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Procoagulant changes in fibrin clot structure in patients with cirrhosis are associated with oxidative modifications of fibrinogen

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Essentials:

- Patients with cirrhosis have hemostatic changes, which may contribute to a risk of thrombosis.
- This in vitro study compares clot formation and structure between patients and healthy subjects.
- Clot formation is delayed in patients; ultimately, however, clot permeability is decreased.
- The thrombogenic structure of fibrin clots may contribute to the thrombotic risk in cirrhosis.

Abstract

Background & Aims: Patients with cirrhosis can be at risk of thrombotic complications due to an imbalance between hemostatic components. However, little is known on how the disease affects clot generation or how alterations in structure of fibrin clots may affect the hemostatic function of these patients.

Methods: We investigated the formation and structure of clots generated with plasma and purified fibrinogen of 42 patients with cirrhosis. Clots generated with plasma and fibrinogen of 29 healthy volunteers were studied for comparison. Clot formation and structure were assessed by turbidity, permeation studies, confocal laser and SEM. The extent of fibrinogen oxidation was assessed by measuring carbonyl content of purified fibrinogen samples.

Results: Tissue factor and thrombin-induced clotting of plasma was delayed in patients. The clotting rate was also decreased, but change in turbidity, fibrin density and fiber thickness were largely comparable to healthy volunteers. Conversely, clot permeability was significantly decreased in patients. When clots were generated with purified fibrinogen, similar differences in clot formation and structure were found as in plasma. The carbonyl content was increased in patient fibrinogen and correlated with disease severity and clot permeability.

Conclusions: Delayed clot formation in cirrhosis ultimately results in decreased clot permeability. Similar alterations in clots generated with purified fibrinogen suggest that modifications of the molecule are (partly) responsible. Taken together, these findings are indicative of hypercoagulable features of clots of patients with cirrhosis, which may explain the increased risk of thrombosis associated with the condition.

Keywords: fibrinogen, hemostasis, liver cirrhosis, oxidative stress, thrombosis.

Introduction

The clinical consequences of hemostatic disorders in patients with chronic and acute liver diseases can vary significantly from bleeding to thrombosis, and the conceptual understanding of the underlying mechanisms have changed considerably over the last decade. The latest proposed paradigm is that the combined effects of the hemostatic changes in cirrhosis produce a rebalanced, yet precarious hemostatic system, which may easily tip toward either a bleeding diathesis or a thrombotic tendency [1,2]. It has now been well established that patients with cirrhosis and abnormal routine coagulation tests do not necessarily have a bleeding tendency, and that thrombotic complications may occur in these patients [3]. In addition, an increasing number of studies suggest a link between thrombosis and progression of liver disease [4,5]. Probably the most striking example is a recent prospective, randomized study demonstrating that low molecular weight heparin therapy, aimed at preventing portal vein thrombosis, also reduced hepatic decompensation and mortality in a cohort of patients suffering from moderate-to-severe cirrhosis [6].

The clinical concept of thrombosis as an important complicating factor of cirrhosis is supported by evidence from the laboratory. *In vitro* studies have demonstrated a disease stage-dependent decrease in the capacity of plasma of cirrhotic patients to regulate thrombin generation [7,8]. In further studies, high levels of the platelet-binding protein Von Willebrand factor were found to result in a supranormal primary hemostatic function in these often thrombocytopenic patients

[9,10]. Recent studies have highlighted abnormal fibrin clot structure and function as a potential risk factor for thrombosis. Acquired or inherited changes in fibrin structure have been associated with venous and arterial thromboembolic events [11-15]. Interestingly, in spite of current advancements in our understanding of the net effects of the changes in primary and secondary hemostasis in chronic liver failure, little is known on the generation and structure of fibrin clots in these patients.

Studies published more than three decades ago suggested qualitative defects in the fibrin clot in patients with cirrhosis, specifically, increased glycosylation of fibrinogen. This hypersialated fibrinogen displayed delayed fibrin polymerization [16,17]. However, in studies conducted by our group [18,19] a normal capacity to generate fibrin clots was found when using plasma of cirrhotic patients indicating that, under more physiological circumstances, qualitative defects in fibrinogen may not necessarily translate to a reduced clot function.

To elucidate the net effect of changes in fibrinogen in cirrhosis, we studied the clot generation process as well the structural properties of clots from a cohort of patients with cirrhosis of various severity and etiology. We examined clots made with plasma as well as with fibrinogen purified from the patients to determine whether changes in plasma composition or in fibrinogen itself underpin any alterations in fibrin function and structure. Finally, we investigated oxidative modifications of fibrinogen, which were recently shown to alter both the structure and function of the molecule [20]. Indeed, as fibrinogen is one of the most abundant plasma proteins, it is a likely target for oxidative stress [21], one of the hallmarks of chronic liver disease [22-25].

Patients and Methods

Patients

Forty-two patients with a clinical diagnosis of cirrhosis were recruited from the Hepatology outpatient clinic or ward of the University Medical Center Groningen between August 2012 and April 2013. These patients were classified according to the Child-Pugh classification [26]. Exclusion criteria

were a documented history of congenital coagulation disorders, recent viral infection (<2 weeks), use of anticoagulant drugs in the past 10 days, pregnancy, HIV positivity, and transfusion with blood products within past 7 days. Plasma samples from 29 healthy volunteers from our laboratory (nine males and twenty females, mean age 34 ±12 years, and two current smokers) were used to establish reference values. Exclusion criteria for the volunteer group were documented history of congenital coagulation disorders, documented history of hepatic disease, recent viral infection (<2 weeks), use of anticoagulant drugs in the past 10 days, pregnancy, and HIV positivity. The study protocol conformed with the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the medical ethics committee of the University Medical Center Groningen, Groningen, The Netherlands. Written informed consent was obtained from each subject before inclusion. Details of blood sample withdrawal and processing have been described previously [27].

Fibrinogen levels

Fibrinogen levels in plasma of patients and healthy volunteers were determined on an ACL TOP 300 analyzer using reagents from Instrumentation Laboratory (Breda, The Netherlands) according to the manufacturer's instructions.

Purification of Fibrinogen and addition of Factor XIII

Fibrinogen was purified from the plasma of patients and volunteers as described [28]. In short, fibrinogen was purified from plasma using IF-1 (calcium dependent antibody) affinity chromatography, during which contaminating proteins were eliminated from the fibrinogen with washing buffers containing 0.3 and 1M NaCl respectively, prior to elution with EDTA. We have previously shown that this method effectively eliminates any Factor (F) XIII bound to the fibrinogen [29], and hence the purified fibrinogen preparations were FXIII-free prior to addition of exogenous FXIII at the controlled concentration of 7.3 µg/ml. Purity of the fibrinogen samples was examined by reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis using a Mini-Protean system

with 4-20% Bis-Tris gradient TGX gels (Bio-Rad, Hercules, CA). Gels were run at 80 V for 10 min and then at 150V for 1 h 15 min in MES buffer (Life Tech., Carlsbad, CA), stained with coomassie blue solution and scanned using a CanoScan 8800 F (Canon, Tokyo, Japan).

Turbidity assays

Fibrin clot formation in plasma or purified fibrinogen was studied by turbidity analysis as described [30]. Investigated parameters were clotting time (time to ½ max OD in min), clotting rate (mOD/min at ½ max OD), and change in turbidity of the clot defined as the difference between the OD at the start and maximum OD. Triplicates were measured for each sample and averaged.

Thrombin generation assay

Thrombomodulin-modified thrombin generation tests were performed by calibrated automated thrombography as previously described [27].

Clot lysis assay

Lysis of a tissue factor–induced clot by exogenous t-PA was studied by monitoring changes in turbidity during clot formation and subsequent lysis essentially as described previously [31].

Clot Permeation

Plasma and purified fibrinogen clotting mixtures for permeation measurements were prepared as described [28,32]. Immediately after mixing, 100 µL of the clotting mixture was carefully transferred to a 4.5 cm plastic tip with a roughened interior surface, which was cut off from a 1 ml Costar pipette tip. The clot mixture was left to consolidate in a humidified chamber at room temperature for 2 h. The plastic tip was then connected through a flexible silicon tube to a syringe containing TBS with a 4-cm pressure drop. Upon connection, TBS was left to permeate through the fibrin clot network for 1.5 h to wash any other, non-fibrin, plasma components, such as albumin. Then,

measurements were performed in duplicate by collecting drops passing through the clot in a pre-weighed eppendorf tube and weighing the total volume of liquid in the tube every 30 minutes for two hours. These measurements were averaged. Clot permeability was ultimately determined by calculating the Darcy's constant (Ks), which is a measure of the pore size of the fibrin network through which liquid may pass, as described [33].

Laser scanning confocal microscopy

Plasma and purified fibrinogen (1 mg/mL) clots for laser scanning confocal microscopy were generated as described [34]. To visualize the clots, AlexaFluor488 FITC labeled fibrinogen (Life Tech, Carlsbad, CA; 50 µg/mL final concentration in plasma and 25 µg/mL in purified experiments) was added during clot formation. Clots were left to form at room temperature in a dark humidified chamber for 1 h. Laser scanning confocal microscopy (LSCM) was then performed using an upright Zeiss LSM-510 META Axioplan2 confocal microscope (Carl Zeiss Ltd, Welwyn Garden City, UK) fitted with a x63 numerical aperture 1.4 oil immersion objective. The scan format was 512x512 pixels with the pinhole set to one Airy unit to obtain maximum resolution in the z-plane. Single optical sections of 230/230µm (x/y) were taken at three different areas throughout the clot to visualize the fibrin network. Fiber density of the clot was analyzed using ImageJ software (National Institute of Health, Bethesda, MD). Fiber density was determined by an in-house designed macro plug-in, which places a 10 x 10 lines grid on individual images and computes the number of fibers intersecting these lines. Fiber densities of three different micrographs per sample were averaged.

Scanning electron microscopy

Plasma clots from 5 patients (3 Child score A and 2 Child B) and 5 healthy individuals were generated and prepared for scanning electron microscopy as described [35]. Subjects were chosen based on clot permeability values closest to the median of their respective groups. Each clot was imaged in five different areas at 20 x 10^3 magnification using a FEI Quanta 200 FEGSEM (FEI, Hillsboro, OR).

Average fiber diameters were measured from 50 random fibers in each sample using ImageJ software.

Fibrinogen γ' levels in plasma

Fibrinogen γ' antigen levels were measured using an in-house ELISA based on the method of Uitte de Willige [36].

Fibrinogen carbonyl content and plasma malondialdehyde levels

Carbonylation of purified fibrinogen samples was quantified using a commercially available ELISA kit (Enzo Life Sciences, Farmingdale, NY) following the manufacturer's instructions. Malondialdehyde (MDA) levels were estimated in plasma as an indication of lipid peroxidation by measuring thiobarbituric acid reactive substances (TBARS) as described [37].

Statistical Analysis

Statistical analysis was performed with the Graphpad InStat (San Diego, CA) software package. Continuous variables are expressed as the mean±SD or median and range. Categorical data are expressed as numbers and percentage. Continuous data were tested for normality and analyzed by Unpaired t-test or the Mann-Whitney *U* test, as appropriate, for comparison between two groups, and by ANOVA with Dunnett's post-test or by Kruskal-Wallis test with Dunn's post-test for multiple group comparisons. In these analyses, values were compared with healthy volunteers' values. Correlations were examined by calculating Pearson's Rho. A P value less than .05 was considered statistically significant.

Results

Demographic, laboratory, and clinical characteristics of patients with cirrhosis and healthy individuals. Patient demographics, including clinical data and laboratory test results are presented in Table 1. Median fibrinogen levels were 2.7 g/L in patients with Child A, 1.8 g/L in Child B and 1.7 g/L in Child C cirrhosis. Median fibrinogen levels were 2.6 g/L in healthy individuals.

Prolonged clotting time and decreased clotting rate, but normal optical density of clots generated with plasma of patients with cirrhosis. Turbidimetric analysis was used to determine fibrin polymerization. As shown in Fig. 1A and 1B, the time to generate a fibrin clot was overall longer when using plasma of patients with cirrhosis (6.1 min [4.5-9.5] (median [range]) when activated with TF and 1.4 min [1.1-2.2] for thrombin-mediated activation) when compared with the reference (5.1 min [3.9-7.3] and 1.2 min [0.9-1.4]). Clotting times in patients or healthy volunteers did not correlate with fibrinogen levels (data not shown). The clotting rate was decreased in patient plasma (median rate 0.3 mOD/min [0.2-0.9] for TF and 0.2 mOD/min [0.1-0.2] for thrombin-mediated activation) when compared to the healthy volunteer group in which the clotting rate was 0.4 mOD/min [0.2-0.7] ($p<0.01$) and 0.3 mOD/min [0.1-0.4] ($p<0.05$) for TF and thrombin-mediated activation, respectively (Figs. 1C and 1D). The generally decreased clotting rate appeared largely the result of significant decreases in the Child B and C subgroups. Clotting rates correlated well with fibrinogen levels within patients ($r=0.86$, $p<0.01$ for TF, and $r=0.35$, $p<0.05$ for thrombin-mediated activation) and volunteers ($r=0.9$, $p<0.01$ for TF and $r=0.58$, $p<0.01$, for thrombin). Thrombin generation assay (TGA) parameters on the other hand did not correlate with turbidity parameters in cirrhosis ($p>0.05$ for every correlation coefficient). In Figs. 1E and 1F, it is demonstrated that, on average, the change in absorbance after fibrin polymerization was comparable between patients and healthy volunteers regardless of the mode of activation, although the change in absorbance was slightly lower than volunteers in the Child B and C groups.

Decreased permeability of clots generated with plasma of patients with cirrhosis. We tested the permeability of clots generated with thrombin. Clots were perfused with TBS and the total volume of fluid passing through the clot in a given time period was measured. A less permeable clot allows less liquid to pass through, resulting in a lower permeability coefficient Ks. As shown in Fig. 2, clots generated with plasma of patients were significantly less permeable (median Ks $6.5x10^{-9}$ [$1.9x10^{-9}$-$1.5x10^{-8}$]) when compared to clots from healthy volunteers (median Ks $9.6x10^{-9}$ [$6.0x10^{-9}$-$2.4x10^{-8}$] ($p<0.01$). Clot permeability negatively correlated with fibrinogen levels in patients, albeit moderately ($r=-0.56$, $p<0.01$). Clot permeability also negatively correlated with clot lysis time in patients (-0.53, $p<0.01$). TGA parameters did not correlate with clot permeability in cirrhosis ($r=0.18$ for Endogenous Thrombin Potential (ETP); $r=-0.04$ for Velocity index; $r=-0.14$ for Lag time; $r=-0,14$ for time-to-peak ($p>0.05$ for every correlation coefficient)).

Normal Fibrinogen γ' levels in plasma of patients with cirrhosis. Previous studies have shown that the proportion of fibrinogen γ' can influence fibrin clot structure *in vitro* and in plasma [38,39]. In Fig 3A, it is shown that fibrinogen γ' are decreased in cirrhosis, however, when adjusted for fibrinogen levels as shown in Fig 3B, the proportion of fibrinogen γ' in plasma was comparable between patients and healthy volunteers (139 µg per mg fibrinogen [50-453] versus 171 µg/mg [87-341] ($p>0.05$)). These data suggest that the observed changes in permeability and clot structure are not caused by any changes in fibrinogen γ' in this study.

Normal fibrin density and fiber diameter within clots generated with plasma of patients with cirrhosis. Fibrin fiber density and diameter was examined by laser scanning confocal microscopy (LSCM) and scanning electron microscopy (SEM), respectively. A representative LSCM image of a section of a fibrin clot generated with plasma of patient with Child B cirrhosis is shown in Fig. 4A. It indicates a normal fiber density in comparison with a representative healthy volunteer clot (Fig. 4B). Fiber density was quantified in Fig. 4C and demonstrated similar median fiber densities in patients

and healthy volunteers, although the fiber density appeared increased in the Child A cirrhosis group (2.45 Fiber/10μm [0.97-8.18] vs. 1.86 [0.66-8.14] in volunteers ($p>0.05$). The fiber diameter also appeared similar in both groups with an average diameter of ~140 nm per fiber, as demonstrated in Fig. 4D. Figs. 4E and 4F show comparable SEM images from individual fibrin fibers within sections of clots of a patient with Child B cirrhosis and a healthy volunteer, respectively.

Prolonged clotting time, but normal clotting rate and optical density of clots generated with fibrinogen purified from patients with cirrhosis. As shown in Fig. 5A, the clotting time at equal fibrinogen concentration was prolonged in the patient group (2.8 min [2.3-3.7] when compared with healthy individuals in which the median was (2.5 min [2.3-3.1] ($p<0.01$). On the other hand, clotting rates as well as changes in absorbance during clotting were similar between patients and healthy volunteers, as demonstrated in Figs. 5B and 5C, respectively.

Decreased permeability, but normal fiber density of clots generated with fibrinogen purified from plasma of patients with cirrhosis. In Fig. 5D, it is shown that clots generated at an equal concentration of fibrinogen were significantly less permeable in the patient group (median Ks $6.1x10^{-9}$ [$1.7x10^{-9}$-$1.1x10^{-8}$]) when compared to clots of healthy volunteers (median Ks $8.1x10^{-9}$ [$4.9x10^{-9}$-$1.4x10^{-8}$] ($p<0.01$). On the other hand, Fig. 5E indicates a normal fiber density of clots generated with fibrinogen of patients in comparison with volunteers. This is visualized in Fig. 5F with representative LSCM images of the fibrin mesh in clots of a patient and a healthy volunteer, respectively.

Increased carbonyl content of fibrinogen purified from plasma of patients with cirrhosis. Fig. 6 shows a significantly increased carbonyl content of fibrinogen isolated from patients when compared with healthy volunteers (median 2.2 nmol/mg [1.4-4.3] versus 1.5 nmol/mg [1.3-2.6], respectively ($p<0.01$). The extent of carbonylation in patients was associated with disease severity

and inversely correlated with clot permeability. The correlation coefficient was r=-0.46; p<0.05 for patients combined, r=-0.69 for the Child A group (p<0.01), r=-0.65 for Child B (p<0.01) and -0.14 for Child C (p>0.05). In addition, it positively correlated with serum MDA levels (r=0.35, p<0.5).

Smoking or co-morbidities in the patient group are not associated with changes in clot permeability. We explored possible relationships between the permeability of clots generated with plasma and smoking, co morbidities and complications in the patient group. We found no differences in clot permeability between smokers and non-smokers or between patients with and without significant co-morbidity (data not shown). Also, no correlation was found between smoking frequency and MDA levels or carbonyl content of fibrinogen (r=-0.05, p>0.05 and r=0.04, p>0.05, respectively). A relationship between clot permeability and thrombosis could not be demonstrated as only three events of thrombotic complications were recorded. Clot permeability in patients with a history of bleeding (mostly variceal bleeds) was not different from patients who had not experienced a bleeding event (Ks $6.5x10^{-9}$ [$2.0x10^{-9}$-1.2 $x10^{-8}$ vs. $6.7x10^{-9}$ [$1.2x10^{-9}$-$1.5x10^{-8}$] (p>0.05; data not shown).

Discussion

This study of fibrin clot structure in patients with cirrhosis shows alterations in clotting kinetics and fibrin structure. We found that although the kinetics of clot formation were decreased in cirrhosis, the clots were ultimately less permeable. This implies that decreased levels or functional defects in fibrinogen in cirrhosis do not necessarily translate into a reduced clot function promoting a risk of bleeding. On the contrary, a decrease in clot permeability may rather indicate an increased risk for thrombosis. Indeed, previous studies have shown decreased fibrin clot permeability to be associated with both arterial and thrombotic diseases [11-15]. In turn, this study supports a potential prothrombotic role of fibrin clots in patients with acute and chronic liver failure opposing the effects of reduced production of coagulation factors [40]. The prolonged clotting times and decreased clot permeability in both plasma and samples of purified fibrinogen suggest that structural alterations of

the fibrinogen molecule itself underpin the changes in fibrin structure. We detected an increase in the carbonyl content of fibrinogen isolated from patients indicating that oxidative modifications of the protein may be responsible for the prolonged clotting time and reduced clot permeability.

We and others are systematically studying consequences of hemostatic defects in patients with cirrhosis. These studies have shown that primary and secondary hemostasis remain functional, and are perhaps even hyperreactive. We first demonstrated that elevated levels of the platelet adhesive protein von Willebrand factor (over)compensate for abnormalities in platelet number and function in patients with cirrhosis [9]. Others have subsequently demonstrated that the thrombin generating capacity in cirrhosis was intact, despite prolongations in routine laboratory tests of coagulation such as the prothrombin time (PT) or international normalized ratio (INR) and activated partial thromboplastin time (APTT) [41]. The data presented here suggest that the final stage of blood coagulation, the formation of a fibrin clot, is also in a rebalanced, perhaps overcompensated state. Despite reduced fibrinogen levels, the fibrin clot is less permeable (and therefore more resistant to permeation of fibrinolytic enzymes into the clot) compared with healthy individuals. The delayed clot formation is in agreement with previous studies in cirrhosis demonstrating that increased fibrinogen glycosylation results in defects in fibrin polymerization [16,17]. Alternatively, slower fibrin polymerization may result from oxidative modifications of the fibrinogen molecule [42-44] and since fibrinogen oxidation can also promote a less permeable fibrin structure [44] it is a potential mechanism to explain our findings.

Fibrin network production is essential for hemostasis. During the course of fibrin polymerization, fibrinogen molecules assimilate to form a 3-dimentional fibrin network. Clot stability is determined by the structure of the network, which is characterized by the density and diameter of fibers, number and nature of the branch points, and number and size of pores [45]. In turn, these structural features may vary depending on a myriad of factors controlling the clot generation

process. These include fibrinogen structure and concentration, available concentrations of pro- and anticoagulants, which in turn dictate the thrombin generation potential, and local cellular effects [46]. By breaking down the process in individual steps we provided insight into how clotting dynamics are affected in cirrhosis. First, we observed that clotting rates of turbidity assays triggered with tissue factor or thrombin are sensitive to the level of fibrinogen in cirrhosis, but less so in the thrombin triggered turbidity assays. This may be explained by the fact that the effects of fibrinogen on thrombin generation [47] are effectively by-passed in the latter and the effect of fibrinogen concentration on clotting rates becomes less dominant. Secondly, we observed increased clotting times when the plasma of patients was activated with either tissue factor or thrombin, independent of the level of fibrinogen. This demonstrated that the complex alterations in coagulation factors associated with cirrhosis were not the only determinant affecting clot generation, and suggested the presence of a defect in the fibrinogen to fibrin conversion step. This was subsequently confirmed when the clotting time remained abnormal when using fibrinogen isolated from patients. As fibrinogen, Factor XIII and thrombin had been adjusted to equal levels between patients and healthy volunteers, the most likely explanation for the rate-limiting step in fibrin polymerization was an alteration in one or more structural properties of fibrinogen.

A recent review on oxidative modifications of fibrinogen revealed different, even opposing effects, depending on the modified site [20]. Here we demonstrated a defect in clot formation kinetics resulting in less permeable clots ultimately, which correlated with an increase in fibrinogen carbonyl content. Interestingly, apart from the changes in clot permeability, other investigated structural parameters were normal (i.e., change in absorbance, fibrin density and fiber diameter) suggesting that the changes in the fibrinogen molecule in patients with cirrhosis only affect fibrin metastructure by reducing pore size or number. This is in contrast with results of turbidity studies conducted by Paton et al. [48], who demonstrated a negative association between fibrinogen carbonyl content and change in absorbance and which may be explained by different experimental conditions, the

most prominent being the lack of added calcium, while fibrinogen is well known to contain functional calcium binding sites.

The lack of association between permeability and other structural parameters, such as fibrin density, may be explained by the fact that it is a combination of modest structural changes that accounts for changes in metastructure, the sum of which leads to a significantly decreased clot permeability. Furthermore, the relationship between permeation and fiber diameter may be influenced by changes in the intrafibrillar composition of fibrin fibers such as protofibril packing [49]. Also, permeability may be governed by more than just structure. Alterations such as oxidation and sialyation might lead to reduced permeability (e.g. by charge/hydrophobicity effects), but not to changes in structure. A similar study using a larger cohort will shed more light on this apparent discrepancy between permeability and other structural parameters in cirrhosis. A limitation of the study is that we cannot rule out that other structural modifications of fibrinogen also may have contributed to a certain extent in the remodeling of the fibrin metastructure, although we did not find increased fibrinogen gamma prime levels in our patients which are thought to prompt the formation of more thrombogenic clots [50]. Nonetheless, the similarly decreased permeability of clots generated with plasma and purified fibrinogen shows that the procoagulant effects associated with changes in fibrinogen structure persist under more physiological conditions and therefore probably translate to the *in vivo* situation.

In recent years, there has been a major shift in our understanding of the clinical consequences of cirrhosis-associated coagulopathy. Expert opinion suggests that bleeding in many (surgical) cases is more likely due to hemodynamic changes in patients with chronic liver disease than to an underlying hemostatic disorder [51]. It also suggests that patients with cirrhosis are not "auto-anticoagulated": they are not protected from thrombotic events even when routine tests of coagulation including PT or INR and APTT are prolonged, or when platelet numbers are low [52]. In

fact, it is increasingly recognized that thrombosis can be a major complicating factor in cirrhosis and may even contribute to disease progression [53,54]. We propose that, similarly to cohorts of smokers, patients with venous thromboembolism and patients with cardiovascular diseases who show comparable changes in fibrinogen and fibrin clot structure [55-58], enhanced fibrinogen carbonylation in patients with cirrhosis prompts the formation of clots with a procoagulant structure. In turn, this contributes to the thrombotic risk in these patients. The mechanism involved in an increased thrombotic risk in patients with less permeable clots may be a reduced disposition of clots to lysis as evidenced by the negative correlation between permeability and clot lysis time demonstrated here. This is the result of both an increased stability of clot structure and reduced capacity of fibrinolytic enzymes to penetrate through the clot [14,59,60]. Ultimately, this may facilitate the development of dangerous thrombi in the (micro)vasculature. Interestingly, clot permeability was not linked to bleeding in the present study. Larger studies will be needed to demonstrate associations with thrombotic events.

In conclusion, the dysfibrinogenemia associated with liver disease has been functionally characterized by an increased clotting time due to impaired fibrin polymerization and was traditionally associated with a risk of bleeding. For the first time, we have shown that impaired clot formation in cirrhosis does not necessarily translate in a decreased hemostatic capacity as clots of patients have thrombogenic features evidenced by reduced permeability. We have attributed this to procoagulant changes in fibrin metastructure resulting from excessive carbonylation of fibrinogen and which appears to compensate for a decrease in fibrinogen level. This study provides further evidence of a rebalanced hemostatic function in patients with cirrhosis and adds to an increasing number of epidemiological, clinical and *in vitro* studies of the presence of an elevated thrombotic risk as a consequence of the condition.

Author Contributions

G. Hugenholtz designed the study, performed the research and the data analysis and wrote the manuscript; F. Macrae, J. Adelmeijer, and S. Dulfer performed part of the research and reviewed the manuscript; R.J. Porte designed the study, and reviewed the manuscript; T. Lisman and R. Ariens designed the study, interpreted the data, and contributed to writing and reviewing of the manuscript. All authors approved the final version of the manuscript.

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Conflict of interest

The authors have no conflict of interest to disclose.

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Characteristics	Child A (n=20)	Child B (n=15)	Child C (n=7)	Patients combined (n=42)	Healthy individuals (n=29)
	Value	Value	Value	Value	Value
Age, years	54 (11)	49 (13)	49 (14)	51 (12)	32 (9)
Male sex	8 {40}	9 {60}	5 {71}	22 {52}	12 {41}
BMI, kg/m^2	25 (4)	29 (5)	30 (6)	27 (5)	23 (3)
Smokers	12 {60}	2 {13}	2 {29}	16 {38}	1 {3}
Cigarettes/day	5 (5)	2 (5)	2 (4)	3 (5)	2
Etiology					
Alcohol abuse	9 {45}	4 {27}	6 {86}	19 {45}	
NAFLD	2 {10}	3 {20}	0	5 {12}	
PSC	3 [15}	1 {6}	0	4 {10}	
AIH	2 {10}	2 {8}	0	4 {10}	
Combination	2 {10}	1 {6}	0	3 {7}	
HCV	0	2 {13}	0	2 {5}	
Indeterminate	0	1 {7}	1 {14}	2 {5}	
Hemochromatosis	0	1 {7}	0	1 {2}	
Wilson's disease	1 {5}	0	0	1 {2}	
PBC	1 {5}	0	0	1 {2}	
Laboratory blood tests					
AST, U/L	48 [36-70]	63 [48-96]	70 [46-81]	56 [43-80]	
ALT, U/L	36 [28-64]	32 [26-60]	29 [19-42]	34 [26-59]	
ALP, U/L	133 [76-191]	112 [92-212]	108 [88-146]	118 [90-172]	
GGT, U/L	112 [56-216]	94 [52-215]	46 [23-91]	93 [48-197]	
LDH, U/L	180 [152-247]	205 (182-235)	220 (200-353)	199 [165-249]	
Total bilirubin, µmol/L	21 [8-26]	46 [25-51]	70 [36-121]	26 [16-51]	
Albumin, g/L	36 [31-43]	33 [31-35]	28 [26-31]	33 [30-41]	
Fibrinogen, g/L	2.7 [2.1-3.7]	1.8 [1.6-2.6]	1.7 [1.5-2.3]	2.2 [1.6-3.0]	2.6 [1.7-3.6]
INR	1.1 (0.2)	1.3 (0.2)	1.5 (0.2)	1.3 [0.2]	
Platelet count, x10^9/L	121 [93-205]	76 [48-133]	75 [44-114]	104 [58-159]	
Hemoglobin	8.1 (1.0)	7.4 (1.0)	6.3 (1.1)	7.6 (1.2)	
WBC, x10^9/L	7.2 (3.3)	4.6 (2.5)	7.3 (3.5)	6.3 (3.2)	
CRP, mg/L	<5 [<5-15]	<5 [<5-14]	7 [<5-18]	6 [<5-15]	
Sodium, mmol/L	138 (3)	139 (3)	132 (2)	137 (4)	
Potassium, mmol/L	3.6 (0.4)	4.0 (0.5)	4.1 (0.5)	4.0 (0.4)	
Creatinine, µmol/L	58 [50-79]	68 [62-91]	85 [47-118]	68 [54-84]	
Urea, mmol/L	4.7 [3.8-6.4]	5.5 [3.5-9.0]	4.5 [4.4-9.4]	5.0 [3.8-7.5]	
Glucose, mmol/L	7.6 (3.2)	6.4 (2.2)	5.6 (0.6)	6.7 (2.5)	
MDA	3.5 [1.8-8.9]	4.5 [3.0-6.8]	5.9 [3.5-10.7]	4.3 [1.8-10.7]	2.8 [2.1-4.8]
Hemostasis tests					
Clot lysis time, min	56 [40-105]	37 [30-65]	40 [30-52]	48 [30-105]	55 [42-72]
TGA, ETP	780 [460-1683]	906 [607-1111]	838 [838-1030]	884 [460-1683]	816 [579-1262]
TGA, Vel Index	63 [26-121]	85 [69-121]	77 [41-97]	61 [26-121]	77 [26-136]
TGA, Lag time	1.7 [1.2-2]	1.3 [1.3-2.0]	1.4 [1.1-5]	1,7 [1.1-2.0]	1.6 [1.3-2.0]
TGA, Peak time	145 [97-248]	182 [139-251]	144 [101-174]	167 [97-248]	162 [102-241]
Complications					
Ascites	5 {25}	9 {81}	7 {100}	21 {50}	
Encephalopathy	2 {10}	1 {6}	5 {71}	8 {19}	
Thrombosis	1 {5}	1 {6}	1 {14}	3 {7}	
Bleeding (mostly variceal)	5 {25}	5 {30}	2 {29}	12 {29}	
Co-morbidities					
Hypertension	4 {20}	4 {27}	0	8 {19}	
AP	1 {5}	1 {6}	0	2 {5}	
DM II	6 {30}	2 {13}	1 {14}	9 {21}	
Chronic kidney disease	1 {5}	1 {6}	0	2 {5}	
Chronic lung disease	2 {10}	0	0	2 {5}	
Chronic bowel disease	2 {10}	1 {6}	0	3 {7}	
Malignancy	1 {5}	2 {13}	0	3 {7}	

Abbreviations: AIH, autoimmune hepatitis; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AP, angina pectoris; AST, aspartate aminotransferase; BMI, body mass index; CRP, C-reactive protein; DM II, diabetes mellitus II; ETP, endogenous thrombin potential; GGT, gamma-glutamyl transpeptidase; HCV, hepatitis C virus; INR, international normalized ratio; LDH, lactate dehydrogenase; MDA, malondialdehyde; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; TGA, thromboelastography. Data are presented as mean (SD), numbers {percentages} or medians [IQR].

Table 1. Demographic, Laboratory, and Clinical Characteristics of the Study Cohorts.

Figure 1. Turbidity parameters of clots generated from plasma of patients with cirrhosis and healthy volunteers. (A) Clotting time upon activation with 5pM TF, and (B) 0.5 IU/ml thrombin. (C) Clotting rate upon activation with TF and (D) thrombin. (E) Changes in optical density of clots upon activation with TF and (F) thrombin. Horizontal bars represent medians. Asterisks indicate significant differences in comparison with healthy volunteer values. *P<0.05; **P<0.01.

Figure 2. Permeability of clots generated with plasma of patients with cirrhosis and healthy volunteers. The permeability coefficient Ks was calculated following Darcy's Law. Horizontal bars represent medians. Asterisks indicate significant differences in comparison with healthy volunteer values. **P<0.01.

Figure 3. Fibrinogen γ' levels in plasma of patients with chronic liver disease and healthy volunteers. (A) Absolute Fibrinogen γ' levels and (B) Fibrinogen γ' levels relative to total fibrinogen. Levels are expressed in microgram per milliter and per milligram fibrinogen. Horizontal bars represent medians.

Figure 4. Representative images of sections of clots generated with plasma of patients with cirrhosis and healthy volunteers. (A) Fibrin mesh in clot of a patient with Child B cirrhosis and (B) of a healthy volunteer clot visualized by laser scanning confocal microscopy (original magnification x630). (C) Fiber density in the fibrin mesh was quantified by counting the number of fibers crossing an arbitrary line drawn through the section. Density is expressed as the number of fibers per 10 μm. (D) Fibrin fiber thickness was quantified by measuring the diameter of individual fibers. The diameter is expressed in nanometers. Horizontal bars represent medians. (E) Fibrin mesh in a clot of a patient with Child B cirrhosis and (F) of a healthy volunteer clot visualized by SEM (original magnification x20.000).

Figure 5. Functional and structural parameters of clots generated with fibrinogen purified from plasma of patients with cirrhosis and healthy volunteers. (A) Clotting time, (B) Clotting rate and (C) Changes in optical density of clots. (D) Permeability and (E) fiber density of clots. For turbidity studies, clotting was initiated

with 0.5 IU/mL thrombin at equal fibrinogen concentrations (1 mg/mL). For permeability and density studies, clotting was initiated with 0.5 IU/mL thrombin at equal fibrinogen and FXIII concentrations (1 mg/mL and 7.3 μg/mL, resp.). (F) Representative LSCM image of fibrin mesh within a clot of a patient with Child B cirrhosis (left panel) and within a healthy volunteer clot (right panel) generated with purified fibrinogen (original magnification x630). Horizontal bars represent medians. Asterisks indicate significant differences in comparison with healthy volunteer values. *P<0.05; **P<0.01.

Figure 6. Carbonyl content of fibrinogen purified from patients with cirrhosis and healthy volunteers. Protein carbonyl content was measured at equal fibrinogen concentrations (20 μg/mL). Horizontal bars represent medians. Asterisks indicate significant differences in comparison with healthy volunteer values. *P<0.05; **P<0.01.