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DOI
10.1016/j.jtha.2023.06.011

Publication date
2023

Document Version
Final published version

Published in
Journal of Thrombosis and Haemostasis

Citation (APA)
Driever, E. G., Muntz, I., Patel, V., Adelmeijer, J., Bernal, W., Koenderink, G. H., & Lisman, T. (2023). Fibrin clots from patients with acute-on-chronic liver failure are weaker than those from healthy individuals and patients with sepsis without underlying liver disease. *Journal of Thrombosis and Haemostasis*, *21*(10), 2747-2758. https://doi.org/10.1016/j.jtha.2023.06.011

Received: 24 February 2023 | Revised: 9 May 2023 | Accepted: 4 June 2023

https://doi.org/10.1016/j.jtha.2023.06.011

ORIGINAL ARTICLE

Fibrin clots from patients with acute-on-chronic liver failure are weaker than those from healthy individuals and patients with sepsis without underlying liver disease

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Funding information
This study was funded in part by a grant from the Dutch Thrombosis Foundation (2018-02) and by a grant from the De Cock-Hadders Foundation (2021-71). I.M. and G.H.K. acknowledge funding from the Convergence program Syn-Cells for Health(care) under the theme of Health and Technology.

Abstract

Background: Previous studies identified decreased clot permeability, without differences in fibrin fiber density in clots, from patients with cirrhosis compared with those from healthy controls (HCs). Fibrinogen hypersialylation could be the reason for this discrepancy.

Objectives: The aim of this work was to study mechanical properties of clots and reassess clot permeability in relation to hypersialylation in patients with stable cirrhosis, acute decompensation, and acute-on-chronic liver failure (ACLF). Sepsis patients without liver disease were included to distinguish between liver-specific and inflammation-driven phenotypes.

Methods: Pooled plasma was used for rheology and permeability experiments. Permeability was assessed with compression using a rheometer and by liquid permeation. Purified fibrinogen treated with neuraminidase was used to study the effects of fibrinogen hypersialylation on liquid permeation.

Results: Mechanical properties of clots from patients with stable cirrhosis and acute decompensation were similar to those of clots from HCs, but clots from patients with ACLF were softer and ruptured at lower shear stress. Clots from sepsis patients without liver disease were stiffer than those from the other groups, but this effect disappeared after adjusting for increased plasma fibrinogen concentrations. Permeability was similar between clots under compression from HCs and clots under compression from patients but decreased with increasing disease severity in liquid permeation. Removal of fibrinogen sialic acid residues increased permeability more in patients than in controls.

Conclusion: Clots from patients with ACLF have weak mechanical properties despite unaltered fibrin fiber density. Previous liquid permeation experiments may have erroneously concluded that clots from patients with ACLF are prothrombotic as

Manuscript handled by: Tetsumei Urano

Final decision: Tetsumei Urano, 05 June 2023

fibrinogen hypersialylation leads to underestimation of clot permeability in this setting, presumably due to enhanced water retention.

KEYWORDS
fibrin, inflammation, liver cirrhosis, permeability, rheology, thrombosis

1 | INTRODUCTION

Patients with liver diseases frequently have hemostatic alterations due to reduced capacity of the liver to produce proteins that are involved in hemostasis [1]. Routine laboratory tests, such as prothrombin time and platelet count, are often disturbed and point to a hypocoagulable state in patients with liver diseases [2]. However, more advanced research-type laboratory assays that are sensitive to both prohemostatic and antihemostatic factors have shown that these patients are in hemostatic rebalance since the prohemostatic and antihemostatic systems change simultaneously [2]. This hemostatic rebalance appears to persist even in the sickest patients [3,4] but may flip toward a prohemorrhagic or prothrombotic state in patients with additional disease complications that alter the hemostatic state. For example, patients with cirrhosis with acute decompensation (AD) or acute-on-chronic liver failure (ACLF) often develop renal failure or have systemic inflammation, complications that are associated with hemostatic changes that have been proposed to increase bleeding risk [5,6].

Despite advances in our understanding of the clinical consequences of altered plasma levels of proteins involved in hemostasis in stable and acutely ill patients with liver disease, there has been little attention to the clot composition and structure. This is of great importance since the composition and structure of the thrombus determine its susceptibility to fibrinolysis and embolization [7,8]. Resistance to fibrinolysis can be assessed by assays that directly probe the lysis time of a clot and tests that measure clot structure or mechanical properties of the clot. For example, abnormally dense fibrin structures have altered mechanical properties, such as increased clot stiffness, and are resistant to degradation by plasmin [9,10]. Such prothrombotic clots have been shown in patients with deep vein thrombosis, coronary artery disease, and stroke [11]. Clots from patients with cirrhosis also have been proposed to have prothrombotic properties, as shown by decreased permeability compared with those from healthy controls [12]. Interestingly, however, no differences were observed in fibrin fiber thickness and density between clots from patients with cirrhosis and those from controls. The discrepancy between decreased permeability and normal fibrin network structure based on analysis of laser scanning confocal microscopy and scanning electron microscopy images was proposed to be related to the presence of posttranslational protein modifications in cirrhotic fibrinogen. These posttranslational protein modifications may affect permeability, without changes in fibrin structure [12]. For example, fibrinogen in patients with liver diseases is hypersialylated [13,14], which has been related to liver disease–induced enhancement of sialyltransferase activity [15]. The consequent increase in negative charge in the fibrin network may decrease permeability as water is better retained within the clot. This hypothesis, however, has never been experimentally verified.

Essentials

- Previous studies showed decreased permeability but normal structure in cirrhotic plasma clots.
- We studied mechanical properties of clots from patients with cirrhosis.
- Clots from the sickest patients are soft and rupture at low shear stress.
- Permeability results varied per experimental setup, likely due to hypersialylation of fibrinogen.

Posttranslational protein modifications of fibrinogen not only affect clot structure but also decrease the fibrin polymerization rate, as was demonstrated for the hypersialylation of fibrinogen [16]. It also affects the mechanical properties of the fibrin network [17], which in turn determines its functionality in wound healing, stability of the fibrin network, and susceptibility to fibrinolysis of the clot [18]. As our previous studies [12,19] identified both prothrombotic and antithrombotic changes in the fibrinogen molecule in patients with cirrhosis using turbidity and permeation assays, the aim of this study was to compare the mechanical properties of clots from patients with stable cirrhosis, AD, and ACLF and reassess clot permeability in relation to hypersialylation in patients with liver diseases. Samples from patients with nonliver sepsis were added to determine whether changes in clot properties in patients with ACLF are directly related to the liver disease or are related to the effects of systemic inflammation on the clot.

2 | METHODS

2.1 | Patients

Citrated plasma samples from patients with mild, stable (Child-Pugh class A or B) cirrhosis, AD cirrhosis, ACLF, and nonliver sepsis as well as healthy controls were collected between June 2017 and August 2021 at King's College Hospital, London, United Kingdom. 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 from participants or, in case of mental incapacity, their personal consultees. Patient characteristics have been published previously [20]. Supplementary Table S1 provides a summary of patient characteristics. Blood samples were obtained on admission to the hospital prior to the administration of blood products or prescription of anticoagulant or antiplatelet agents. Blood samples were taken into sodium citrate tubes and centrifuged at 2000 *g* at 18 °C for 10 minutes and subsequently at 10 000 *g* for 10 minutes and were stored at −80 °C until use. Plasma from 10 randomly selected patients or controls from each group was pooled, and the fibrinogen concentration was determined by the Clauss method on an automated coagulation analyzer (StaCompact 3; Stago) using reagents and protocols from the manufacturer.

2.2 | Rheology

Rheology experiments were performed using an Anton Paar rheometer (MCR501; Graz) equipped with a 30-mm stainless steel cone plate geometry. The rheometer bottom plate and incubator hood (H-PTD200; Anton Paar) were prewarmed to 37 °C and kept at a constant temperature of 37 °C using a Peltier device during the experiments. The geometry and bottom plate were precoated with 0.5-mg/mL (0.024-mg/cm^2) human fibrinogen (FIBI; Enzyme Research Laboratories) for 30 minutes to enhance the attachment of clots to the plates [21]. Clots were prepared from pooled plasma from each patient group by adding 1-U/mL thrombin (human α-thrombin; Sekisui Diagnostics) and 17mM calcium chloride ($CaCl_2$; Sigma Aldrich) (final concentrations) to the samples. Evaporation was prevented by adding a layer of mineral oil (Sigma Aldrich) around the sample edge.

Polymerization of the clots was monitored by applying shear strain oscillations with an amplitude of 0.5% and a frequency of 0.5 Hz for 1.5 hours. The storage (G') and loss (G") moduli during polymerization were recorded. Nonlinear rheology was studied with a stress ramp protocol: shear stress was gradually increased logarithmically from 0.01 Pa to 10 000 Pa with 20 points per decade (10 seconds per point) until the fibrin network ruptured, which was observed as a sudden drop in differential modulus (K'), defined as the tangent of the stress-stress curve.

2.3 | Permeability

Permeability for each group was assessed under compressive force using the rheometer and under the force of gravity by liquid permeation. To assess the permeability of the clots from each group under a compressive force, we conducted compression tests using a plate-plate geometry with noncoated 20-mm parallel rheometer plates. Directly after transferring the clotting mixture to the bottom plate of the rheometer, the top plate was lowered to a gap of 0.5 mm. After polymerization for 1.5 hours under a small oscillatory strain while recording the storage and loss moduli, compression was achieved by lowering the top plate by 1 μm/s in steps of 100 μm, thereby measuring the resistant force of the sample. The gap was held constant between each compression step to allow the normal force to equilibrate. Stepwise compression was continued until the gap between the plates was reduced to 0.2 mm. Supplementary Figure S1 shows an example of a normal vs time plot and the gap between the plates vs time plot. The permeability coefficient K_s was calculated with the following equation:

$$K_s = \frac{\eta a^2 \dot{\varepsilon}}{8C}$$

where η is the viscosity of the fluid phase as 0.001 Pa*s; a is plate radius as 0.01 m; $\dot{\varepsilon}$ is the axial strain rate in v/h, where v is the velocity of the upper plate and h the initial height of the fibrin gel; and C is the intercept of a straight-line fit of normal force vs time [22]. Permeability was also measured with an assay that allows permeation of Tris-buffered saline (TBS) by liquid permeation under the force of gravity, as previously described [23]. The permeability of fibrin clots under the force of gravity was assessed with plasma samples and with isolated fibrinogen that was treated with neuraminidase and nonneuraminidase-treated fibrinogen (see below). The permeability coefficient K_s was calculated following Darcy law: K_s = (Qlη) / (TAΔP), where Q indicates the volume of the liquid, l indicates clot length (1.7 cm), η indicates viscosity, T indicates time, A indicates the cross-sectional area of the clot (π0.3 cm^2), and ΔP indicates pressure drop. As the plasma fibrinogen concentration partially determines the clot permeability, the K_s values were normalized by the fibrinogen concentration divided by a reference concentration of 2 mg/mL to the power of −2.2, which was described by Punter et al. [22] and is applicable to homogeneous and isotropic fibrous network structures, which was validated using confocal microscopy (see below).

2.4 | Purification of fibrinogen and neuraminidase treatment

Fibrinogen was purified from pooled patients and control plasma via ethanol precipitation [24]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to determine purity, and the Pierce BCA Protein Assay (Thermo Fisher Scientific) was used to determine the fibrinogen concentration. Purified fibrinogen was incubated with neuraminidase to cleave sialic acid residues from fibrinogen. A concentration of 0.02 U of neuraminidase (Sigma Aldrich) per milligram fibrinogen was added to the purified fibrinogen and incubated for 3 hours at 37 °C [25,26]. Nontreated samples were incubated with the same volume of TBS. After incubation, the samples were dialyzed against TBS-EDTA (25mM Tris, 150mM sodium chloride [NaCl], and 10mM EDTA) overnight and subsequently against TBS (25mM Tris, 150mM NaCl, pH 7.4). Purified fibrinogen preparations were diluted with TBS to a concentration of 1.1 mg/mL. Factor XIII (Cluvot; CSL Behring) was added at a final concentration of 8.7 μg/mL. These samples were then used for liquid permeation (see above).

2.5 | Laser scanning confocal microscopy

Pooled plasma was mixed with thrombin (final concentration, 1 U/mL; human α-thrombin; Sekisui Diagnostics), $CaCl_2$ (final concentration, 17 mM), TBS, and Alexa Fluor 488-labeled fibrinogen (final concentration, 30 μg/mL; Thermo Fisher Scientific). After mixing, the samples were immediately transferred to a CoverWell perfusion chamber (Sigma Aldrich). Clots were formed in the dark in humidified chambers (wet tissue in a closed petri dish) for 1.5 hours. Clots were visualized with a Leica TCS SP8 confocal laser scanning microscope equipped with a 63×/1.40-numerical–aperture oil objective (Leica Microsystems). Alexa Fluor-488 fibrinogen was excited at 488 nm using an argon laser. Three samples per condition were visualized, with 2 images per sample (70 × 70 μm). Number of pores and pore sizes were determined using a custom-written script in Python based on a study by Münster and Fabry [27]. Confocal images were first denoised using the total variation minimization method [28] on a slice-by-slice basis. A local threshold was then applied to obtain a binary confocal stack. The Euclidean distance map (EDM) was determined, and a Gaussian filter was applied to the EDM. Finally, the local maxima of the EDM were determined, which represent the furthest distance from a fiber in the image. We selected these distances as half of the pore size.

2.6 | Statistical analysis

GraphPad Prism, version 8.4.2 (San Diego), was used for data presentation and analysis. Data are presented as mean ± SD. Statistical significance was tested using 1-way or 2-way analysis of variance, with Dunnett multiple comparisons test to test for statistically significant differences between healthy controls and patient groups. The Student's *t*-test was used to test for statistically significant differences between samples with or without neuraminidase. A P value of $<.05$ was considered statistically significant.

3 | RESULTS

The mechanics and structure of clots formed from pooled plasma of patients with stable cirrhosis, AD cirrhosis, ACLF, and nonliver sepsis as well as healthy controls were measured. Plasma levels of fibrinogen in these plasma pools were comparable between controls and patients with liver diseases (2.6 mg/mL in healthy controls vs 2.2-3.1 mg/mL in patients with liver diseases; Table 1) but were markedly increased in sepsis patients without liver disease (6.6 mg/mL; Table 1).

TABLE 1 Fibrinogen concentration in pooled plasma from included patients was measured by the Clauss method.

Patient group	Fibrinogen (mg/mL)
HC	2.6
SC	2.9
AD	3.1
ACLF	2.2
NLS	6.6

ACLF, acute-on-chronic liver failure; AD, acute decompensated; HC, healthy control; NLS, nonliver sepsis; SC, stable cirrhosis.

3.1 | Clots from patients with ACLF are softer than clots from other liver patients or patients with nonliver sepsis

To assess the viscoelastic properties of pooled plasma clots, we measured G' and G'' during fibrin polymerization while small-shear–strain oscillations were applied. G' gives information about the elastic properties of the clot and is also referred to as stiffness, while G'' gives information about the viscous properties of the clot [29]. The moduli reached steady-state values after ∼30 minutes for healthy controls and patients with liver diseases and 50 minutes for patients with nonliver sepsis (Supplementary Figure S2). In steady state, plasma clots from patients with ACLF were softer than clots from the other patient groups and healthy controls, with a 3-fold lower G' compared with those from healthy controls (Figure 1A). Plasma clots from patients with stable cirrhosis and AD showed viscoelastic properties comparable with those of healthy controls, whereas clots from plasma of patients with nonliver sepsis were stiffer, with a 5-fold higher G' compared with those from healthy controls (Figure 1A). The viscous moduli G'' showed similar trends as G' (Figure 1B).

G' is known to increase with increasing plasma fibrinogen concentration [30,31]. Since patients with nonliver sepsis had markedly increased fibrinogen levels, we assessed whether variations in fibrinogen concentration alone could explain the differences in G'. Therefore, we normalized the G' values by the square of fibrinogen concentration, which is the concentration dependence expected based on theoretical models and experiments on clots made of purified fibrinogen [30,31]. Figure 1C shows that the higher G' of plasma clots from patients with sepsis is (partially) explained by enhanced fibrinogen concentration as the normalized G' values are similar between patients with nonliver sepsis and healthy controls. The G' of plasma clots from patients with ACLF was almost 2-fold lower than that for the other groups after normalization for fibrinogen concentration, indicating that plasma fibrinogen concentration does not completely explain the softer clot properties in these patients.

The loss tangent (Figure 1D), defined as the ratio between G'' and G' ($\tan\delta = G''/G'$), describes whether a material has more solid-like or more liquid-like behavior. The loss tangent was far <1 for all plasma clots, indicating solid-like behavior. It was higher in patients with ACLF, which indicates more liquid-like behavior of clots from ACLF samples compared with those from the other groups (Figure 1D). Plasma clots from patients with nonliver sepsis had lower loss tangent values than plasma clots from patients with liver diseases and healthy controls, indicating more solid-like behavior (Figure 1D).

Next, the response of plasma clots to large shear deformations was measured by increasing the shear stress. Figure 2A shows that

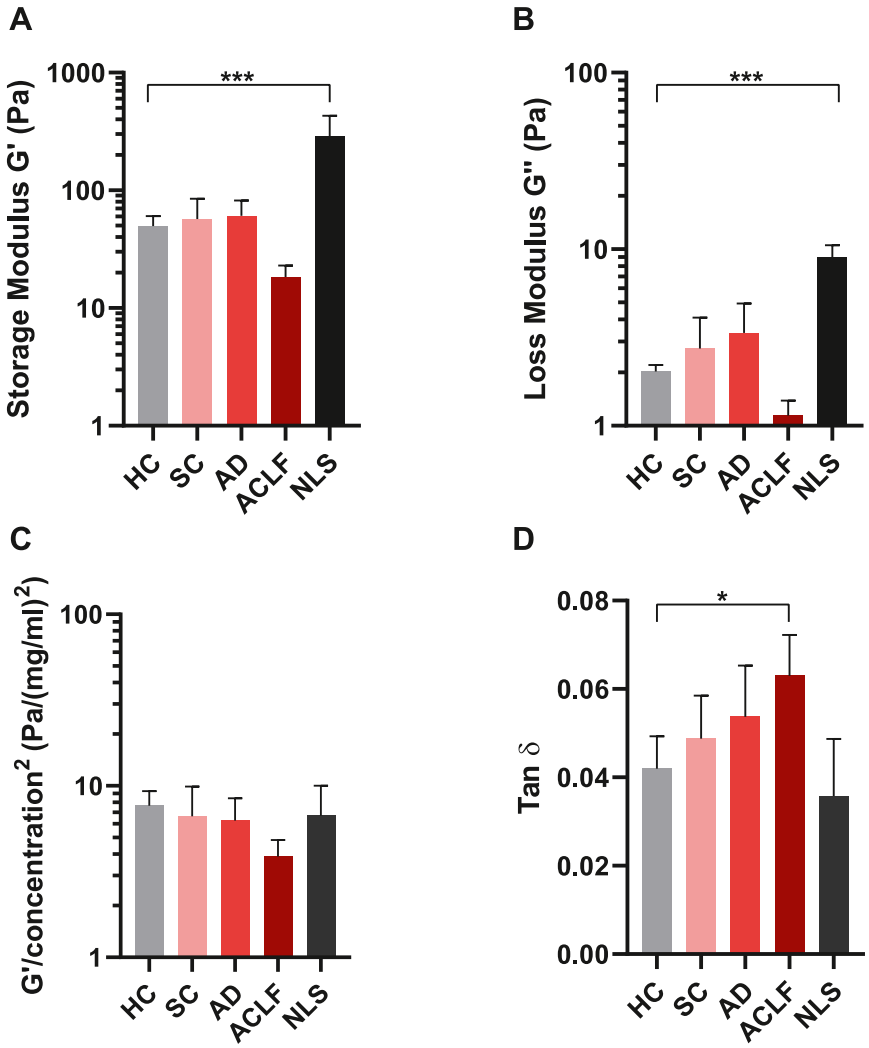

FIGURE 1 Linear viscoelastic properties of clots prepared from pooled plasma samples from healthy controls and patients with stable cirrhosis, acute decompensation, acute-on-chronic liver failure, and nonliver sepsis. (A) Linear storage modulus G'. (B) Linear loss modulus G''. (C) Normalized storage moduli, obtained by rescaling by the square of the fibrinogen concentration. (D) Loss tangent, defined as tan δ = G''/G'. Data are presented as mean ± SD. $N = 4$ for all groups. Comparisons were made using 1-way analysis of variance using the Dunnett multiple comparisons test. *Indicates a statistical significant difference between healthy controls and patient groups. *$P < .05$; **$P < .001$; ***$P < .0001$. ACLF, acute-on-chronic liver failure; AD, acute decompensation; HC, healthy control; NLS, nonliver sepsis; SC, stable cirrhosis.

clots from all groups were strongly stress stiffened. This response is dependent on the fibrinogen concentration [30], which explains a stronger strain-stiffening response in clots from patients with nonliver sepsis. After normalization of the differential modulus K' for the linear modulus K_0 (determined by taking the average of the K' values until stress-stiffening starts) and of the shear stress σ for the onset stress σ_0, strain stiffening was similar for all clots. The onset stress σ_0 where strain stiffening sets in (determined by taking the minimum value of $K'/\sigma^{0.5}$ vs σ plot [32]) was almost 3-fold lower for clots from patients with ACLF than for those from healthy controls (Table 2) and almost 5-fold higher for clots from patients with nonliver sepsis than for those from healthy controls (Table 2). The maximum differential modulus K_{max}, the maximum value of K' before the fibrin network ruptures under increasing stress, was 1.3-fold lower in clots from patients with ACLF than in those from healthy controls, but this difference was not statistically significant (Table 2). Conversely, the K_{max} was 3-fold higher in clots from patients with nonliver sepsis than in those from healthy controls (Table 2).

3.2 | Permeability of clots under the force of compression and force of gravity

The permeability of the plasma clots was assessed by measuring the normal force exerted by the clots during compression in a parallel-plate geometry experiment (Figure 3A and Supplementary Figure S1) and by liquid permeation (Figure 3B). The liquid permeation method measures the filtration of TBS under the force of gravity

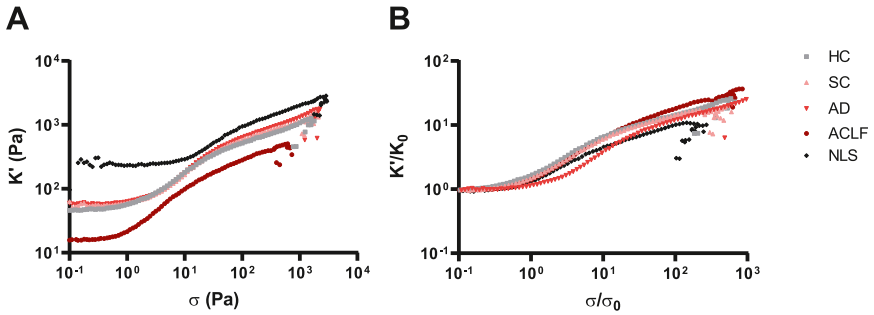

FIGURE 2 Stress-stiffening response of clots prepared of pooled plasma from healthy controls and patients with stable cirrhosis, acute decompensation, acute-on-chronic liver failure, and nonliver sepsis. (A) The differential elastic modulus (K') is shown as a function of applied shear stress (σ), revealing stress-stiffening behavior for all plasma clots. (B) Stress-stiffening curves with K' normalized by the linear modulus, K_0, and shear stress normalized by the onset stress where strain stiffening sets in (σ_0). Curves represent mean curves of 4 measurements for each group. ACLF, acute-on-chronic liver failure; AD, acute decompensation; HC, healthy control; NLS, nonliver sepsis; SC, stable cirrhosis.

through plasma clots that were formed in a cutoff pipette tip and connected to a syringe containing TBS. Figure 3C shows that permeability under the force of compression was similar between patients with liver diseases and healthy controls but that permeability was lower in clots made with plasma from patients with nonliver sepsis. Figure 3D shows that under the force of gravity, permeability decreased from patients with stable cirrhosis to patients with AD cirrhosis and ACLF. The lowest permeability was measured in patients with nonliver sepsis. Since the plasma fibrinogen concentration was much higher in patients with nonliver sepsis, we next normalized the permeability constants for fibrinogen concentration following the behavior expected for a uniform fibrous network [22]. Figure 3E, F shows that after normalization for fibrinogen concentration, the permeability of clots from patients with ACLF was lower than the permeability of clots from healthy controls, other patients with liver diseases, or patients with nonliver sepsis. The normalized permeability of clots from patients with nonliver sepsis was 3 to 6 times higher than the normalized permeability of clots from healthy controls and patients with liver diseases, indicating that the intrinsic fibrin network structure of plasma clots from patients with nonliver sepsis was more open but that high plasma fibrinogen levels may compensate for this effect, resulting in less permeable plasma clots.

For the measurements of permeability under the force of compression, plasma clots were formed between 2 parallel plates of the rheometer to complete fibrin polymerization. Then, the gap was decreased in 3 subsequent steps with an axial compressive strain of 20% per step. Figure 4 shows that the permeability of the clots of healthy controls and patients with stable cirrhosis and AD increased when the gap between the 2 plates became smaller, indicating that the pore size increased. This effect was not observed in clots from patients with ACLF and nonliver sepsis. The increase in permeability in healthy controls and patients with stable cirrhosis and AD suggests that these samples remodel more under compression than clots from patients with ACLF and sepsis.

3.3 | Sialic acid content of fibrinogen in patients with liver diseases contributes to a decrease in permeability under the force of gravity

Previous work showed that fibrinogen in patients with liver disease has an increased sialic acid content [12]. We previously hypothesized that the negative charge caused by these sialic acid residues retains fluid, thereby decreasing the permeability constant K_s as this

TABLE 2 Strain-stiffening parameters of clots from pooled plasma of healthy controls, patients with liver diseases, and patients with nonliver sepsis.

Parameter	HC	SC	AD	ACLF	NLS
σ_0	3.0 ± 1.2	2.7 ± 1.2	1.6 ± 1.3	1.1 ± 0.4	14.5 ± 8.0[a]
σ_{max}	2531 ± 703	3300 ± 2416	3841 ± 1716	1613 ± 1267	6275 ± 2899[b]
K_0	49.2 ± 9.5	56.6 ± 28.2	58.2 ± 20.1	17.4 ± 4.9	296.7 ± 132.3[a]
K_{max}	1525 ± 521	2169 ± 1758	2335 ± 1059	971 ± 847	4497 ± 2156[b]

Data are presented as mean ± SD. Comparisons were made using 1-way analysis of variance with the Dunnett multiple comparisons test.

ACLF, acute-on-chronic liver failure; AD, acute decompensation; HC, healthy control; K_0, linear modulus; K_{max}, maximum differential storage modulus; NLS, nonliver sepsis; SC, stable cirrhosis; σ_0, onset stress of strain stiffening; σ_{max}, stress at K_{max}.

[a] $P < .0001$, statistically significant differences between healthy controls vs patient groups.

[b] $P < .05$, statistically significant differences between healthy controls vs patient groups.

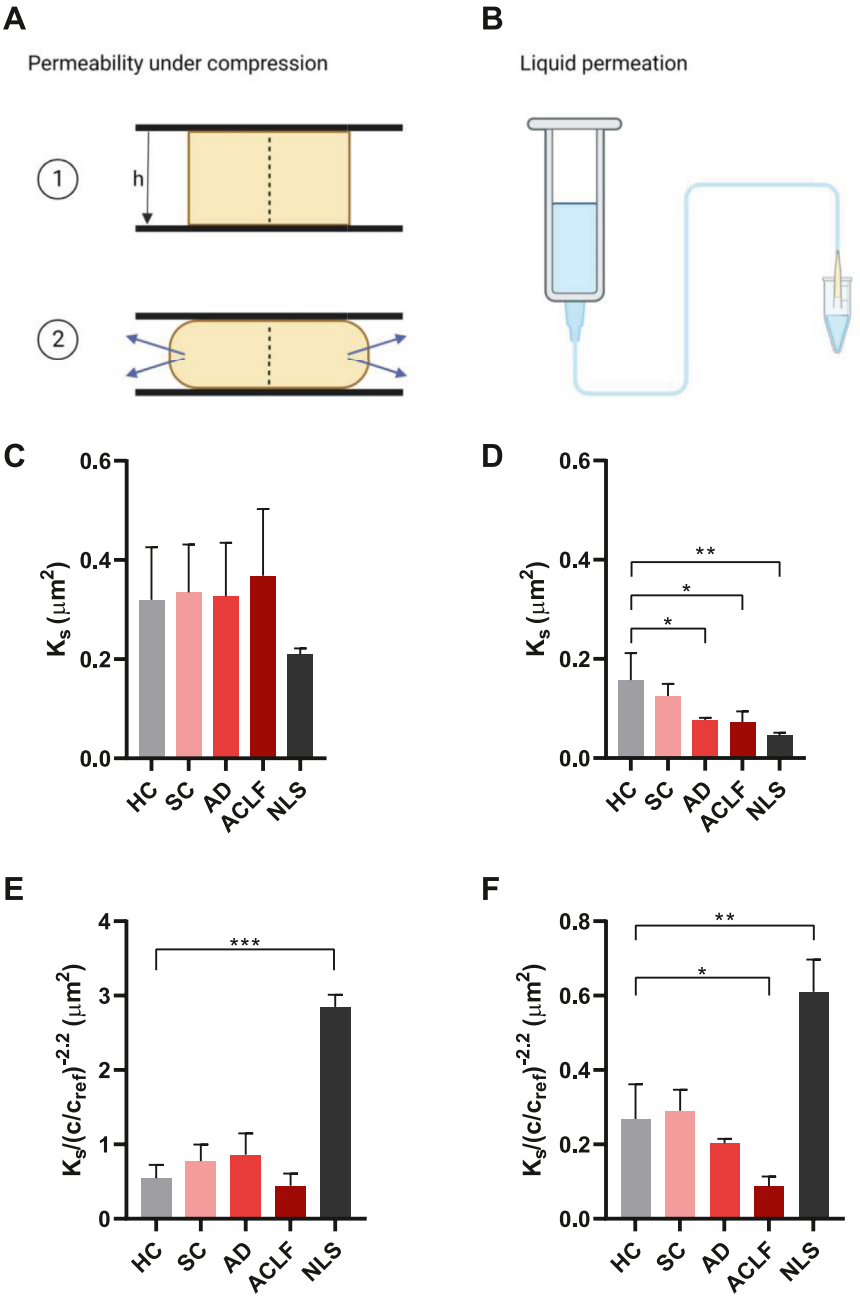

FIGURE 3 Permeability coefficient K_s measured under the force of compression and by liquid permeation. Schematic overview of permeability measurement (A) under the force of compression and (B) by liquid permeation. Permeability of clots from plasma of healthy controls, patients with liver diseases, and patients with nonliver sepsis measured (C) under the force of compression and (D) with liquid permeation. Permeability (E) under the force of compression and (F) by liquid permeation corrected for plasma fibrinogen concentration. The reference concentration is 2 mg/mL (Punter et al. [22], 2020). Data are presented as mean ± SD. *N* = 4 for all groups. Comparisons were made using 1-way analysis of variance with the Dunnett multiple comparisons test. *Indicates a statistical significant difference between healthy controls and patient groups. **P* < .05; ***P* < .001; ****P* < .0001. ACLF, acute-on-chronic liver failure; AD, acute decompensation; HC, healthy control; NLS, nonliver sepsis; SC, stable cirrhosis.

measures the amount of fluid that flows through the clot [12]. We isolated fibrinogen from plasma of healthy controls, patients with liver disease, and patients with nonliver sepsis (average purity, 86%) and treated it with neuraminidase to remove sialic acid residues from the fibrinogen molecules. We then repeated the permeability experiments by liquid permeation with neuraminidase-treated and nontreated fibrin clots, with fibrinogen in the same concentration for each patient group (1 mg/mL). Figure 5 shows that permeability increased after removing sialic acid residues by neuraminidase treatment from the fibrinogen molecule. The effect of neuraminidase was small in healthy

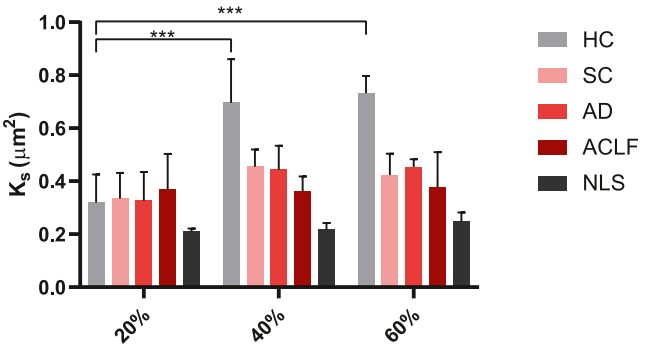

FIGURE 4 Permeability of plasma clots under the force of compression with a stepwise decrease in the gap distance between the 2 parallel plates of the rheometer. The x-axis represents the increasing compressive strain. The permeability constant K_s increases in clots from healthy controls after the second compression compared with the value measured after the first compression and stays constant after the third compression. This effect is not observed in clots from patients with liver diseases and patients with nonliver sepsis. Data are presented as mean ± SD. *N* = 4 for all groups. Comparisons were made using 2-way analysis of variance with the Dunnett multiple comparisons test. ***Indicates a statistical significant difference ($P < .0001$) compared with the first compression. ACLF, acute-on-chronic liver failure; AD, acute decompensation; HC, healthy control; NLS, nonliver sepsis; SC, stable cirrhosis.

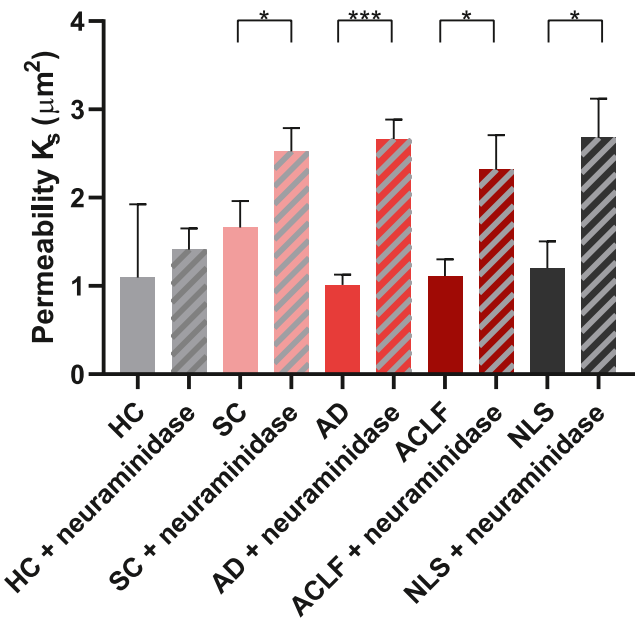

FIGURE 5 Permeability of purified fibrinogen treated with neuraminidase and nontreated fibrinogen, measured using liquid permeation. Samples treated with neuraminidase are presented as dashed lines in the bars. Permeability is higher in clots where sialic acid residues were removed by treatment with neuraminidase. Data are presented as mean ± SD. *N* = 2 for all groups. Comparisons were made using the Student's *t*-test. *Indicates a statistical significant difference between nontreated fibrinogen and fibrinogen treated with neuraminidase within a group; *$P < .05$; ***$P < .0001$. ACLF, acute-on-chronic liver failure; AD, acute decompensation; HC, healthy control; NLS, nonliver sepsis; SC, stable cirrhosis.

controls, but it caused a significant increase in permeability in patients, indicating that increased sialic acid content in fibrinogen from patients contributes to the decrease in permeability under the force of gravity. In addition, fibrin clots without sialic acid residues from patients were more permeable than those from healthy controls, which indicates that other posttranslational protein modifications cause weaker fibrin network structures in fibrin clots from patients than in those from healthy controls.

3.4 | Fibrin networks in clots generated with plasma from healthy controls, patients with liver diseases, and patients with sepsis have similar homogeneous and isotropic structures

Finally, we used confocal laser scanning microscopy to assess the structure of fibrin fibers in clots made with plasma of healthy controls, patients with liver diseases, and patients with nonliver sepsis. Figure 6 shows that the fibrin networks within the clots from controls, patients with liver diseases, and patients with sepsis had a homogeneous and isotropic structure. The number of pores per cubic micrometer of the fibrin networks and the average pore size in micrometer were measured from 3-dimensional confocal stacks using an automated image analysis and showed similar pore size distribution between the samples (Figure 6B). The number of pores per cubic micrometer and average pore sizes were also similar between healthy controls, patients with liver diseases, and patients with nonliver sepsis (Figure 6C, D). To account for effects of variations in plasma fibrinogen concentration, we normalized the pore size by fibrinogen concentration to the power of −0.5, the concentration dependency expected for a homogeneous and isotropic fibrin network [33]. After this normalization, pore size remained similar between healthy controls and patients with liver diseases but significantly increased for clots from patients with nonliver sepsis (Figure 6E). Again, this indicates that a more open structure of the fibrin network in clots generated with plasma from patients with nonliver sepsis is compensated by a high plasma fibrinogen concentration.

4 | DISCUSSION

In this study, we investigated the mechanical properties of clots from patients with stable cirrhosis, AD cirrhosis, ACLF, and nonliver sepsis. We showed that the mechanical properties of clots from patients with stable cirrhosis and AD cirrhosis are similar to those of clots from healthy controls but that clots from patients with ACLF are softer, have a more liquid-like response, and rupture at lower shear stress. Conversely, clots from patients with nonliver sepsis are stiffer, have more solid-like behavior, and require higher shear stress to rupture

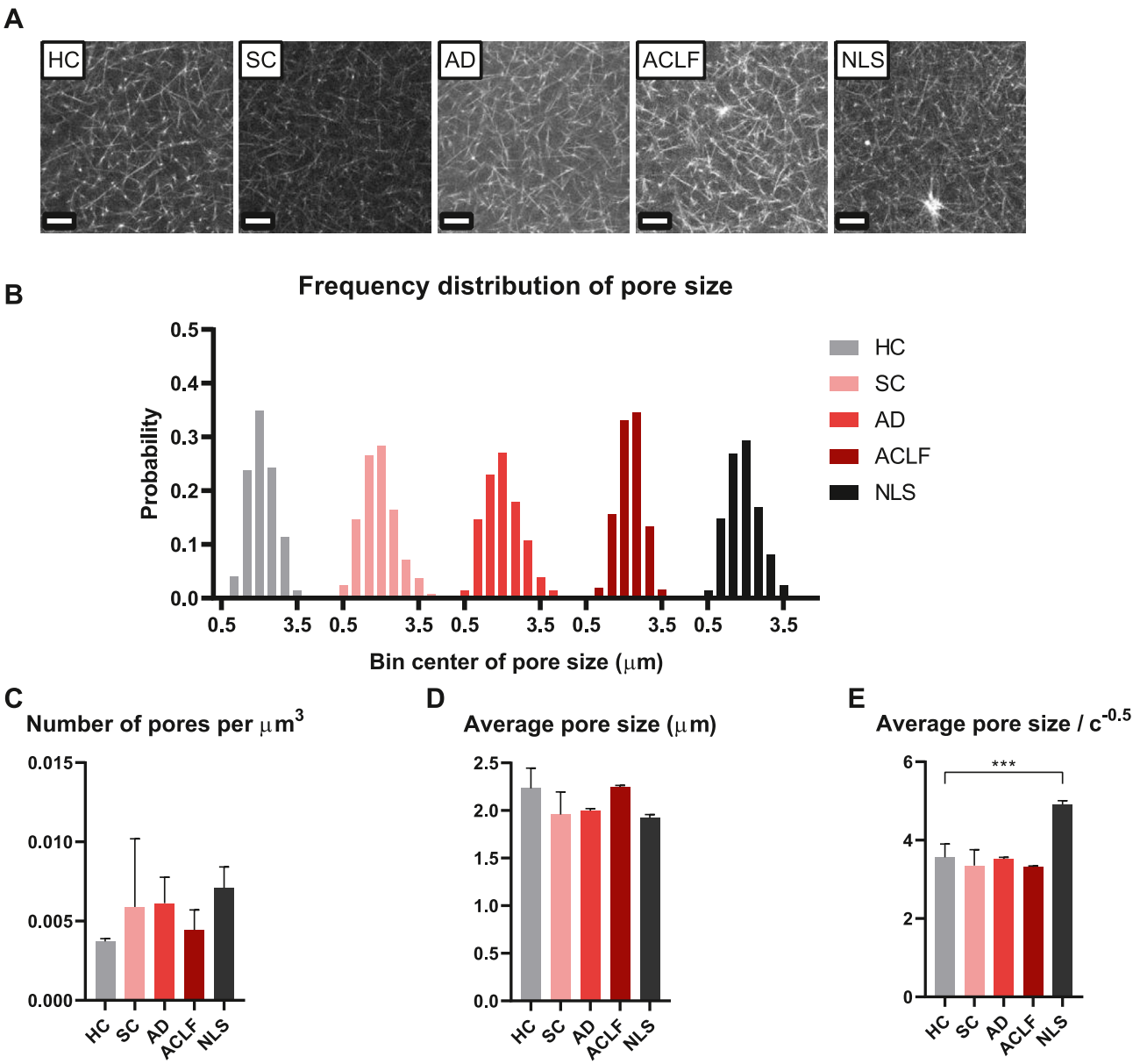

FIGURE 6 Structural characterization of plasma clots. (A) Confocal microscopy images of clots made with plasma. Each sample shows an isotropic and homogeneous fibrin fiber network structure. The white scale bars represent 10 μm. (B) Frequency distribution of pore size in the clots. (C) Number of pores per cubic micrometer. (D) Average pore sizes in micrometer within the clots. (E) Average pore size normalized for the fibrinogen concentration to the power of −0.5. Data are presented as mean ± SD. $N = 2$ for all groups. Comparisons were made using 1-way analysis of variance with the Dunnett multiple comparisons test. *Indicates a statistical significant difference between healthy controls and patient groups. *$P < .05$; **$P < .001$; ***$P < .0001$. ACLF, acute-on-chronic liver failure; AD, acute decompensation; HC, healthy control; NLS, nonliver sepsis; SC, stable cirrhosis.

the fibrin network. Interestingly, although clots from patients with nonliver sepsis have stiffer and more rigid mechanical properties, we found that enhanced plasma fibrinogen levels in these patients may compensate for an actual weaker and more open fibrin network structure.

Qualitative and functional alterations in fibrinogen and fibrin networks are common findings in patients with liver diseases [34]. Results of rotational thromboelastometry, a viscoelastic assay that measures the kinetics of clot formation, have shown that, specifically, patients with ACLF have a hypocoagulable profile [35,36]. These hypocoagulable results include delayed fibrin formation and decreased maximum clot firmness and were more frequently observed in patients with ACLF with systemic inflammation [35,36]. In the present study, we used rheology experiments to investigate the mechanical properties of plasma clots and found that clots from patients are softer and rupture at lower applied shear stress. Based on these results, one could expect that fibrin networks in patients with ACLF are more prone to embolization and more susceptible to fibrinolysis as the biomechanical properties of fibrin have a critical impact on clot stability [37]. However, our previous work has

demonstrated profound resistance against fibrinolysis in many patients with ACLF, which is related to profoundly elevated levels of plasminogen activator inhibitor type-1, which may counteract the weak biomechanical properties [3,19,38]. Embolization and fibrinolysis are also affected by other factors, including shear stress, vascular smooth muscle contractions, platelet-driven clot contraction, fibrin fiber cross-linking, and fibrin fiber density [39–41]. Here, we showed similar fibrin fiber densities in patients with ACLF and healthy controls, but how other factors contribute to the risk of embolization and susceptibility to fibrinolysis and how fibrin biomechanical properties are associated with thrombus stability in acutely ill patients with liver diseases should be subject to future research. We have previously demonstrated that *in vitro* addition of fibrinogen concentrate improves the quality of fibrin clots in plasma from patients with ACLF [42], which suggests that fibrinogen administration to patients with ACLF may be effective in treating or preventing bleeding. However, a recent retrospective clinical study showed no benefit of fibrinogen administration on bleeding or mortality in critically ill patients with cirrhosis [43].

Inflammation and oxidative stress are important aspects of liver disease [44] and affect fibrinogen and fibrin functions [5]. In particular, patients with ACLF often experience systemic inflammation [45]. In this study, we included patients with nonliver sepsis to determine whether altered mechanical properties of clots from patients with liver diseases are directly related to the liver disease or are related to the effects of critical illness and systemic inflammation on clots. Posttranslational protein modifications are often driven by inflammation. For example, oxidation and nitration of fibrinogen can result from oxidative stress during inflammatory responses [46]. These posttranslational protein modifications have been associated with decreased clot stiffness and decreased fibrinolysis [47,48], which have also been described in patients with liver diseases [17,49]. In our rheology experiments, we found that clots from patients with ACLF, who often have systemic inflammation [50,51], had weaker mechanical properties than the other groups. Clots from patients with nonliver sepsis had stiffer clot properties, with higher storage, loss moduli, and loss tangent. However, these characteristics appeared to be fibrinogen concentration dependent. The plasma fibrinogen concentration in patients with nonliver sepsis was very high. After normalization of the mechanical parameters for fibrinogen concentration, the mechanical properties of nonliver septic clots were similar to those of clots from healthy controls and patients with liver diseases. These results indicate that increased fibrinogen concentration may compensate, at least in part, for weaker fibrin clot properties. In patients with severe liver disease, the liver likely does not have the capacity to produce excess fibrinogen; thus, there is no compensation for weak fibrin clot properties in these patients. Interestingly, when normalized for fibrinogen concentration, clots from patients with ACLF were softer but less permeable than clots from healthy controls, indicating that other factors in the fibrin network contribute to clot stability in these patients. Future studies in acutely ill patients with liver diseases should focus on the effects of (treatment of) inflammation on thrombotic and bleeding complications.

In addition to effects on clot stiffness and fibrinolysis, oxidation and glycosylation of fibrinogen have also been related to permeability of clots. Previous work [12,16] conducted by our group showed delayed clot formation in patients with cirrhosis, which previous studies have shown to be related to hypersialylation. When clots were ultimately formed, we showed decreased clot permeability, which was proposed to be related to increased oxidation of the fibrinogen molecule [12]. Here, we performed permeability experiments with plasma using 2 methods: a rheometer, which measures the normal force during compression as a measure of permeability [22], and liquid permeation, which measures the flow rate of a physiologic buffer through a clot under the force of gravity. We found similar permeability results between patients with liver diseases and healthy controls in the compression test but decreased permeability in patients with more severe liver disease in the liquid permeation test. We hypothesized that posttranslational protein modification with sialic acid residues on fibrinogen causes increased negative charge in the clot that retains water [12,16]. Our experiments using purified fibrinogen indeed revealed that permeability increases to a much larger extent in patients than in controls after sialic acid residues are removed. In addition, the pore sizes within the fibrin networks were similar between clots from healthy controls and those from patients, indicating that factors other than pore size cause differences in liquid permeation results. The lower permeability under the force of gravity in plasma clots thus does not truly reflect a more thrombogenic clot but reflects a higher net negative charge. In studies under compressional force, the electrostatic repulsion may not be strong enough to retain water in the fibrin network. In our rheology compression experiments, we found that deformation of the fibrin network in healthy controls in subsequent compression steps caused an increase in the permeability constant. This indicates that the fibrin network becomes more porous when it is more compressed. We expected the fibrin networks to become less porous as the density of fibrin fibers increases upon compression [52]. However, at lower degrees of compression, fibrin networks soften [53], which may cause an increase in permeability. In addition, densification of fibrin fibers is unequally distributed across the fibrin network [54], and it could be that the less homogenous distribution of pore sizes results in an increase in permeability in clots from healthy controls. Increasing permeability at a higher degree of compression was not or less observed in clots from patients. Future studies are required to gain insight into the response to compressive forces of fibrin fibers and their structure in clots from patients with liver diseases.

We acknowledge some limitations to our conclusions. First, we performed experiments with plasma, omitting the contributions of red cells and platelets to the mechanical properties of clots. Given the changes in red cell and platelet counts in patients, this simplification was both justified and required. Second, we used pooled plasma as relatively large volumes of plasma were required for these experiments and could, therefore, not relate results to specific patient characteristics.

In conclusion, we demonstrated that clots from patients with ACLF have weak biomechanical properties and decreased clot

permeability. These findings could be related to posttranslational protein modifications as a result of inflammation and oxidative stress. In patients with nonliver sepsis, we found that weaker fibrin biomechanical properties are compensated by high plasma levels of fibrinogen. Furthermore, we demonstrated that the fibrin fiber density in clots from patients with liver diseases is similar to the density in clots from healthy controls and that the decreased permeability in patients with liver diseases in liquid permeation experiments is caused by increased sialic acid content of fibrinogen. How these results and other factors that contribute to thrombus stability are associated with thrombotic and bleeding complications in patients with liver diseases requires further study. In addition, the potential interest of anti-inflammatory treatment in acutely ill patients with liver diseases to prevent thrombotic or bleeding complications should be subject to future research.

AUTHOR CONTRIBUTIONS

E.G.D. performed experiments, analyzed the data, and wrote the manuscript. I.M. analyzed and interpreted the data and revised the manuscript. V.P. and W.B. collected samples, supervised sample collection, and revised the manuscript. J.A. performed experiments and revised the manuscript. G.H.K. conceived the project, supervised experiments, interpreted the data, and revised the manuscript. T.L. conceived the project, supervised experiments, interpreted the data, and wrote the manuscript.

DECLARATION OF COMPETING INTERESTS

There are no competing interests to disclose.

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SUPPLEMENTARY MATERIAL

The online version contains supplementary material available at https://doi.org/10.1016/j.jtha.2023.06.011