–	–	–	–	–
Alanine aminotransferase (IU/L)	N/A	26 [10-105]	43 [11-401]	37.5 [10-371]	2035 [60-5511]	35 [17-1120]
Albumin (g/L)	N/A	40.0 ± 5.1	29.2 ± 4.3	31.5 ± 5.8	26.1 ± 4.3	28.4 ± 3.9
Alkaline phosphatase (IU/L)	N/A	105 [5-856]	145 [1-837]	104 [1-415]	96.5 [46-303]	91 [42-163]
Aspartate aminotransferase (IU/L)	N/A	31 [6-109]	68 [22-581]	80 [12-1292]	629.5 [86-6369]	81 [13-3718]
Bilirubin (total) (mg/dL)	N/A	0.9 ± 0.5	4.8 ± 3.1	13.0 ± 12.5	13.3 ± 10.1	1.1 ± 1.0
Creatinine (mg/dL)	N/A	0.8 [0.6-1.3]	0.8 [0.3-1.8]	1.3 [0.0-9.7]	1.3 [0.4-3.8]	1.1 [0.0-6.3]
Fibrinogen (g/L)	N/A	3.5 ± 0.8	3.3 ± 3.6	2.3 ± 1.2	2.0 ± 1.5	4.8 ± 0.9
Gamma-glutamyl transferase (IU/L)	N/A	76 [9-2687]	78 [11-938]	54 [15-661]	120 [29-340]	52 [11-291]
Hemoglobin (g/dL)	N/A	134 [63-163]	103 [79-146]	85.5 [66-138]	106 [84-153]	92 [75-133]
International normalized ratio	N/A	1.2 [1.0-1.5]	1.6 [1.1-2.4]	2.0 [1.1-4.7]	2.3 [1.0-7.7]	1.2 [1.0-6.2]
Na+ (mmol/L)	N/A	138.2 ± 3.7	133 ± 4.1	137.6 ± 7.4	139.5 ± 5.7	140.3 ± 5

(Continues)

TABLE (Continued)

Variable	Control (n = 41)	Stable cirrhosis (n = 39)	Decompensated cirrhosis (n = 63)	ACLF (n = 50)	ALI/ALF (n = 16)	Sepsis (n = 23)
Platelet count (x10^9/L)	N/A	158 [45-359]	105 [23-253]	86 [20-271]	127.5 [27-837]	207 [52-669]
Urea (mmol/L)	N/A	5 [2-11]	4.9 [1-15]	10.1 [2-37]	5.9 [0-15]	9.6 [4-40]
White blood cell count (×10^9/L)	N/A	5 [1-10]	5 [1-16]	9.1 [2-31]	8 [4-39]	13 [5-48]
Interventions during admission	–	–	–	–	–	–
Use of pressors	N/A	0	0	20 (40)	5 (31)	19 (83)
Outcome						
Transplanted	N/A	9 (23)	24 (38)	9 (18)	6 (38)	N/A
3-mo mortality	N/A	1 (3)	9 (14)	18 (36)	2 (13)	5 (22)

Mean ± SD or, if appropriate, medians with [minimum and maximum] are reported for continuous variables and count with percentage for categorical variables.

[a] Other etiologies for ALI/ALF include paracetamol overdose, hypotension, necrotizing pancreatitis, and rhabdomyolysis.

ACLF, acute-on-chronic-liver failure; ALI/ALF, acute liver injury/failure; BMI, body mass index; CLF-C, Chronic Liver Failure – Consortium; CLF-C-OF, CLF-C Organ Failure score; CLF-C-AD, CLF-C Acute decompensation; MELD, Model of End Stage Liver Disease; NAFLD/NASH, non-alcoholic fatty liver disease/non-alcoholic steatohepatitis.

3.3 | No evidence of activation of the extrinsic pathway of coagulation in patients with liver disease

We assessed whether *in vivo* thrombin generation in liver disease was associated with the activation of the extrinsic pathway of coagulation, as assessed by plasma levels of FVIIa-AT complexes and free FVIIa. As depicted in Figure 2A and B, FVIIa-AT and FVIIa were both reduced in all liver etiologies, but not in patients with sepsis, compared with healthy controls, and median levels decreased proportional to disease severity. Furthermore, levels of FVIIa-AT complexes or free FVIIa were not correlated to levels of F1+2, TAT, and D-dimers (Supplementary Tables S1 and S2).

3.4 | No evidence of activation of the intrinsic pathway of coagulation in patients with liver disease

We next investigated whether *in vivo* thrombin generation in liver disease was linked with *in vivo* activation of the intrinsic pathway of coagulation by assessing components of the contact system. We analyzed complexes of activated intrinsic coagulation factors with C1inh, specifically C1inh-FXIIa, C1inh-FXIa, and C1inh-PKa complexes and levels of free factor XIIa as markers of activation of the intrinsic pathway. We observed similar levels of plasma levels of free FXIIa (Figure 3A) and C1inh-FXIa complexes (Figure 3C) in patients compared to controls. In contrast, both, C1inh-FXIIa complexes

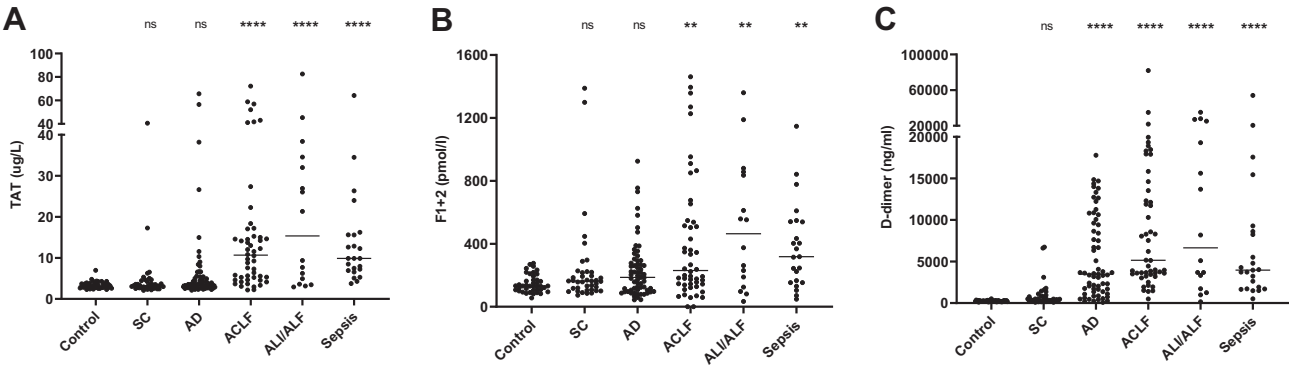

FIGURE 1 Plasma levels of (A) TAT, (B) F1+2, and (C) D-dimers were measured in patients with SC (n = 39), AD (n = 63), ACLF (n = 50), ALI or ALF (n = 16), or sepsis (n = 23) and compared with healthy controls (n = 41). Horizontal lines indicate medians. ns = not significant, $^*p < .05$, $^{**}p < .01$, $^{****}p < .0001$, all vs control. TAT, Thrombin-antithrombin-complex; F1+2, Prothrombin fragment F1+2; SC, stable cirrhosis; AD, decompensated cirrhosis; ACLF, acute-on-chronic-liver failure; ALI/ALF, acute liver injury/failure.

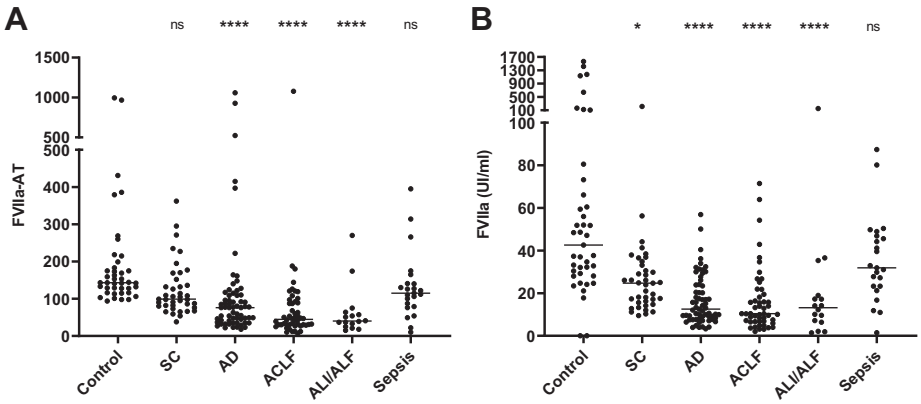

FIGURE 2 Plasma levels of (A) FVIIa-AT complexes and (B) FVIIa were measured in patients with SC (n = 39), AD (n = 63), ACLF (n = 50), ALI or ALF (n = 16) or sepsis (n = 23) and compared to healthy controls (n = 41). Horizontal lines indicate medians. ns = not significant, *p < .05, ***p < .001, ****p < .0001, all vs control. FVIIa-AT, activated factor VII-antithrombin complex; SC, stable cirrhosis; AD, decompensated cirrhosis; ACLF, acute-on-chronic-liver failure; ALI/ALF, acute liver injury/failure.

(Figure 3B) and C1Inh-PKa complexes (Figure 3D) were significantly lower in all liver etiologies and sepsis compared to healthy controls and decreased proportional to disease severity.

3.5 | Reduced plasma levels of pro- and anticoagulants in patients with liver disease

Thus, although we found clearly elevated levels of *in vivo* markers of thrombin generation, we did not find elevated levels of markers of activation of the extrinsic or intrinsic coagulation pathway in patients with liver disease and patients with sepsis without underlying liver disease. However, levels of these markers may be difficult to compare between patients with liver disease and controls as plasma levels of their respective zymogens may be much lower in patients with liver disease compared with controls. Therefore, we measured plasma levels of FXII, FXI, FVII, PK, and HK (Figure 4) and found decreased plasma levels of all 5 zymogens in patients with liver disease proportional to disease severity. Also, in patients with sepsis without underlying liver disease, plasma levels of intrinsic pathway zymogens were decreased compared with controls. As shown in Figure 5A and C, the anticoagulant proteins AT and PC were also significantly reduced in liver disease, notably AD, ACLF, and ALI/ALF, compared with those in healthy controls. We found no statistically significant decrease in PS levels in patients, except in patients with ALI/ALF. Nonetheless, a mild reduction in plasma levels proportional to the severity of liver disease was observed (Figure 5B). We next assessed whether the reduced levels of AT and PC may be responsible for the amplification of a small initiating coagulation trigger *in vivo*, and thereby explain the discrepancy between elevated markers of activation of coagulation without apparent activation of the intrinsic or extrinsic pathways. We selected 15 samples from our cohort in which both AT and PC levels were below 50% and performed thrombin generation tests in these samples in the absence and presence of purified AT and PC that was added to obtain AT and PC levels of 100% in each sample as described previously [31]. As shown in Supplementary Figure S1, thrombin generation was slightly higher in patients compared with 10 healthy controls without AT and PC normalization. When both AT and PC levels were normalized to 100%, thrombin generation was profoundly inhibited.

3.6 | Ratios of activation markers with their respective zymogens

We next calculated and analyzed the ratios of markers of activation of extrinsic or intrinsic activation of coagulation with their respective zymogen levels to correct for the decreased zymogen levels in patients (Figure 6). Although free FXIIa levels corrected for FXII zymogen levels and C1in-FXIa corrected for FXI were (slightly) elevated in patients with ACLF, ALI/ALF, and sepsis, C1inh complexes with FXIIa and PK were not elevated in patients when corrected for zymogen levels. Free FVIIa levels corrected for FVII zymogen were mildly elevated in ACLF patients. FVIIa-AT complexes corrected for FVII zymogen levels were slightly elevated in patients with AD.

3.7 | Comparisons between subgroups of liver disease

Finally, we compared values of all analytes assessed between the different subgroups of patients. We found significantly higher plasma levels of TAT and D-dimers in patients with more advanced disease compared with stable cirrhosis (Supplementary Table S3). However, levels of these markers were not significantly different between decompensated cirrhosis and stable cirrhosis. In contrast, all markers of extrinsic activation, intrinsic activation, and all zymogens and natural anticoagulants measured were significantly higher in patients with stable cirrhosis compared with decompensated cirrhosis, ACLF, and ALI/ALF, but not statistically different between decompensated cirrhosis and more advanced liver disease.

4 | DISCUSSION

In this study, we found substantially elevated markers of *in vivo* generation of thrombin in patients with chronic liver diseases, ALF, and nonliver sepsis without evidence of concurrent activation of the intrinsic or extrinsic pathway of coagulation. We propose this finding to result from very low-grade activation of coagulation either via the intrinsic or extrinsic pathway that is amplified to substantial thrombin

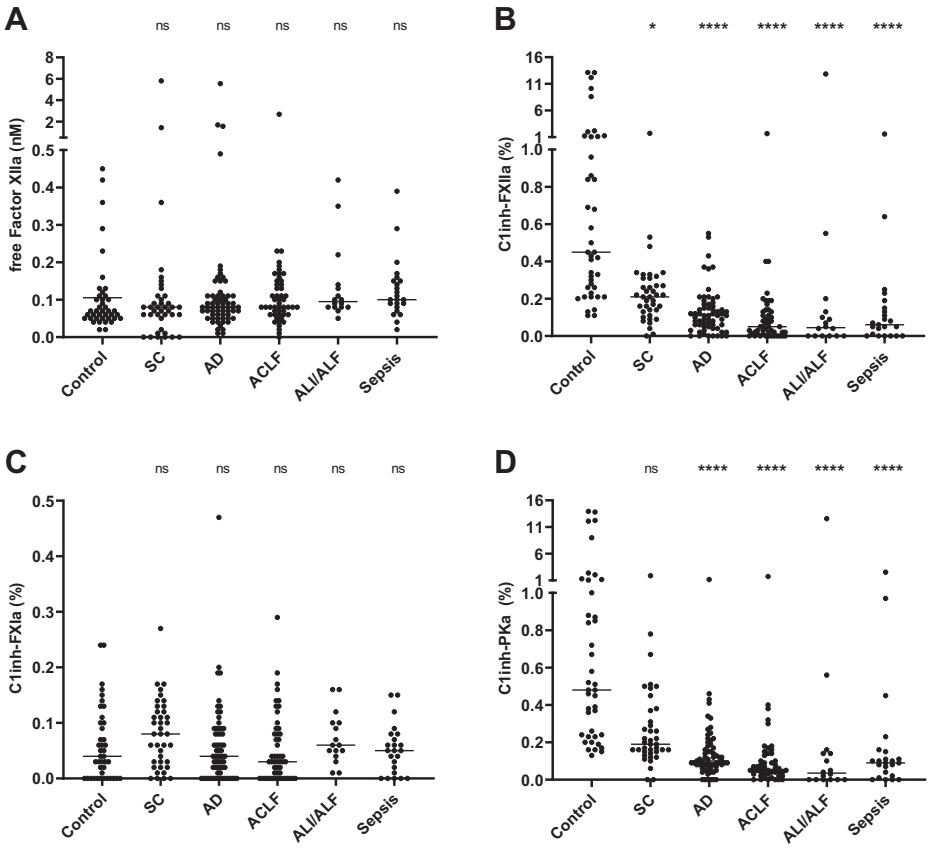

FIGURE 3 Plasma levels of (A) Factor XIIa (free), (B) C1inh-FXIIa complexes, (C) C1inh-FXIa complexes, and (D) C1inh-PKa complexes were measured in patients with SC (n = 39), AD (n = 63), ACLF (n = 50), ALI or ALF (n = 16) or sepsis (n = 23) and compared to healthy controls (n = 41). Horizontal lines indicate medians. Asterisks with bars indicate significance compared to control (ns = not significant, $^{*}p < .05$, $^{****}p < .0001$). C1inh: C1-Esterase-Inhibitor; SC, stable cirrhosis; AD, decompensated cirrhosis; ACLF, acute-on-chronic-liver failure; ALI/ALF, acute liver injury/failure.

generation because of defective regulation of coagulation by deficiencies of the natural anticoagulants AT and PC. Indeed, deficiencies in natural anticoagulants have been well established as contributors to the normal to enhanced *in vitro* thrombin-generating capacity in patients with cirrhosis [3,31–33]. In addition, plasma levels of AT and PC are important determinants of thrombin-generating capacity in healthy individuals [34]. Our data confirm previous findings on enhanced *in vivo* thrombin generation in liver disease [1,3,8,9,11] while providing a novel insight into the mechanisms underlying activation of coagulation in these patients.

Profound thrombin generation, as reflected by increased levels of TAT, F1+2, and D-dimers, primarily occurred in the sickest patients studied. Indeed, previous studies found no evidence for enhanced *in vivo* thrombin generation in patients with stable cirrhosis [32], whereas other studies did find elevations of TAT, F1+2, and D-dimers in patients with more advanced (Child B and C) cirrhosis [8,11]. It is tempting to speculate that the lack of activation of coagulation in stable cirrhosis relates to the relatively preserved levels of natural anticoagulant proteins, but it may also be that in these stable patients, the initiating trigger for activating coagulation is absent. Furthermore, activation of coagulation was significantly higher in acutely ill patients compared with patients with stable decompensated disease. This suggests a transition to a more hypercoagulable state in patients in whom acute complications develop.

Despite markedly elevated levels of *in vivo* generation of thrombin in the sickest patients, we did not find detectable activation of the extrinsic or intrinsic pathway, as measured by various markers of *in vivo* activation of these pathways, even after adjustment for decreases in zymogen levels. Only levels of free FXIIa corrected for FXII zymogen levels were elevated in AD, ACLF, ALF, and nonliver sepsis. However, given its rapid inactivation by inhibitors in plasma, free FXIIa levels may be a less reliable marker than FXIIa bound to C1inh, which were not significantly elevated in any group. Furthermore, we observed an elevation of C1inh-FXIa corrected for FXI in ALI/ALF and an increase in FVIIa corrected for FVII in ACLF. Whether this inconsistent increase in free FXIIa, C1inh-FXIa, and FVIIa is a signal for involvement of the intrinsic or extrinsic pathway or a chance finding requires further study. Interestingly, one study in patients with ALF has suggested that activation of coagulation in these patients primarily proceeds through the extrinsic pathway as zymogen levels of extrinsic factors were much lower compared to zymogen levels of intrinsic factors [35]. In that same study, zymogen levels of intrinsic and extrinsic factors were similarly reduced in patients with cirrhosis, and it was suggested that whereas in ALF, coagulation factor levels are primarily decreased because of a tissue-factor–driven consumption coagulopathy, in cirrhosis defective hepatic synthesis primarily explains low factor levels. In our study, we confirm these earlier observations, with much higher levels of intrinsic zymogen levels in our small ALI/ALF cohort compared with zymogen FVII levels. We also observed similarly decreased levels of zymogen levels of intrinsic factors compared with zymogen FVII levels in the cirrhosis patients. However, because we do not find direct evidence of the activation of

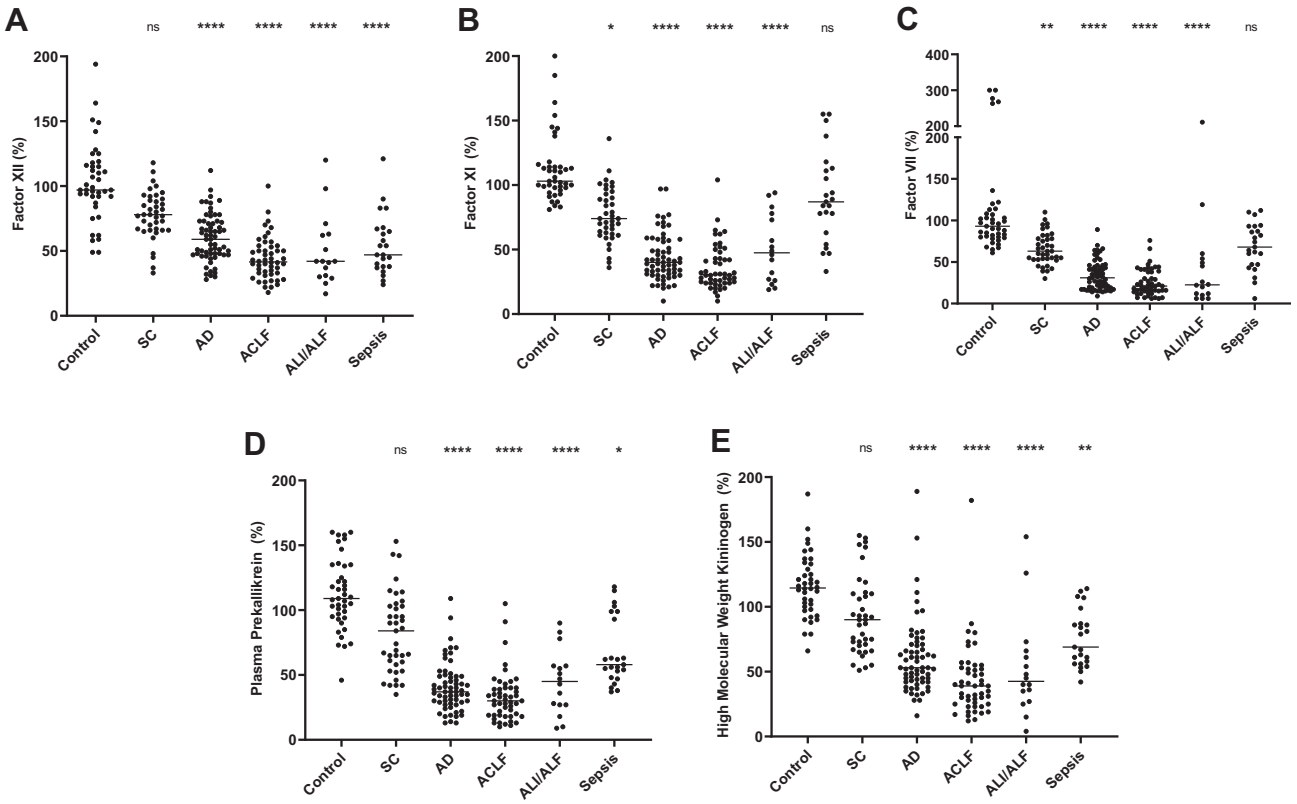

FIGURE 4 Plasma levels of (A) FXII, (B) FXI, (C) FVII, (D) PK, and (E) HK were measured in patients with SC (n = 39), AD (n = 63), ACLF (n = 50), ALI or ALF (n = 16) or sepsis (n = 23) and compared with healthy controls (n = 41). Horizontal lines indicate medians. ns = not significant, $^{*}p < .05$, $^{**}p < .01$, $^{****}p < .0001$, all vs control. F, factor; PK, prekallikrein; HK, high-molecular-weight-kininogen; SC, stable cirrhosis; AD, decompensated cirrhosis; ACLF, acute-on-chronic-liver failure; ALI/ALF, acute liver injury/failure.

the extrinsic pathway in our ALI/ALF cohort, as evidenced by low FVIIa-AT levels, we feel that the conclusion that coagulation activation in ALI/ALF is tissue-factor–driven is incompletely supported by the data.

Similar to patients with liver disease, patients with sepsis did not show activation of the intrinsic or extrinsic system. In line with this finding, in a small study in which sepsis was modeled in healthy individuals by infusion of endotoxin, *in vivo* generation of thrombin was demonstrated, without evidence of activation of the intrinsic pathway as evidenced by a lack of increase in C1inh-FXIIa and C1inh-PKa complexes [36]. However, in a subsequent study using the same model, inhibition of the tissue factor-FVIIa complex (TF/FVIIa) blocked endotoxin-induced thrombin generation, measured by F1+2 levels, which contrasts with our findings [37].

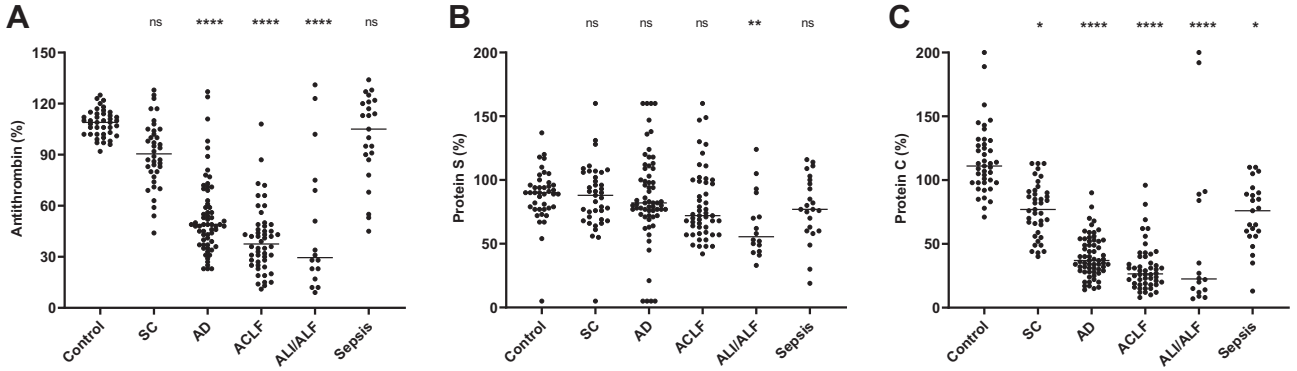

FIGURE 5 Levels of (A) antithrombin, (B) Protein S, and (C) Protein C were measured in plasma of patients with SC (n = 39), AD (n = 63), ACLF (n = 50), ALI or ALF (n = 16) or sepsis (n = 23) and compared with healthy controls (n = 41). Horizontal lines indicate medians. ns = not significant, $^{*}p < .05$, $^{****}p < .0001$, all vs control. AT, antithrombin; PS, protein S; PC, protein C; SC, stable cirrhosis; AD, decompensated cirrhosis; ACLF, acute-on-chronic-liver failure; ALI/ALF, acute liver injury/failure.

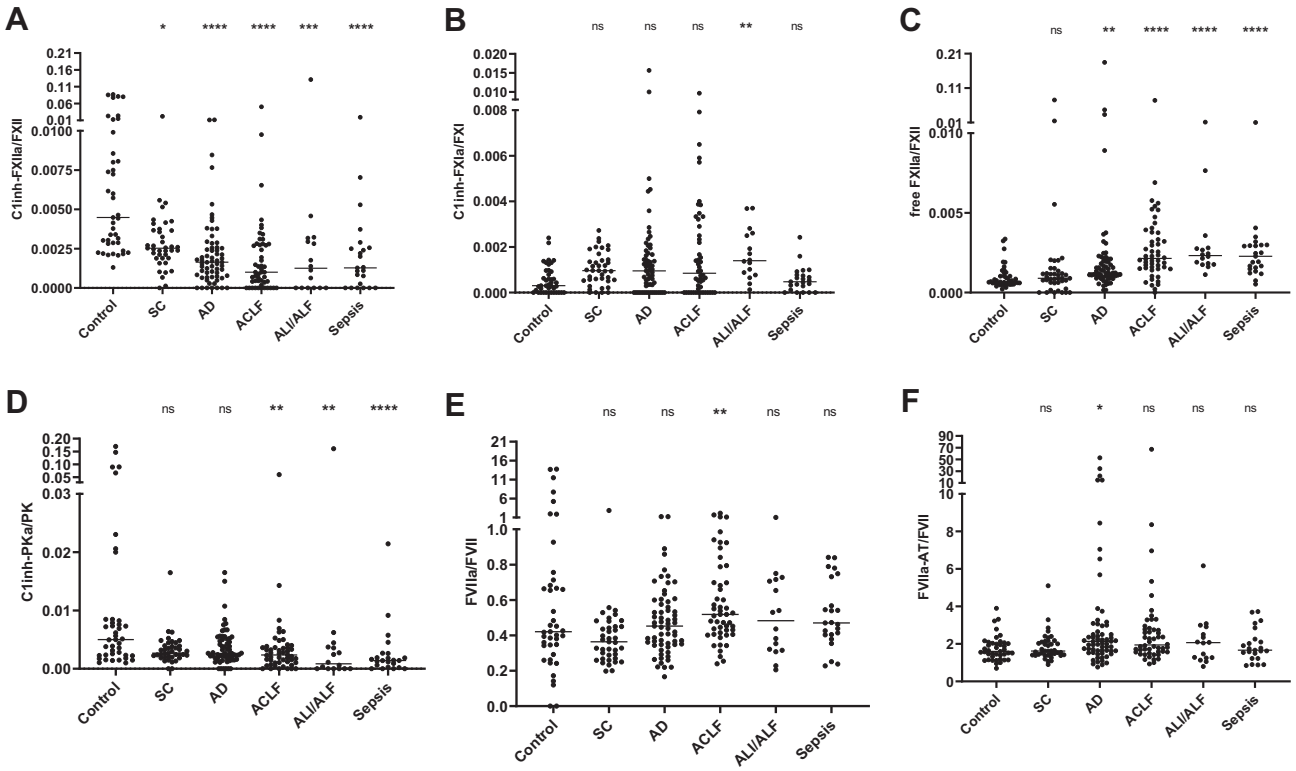

FIGURE 6 Ratios of plasma levels of coagulation factors and their respective zymogens (A–F) in patients with SC (n = 39), AD (n = 63), ACLF (n = 50), ALI or ALF (n = 16) or sepsis (n = 23) and compared with healthy controls (n = 41). Horizontal lines indicate medians. ns = not significant, *p < .05, **p < .01, ***p < .001, ****p < .0001, all vs control. SC, stable cirrhosis; AD, decompensated cirrhosis; ACLF, acute-on-chronic-liver failure; ALI/ALF, acute liver injury/failure.

We acknowledge an important difficulty in interpreting plasma levels of markers of activation of coagulation in patients with liver diseases. This caveat being the fact that these markers are cleared by the liver. For example, we have recently demonstrated that levels of D-dimer and thrombin-antithrombin complexes are much lower in samples taken from the hepatic vein compared to samples taken from the portal vein or systemic circulation [38]. As clearance of these markers may be reduced when the functional capacity of the liver to clear proteins decreases, plasma levels of TAT, F1+2, and D-dimer may in part reflect the lack of clearance and therefore may overestimate the activation of coagulation because of accumulation of these proteins in the circulation. Nevertheless, such an accumulation mechanism would be in play for other markers, such as C1inh complexes and FVIIa-AT complexes. As levels of these proteins are very low, even in the sickest patients with liver disease, it may be that the accumulation issue is not very relevant, and that there is indeed a substantial generation of thrombin as suggested by TAT, F1+2, and D-dimer levels.

To conclude, in this study, we demonstrated enhanced *in vivo* thrombin generation in liver disease, without detectable activation of the intrinsic or extrinsic pathways. We propose that low-grade activation of coagulation is highly amplified because of improper regulation of coagulation by deficiencies in natural anticoagulants AT and PC. Indeed, thrombin generation experiments confirm the profound effect of AT and PC deficiency on thrombin generation in plasma in patients with liver disease. Future studies, for example, using anticoagulants that specifically target the intrinsic (FXIIa inhibitors) or extrinsic (active site-inactivated FVIIa) system, should explore whether thrombin generation in these patients or in animal models can be effectively downregulated. Such experiments would show whether activation of the intrinsic or extrinsic pathway is responsible for the generation of thrombin. Adequately targeted anticoagulants may then be used to prevent or treat thrombotic complications of liver diseases.

AUTHOR CONTRIBUTIONS

Conception and design: F.L.E., W.B., V.C.P., C.M., and T.L. Data acquisition: F.L.E., J.A., M.S., D.J., W.B., and V.C.P. Data analysis and interpretation: F.L.E., W.B., V.C.P., C.M., and T.L. Drafting of the manuscript: F.L.E. and T.L. Revision of the manuscript: M.S., J.A., D.J., W.B., C.M., V.C.P., and T.L. Supervision: T.L.

DECLARATION OF COMPETING INTEREST STATEMENT

There are no competing interests to disclose.

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SUPPLEMENTARY MATERIAL

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