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Altered clot formation and lysis are associated with increased fibrinolytic activity in ascites in patients with advanced cirrhosis

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Abstract
Analysis of coagulation disorders and assessment of rebalanced hemostasis with the use of traditional coagulation assays is challenging in cirrhotic patients. Therefore, alternative tests are under investigation for the evaluation of coagulopathy in this specific setting. Aim of this study was to analyze the modifications of clot structure and function in cirrhotic patients with different degrees of severity. Cirrhotic patients referred to our Unit were consecutively enrolled. Global test measurements, including clot and lysis assays, clot lysis time, and determination of other fibrinolytic parameters, were performed. Analyses of clot formation, morphology, and lysis were performed with a turbidimetric clotting and lysis assay (EuroCLOT). Lysis of a tissue factor-induced clot by exogenous tissue plasminogen activator was analyzed by studying the modifications of turbidity during clot formation and the following lysis. We evaluated coagulative and fibrinolytic parameters in both plasma and ascites. Urokinase plasminogen activator (uPA) and gelatinase activity in ascites were also measured. We analyzed data from 33 cirrhotic patients (11 in Child–Pugh class A; 22 in class B or C and with ascites) and 21 healthy subjects (HS). In class B/C patients prolonged latency time, a decline in clotting absorbance, and decreased fibrin formation were observed in comparison with class A and HS. Generated curves and Thrombin-Activatable Fibrinolysis Inhibitor (TAFI) progressively declined from HS to class C patients, whereas levels of plasminogen activator inhibitor-1 and tissue plasminogen activator increased. D-dimer levels were markedly increased in ascites, together with significantly smaller levels of TAFI, αlfa2-antiplasmin, and plasminogen. Caseinolytic activity was also present. Class C patients showed smaller amount of uPA and significantly lower levels of matrix metallopeptidases (MMP)2 in ascites in comparison with Class B subjects. Clot formation and lysis are altered in cirrhosis and fibrinolysis is activated in ascites. Ascitic levels of uPA and MMP2 are reduced and inversely related to the severity of liver disease.

Keywords Liver cirrhosis · Fibrinolysis · Clot formation/lysis · Ascites · Coagulopathy

Professor Roberto Giulio Romanelli prematurely died in May 2019.

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Introduction

Liver is the main site of synthesis and degradation of both coagulation factors and fibrinolytic molecules. Notably, liver diseases are associated with decreased levels of procoagulant factors, thrombocytopenia and alterations in the fibrinolytic system [1]. In the past, a globally reduced procoagulant state in these patients has been suggested. However, Tripodi et al. [1] provided evidence that plasma from patients with liver cirrhosis could generate, in the presence of thrombomodulin, similar, or even greater, amounts of thrombin than healthy subjects. Indeed, blood coagulation in such patients is rebalanced, owing to the simultaneous reduction of procoagulant and anticoagulant factors [1, 2]. Traditional coagulation assays provide limited information on the status of rebalanced hemostasis in patients with cirrhosis. Therefore, alternative tests such as thrombin generation assay and viscoelastic testing are under evaluation for the assessment of coagulopathy in this specific setting [3, 4].

The EuroCLOT consortium has developed a new turbidimetric assay for the study of clot formation, morphology and fibrinolysis as dynamic processes [5]. This assay allows a high throughput analysis of samples with an improvement of reproducibility of the lag time estimations with poor interassay variability and bias in the estimates of components of variation [5]. However, coagulation assays using the EuroCLOT system have not yet been performed in patients with liver disease.

Another peculiar aspects of patients with decompensated cirrhosis, is that ascites is reabsorbed into systemic circulation via thoracic duct and several substances are transported back to plasma by different routes and mechanisms [6]. In addition, mesothelial cells produce both tissue plasminogen activator (tPA) and PAI-1. When the urokinase-Plasminogen-Activator-Receptor (uPAR)/uPA/Plasminogen cascade is activated, it generates plasmin, an activator of pro-matrix metallopeptidases (MMPs), including gelatinases. Interestingly, there are multiple interactions between haemostasis, plasminogen activators and MMPs. Both plasminogen activators (uPA and tPA) and thrombin can activate latent MMP2. Therefore, both thrombin and fibrinolytic enzymes might participate in the regulation of MMP2-mediated proteolysis [7].

Based on the above background, this study was undertaken to evaluate the alterations in clot structure/function in cirrhotic patients with different degree of severity, and to analyze the components of the fibrinolytic system in plasma. In addition, we assessed the fibrinolytic parameters and plasminogen activator (uPA) in ascites to establish whether ascites fluid, once reabsorbed into systemic circulation, might affect the hemostatic balance. Considering the interactions between hemostasis and MMPs generation, gelatinase (MMP2, MMP9) activities were also measured in ascites.

Patients and methods

Study design and population

We consecutively enrolled cirrhotic patients referred to our clinical Unit from January to December 2018. Diagnosis of cirrhosis was based on patient's history, physical examination, liver ultrasound, laboratory findings, endoscopy, and, when not contraindicated, liver biopsy. Ascites was detected by physical examination and confirmed by ultrasound. Exclusion criteria were as follows: alcohol use (last 3 months), drugs interfering with the hemostatic system including antithrombotic prophylaxis, organic cardiovascular/kidney/pulmonary involvement, diabetes mellitus, hepato-renal syndrome, spontaneous bacterial peritonitis, recent infections (last 3 months), past or current diagnosis of vein thrombosis (including portal thrombosis), presence of malignancies (including hepatocellular carcinoma), overt hepatic encephalopathy [8], or recent gastrointestinal bleeding (last 3 months) [8]. Child–Pugh/MELD scores were employed to classify patients according to the severity of cirrhosis [8]. Patients with ascites were treated with diuretics and received a low-sodium diet (88 mmol/day). In all patients with ascites diagnostic/therapeutic paracentesis was performed to exclude spontaneous bacterial peritonitis. Controls were age- and sex-matched healthy subjects (HS) and included volunteers from the staff of the University of Florence. The Local Ethical Committee approved the study, which conformed to the principles outlined in the Declaration of Helsinki. All participants gave their informed written consent before entering the study.

After overnight fasting, between 7:00 and 9:00 a.m., blood samples were obtained from patients or HS by venipuncture of an antecubital vein with minimal venous stasis, with a 21 gauge needle. The first tube was used for measurements other than coagulation/fibrinolysis. Blood samples were drawn in vacutainer tubes (Beckton Dickinson, Mayland, USA), containing 0.109 M trisodium citrate (final ratio with blood 1:10). Samples were immediately centrifuged at 4 °C (2000*g* for 15 min) and plasma samples were snap frozen and stored in small aliquots at − 80 °C, until further assay.

Ascitic fluid was collected during diagnostic paracentesis in sterile conditions directly in Falcon tubes containing 0.109 M concentration of trisodium citrate (final ratio with ascitic fluid 1:10), and then centrifuged at 2000*g* for 15 min at 4 °C. Ascitic fluid supernatants were snap frozen and stored at − 80 °C, until further assay.

Analysis of clot formation, morphology and lysis

Global test measurements, including clot and lysis assays, clot lysis time (CLT), and determination of other fibrinolytic parameters, were performed at the Thrombosis Centre, University of Florence. We employed two sensitive turbimetric assays.

a. Analyses of clot formation, morphology, and lysis were performed by using turbidimetric clotting and lysis assay, according to Carter et al. [5], with minor modifications and spectrophotometrical readings, as previously described [9]. To facilitate analysis of data from 96-well plates, we used a simple algorithm built in R language to analyze the large amount of raw data generated, obtaining the same parameters described by Carter et al. [5]. Our interassay coefficients of variation (CV) were as follows: LagC 11%, MaxAbsC 4%, crude rate of clot formation (CRC) 9%, Lys50t0 15%, LysT 14%, LR 12%, and AUC 15%.
b. *CLT* The lysis of a tissue factor-induced clot by exogenous tPA was studied by monitoring changes in turbidity during clot formation and subsequent lysis according to previous data [10], with some modifications in tPA concentrations employed and CLT calculation model, as previously described [11]. For each sample, CLT was determined in duplicate. CLT was defined as the interval between the maximum rate of clot formation and the maximum rate of clot lysis of CLP (clot lysis profile) analysis. The intra-assay and inter-assay CVs were 4% and 7%, respectively.

Other laboratory assays

To assess the contribution of thrombin-activatable fibrinolysis inhibitor (TAFI) activation to CLT, the assay was repeated in the presence of carboxypeptidase inhibitor (CPI) (25 µl/ml), a specific inhibitor of activated TAFI. The contribution of TAFI to the CLT was quantified by calculating the Clot Lysis Ratio (CLR), which is defined as the ratio between CLT measured in the absence/presence of CPI [12]. Plasma TAFI ag level was measured with a commercially available ELISA kit (Asserachrom TAFI, Diagnostica Stago, Asnieres, France). Plasminogen activator inhibitor-1 (PAI-1) and tissue plasminogen activator (tPA) levels were determined by using commercially available ELISA kits (Asserachrom PAI-1 and Asserachrom t-PA, Diagnostica Stago, Asnieres, France); αlfa2-antiplasmin (α2-AP) and plasminogen (PLG) were measured by photometric determinations, by using Behrichrom assays (Siemens, Marburg, Germany). Fibrinogen was evaluated by clotting assay according to Clauss (Fibrinogen C) and D-dimer by using Hemosil assays (IL, USA).

Zymography and plasminogen activators

Aliquots of ascitic fluids were subjected to SDS–PAGE (10%) and subsequent zymography, according to a described protocol [13]. Zymography was also performed in the presence of 2 mM amiloride, which specifically inhibits uPA, within the casein layer [14].

Gelatin zymography

Aliquots of ascitic fluids were subjected to SDS-PAGE (8%, containing 0.13% gelatin) and were subsequently to zymography as described [15]. The bands containing gelatinolytic activity of the pro- and active forms of MMP2 and MMP9, respectively, appeared transparent and were evident in the otherwise homogeneous blue gel. Bands were quantified using ImageJ software [15].

Statistical analyses

A physician trained in statistics encoded all samples into a dedicated database in an anonymous form. Data are expressed as the mean (± standard deviation) or median (with range) as applicable. Confidence interval (CI) is presented where appropriate. A full descriptive analysis was performed of all considered variables. Student's *t* test was used for paired data. Analysis of variance-ANOVA and, when the F test was significant, the Duncan's test were used for comparison among groups. Continuous ordinal data were compared with Mann–Whitney *U* test. Spearman's correlation coefficients were used to study the correspondences between variables.

A significance level of 0.05 was considered for all tests. SPSS® software version 21.0 (MJ Norusis, Chicago, US) was used for all statistical analyses.

Results

The demographic and clinical characteristics of patients and HS are reported in Table 1. All subjects were enrolled as outpatients. No significant differences in age and gender were observed between patients and HS. As shown in the Table B and C, cirrhotic patients showed statistically significant differences in respect to HS for several parameters.

Plasma coagulation and fibrinolysis parameters

Analyses of clot formation, morphology and lysis of the studied groups are shown in Table 2. All these parameters, except lysis rate (LR), were significantly altered in Child–Pugh C cirrhotic patients compared to HS (from $p = 0.013$ to $p < 0.001$). In Child–Pugh B patients, the

Table 1 Baseline characteristics of the study sample

	Healthy subjects ($n=21$)	Child–Pugh class A ($n=11$)	Child–Pugh class B ($n=9$)	Child–Pugh class C ($n=13$)
Gender (M/F)	10/11	6/5	5/4	9/4
Age (mean ± SD, years)	68 ± 14	65 ± 11	69 ± 17	70 ± 12
Etiology				
HBV	–	–	1	1
HCV	–	8	5	5
ALD	–	–	–	1
HCV + ALD	–	2	3	6
Cryptogenic	–	1	–	–
MELD score[a]				
< 17	–	11	8	5
≥ 17	–	–	1	8
HR (bpm)	80 ± 7	79 ± 1	80 ± 2	77 ± 2*
SBP (mmHg)	129 ± 9	118 + 9*	111 ± 13***	103 ± 8.0***
DBP (mmHg)	80 ± 8	69 + 7*	69 ± 13*	64 ± 8***
Hb (mmol/L)	14.7 ± 1.8	14.3 + 1.8	10.7 ± 1.1***	11.0 ± 1.6***
aPTT (s)	30.4 ± 6.0	28.4 + 2.5	33.7 ± 6.2	34.1 ± 9.3
PLT (× 109/L)	268 ± 72.0	134 + 55.0*	129 ± 94.0**	114 ± 93.0***
AST (U/L)	26 ± 10.0	64 ± 51.0*	63 ± 83.0*	62 ± 44.0**
ALT (U/L)	25 ± 9.0	59 ± 48.0*	47 ± 59.0*	63 ± 51.0**
γGT (U/L)	24 ± 10.0	56 + 62	143 ± 230**	86 ± 72**
Bilirubin (mg/dL)	0.7 ± 0.2	1.1 + 1.2	2.3 ± 1.4**	4.9 ± 5.8**
Albumin (g/dL)	4.0 ± 0.7	3.85 + 0.2	3.1 ± 0.7**	2.7 ± 0.4**

Descriptive statistics are given as the mean ± standard deviation (SD)

M males, *F* females, *SD* standard deviation, *HBV* hepatitis B virus, *HCV* hepatitis C virus, *ALD* alcoholic liver disease, *MELD* Model for End Stage Liver Disease, *HR* heart rate, *DBP* diastolic blood pressure, *SBP* systolic blood pressure, *Hb* hemoglobin, *PLT* platelets, *ALT* alanine transaminase, *AST* aspartate transaminase, *γGT* Gamma-glutamyl transferase

[a]Fisher's exact test between pts

Patients vs. healthy subjects: ***$p \le 0.001$, **$p \le 0.01$, *$p \le 0.05$

Table 2 Analyses of clot formation, morphology and lysis

	Healthy subjects ($n=21$)	Child–Pugh class A ($n=11$)	Child–Pugh class B ($n=9$)	Child–Pugh class C ($n=13$)
LagC (s)	399 (235–578)	426 (328–534)	461 (229–701)	546***/^{aa} (346–726)
MaxabsC (au)	0.222 (0.131–0.327)	0.185 (0.115–0.263)	0.096* (0.030–0.569)	0.088***/^{aaa} (0.029–0.211)
CRC 10^{-4} (au/s)	2.48 (1.3–4.7)	2.68 (1.6–4)	2.09 (0.7–1.4)	1.08**/^{aa} (0.5–1.4)
$Lys50_{t0}$ (s)	2522 (1592–4622)	2142 (1322–3036)	2424 (1133–4005)	1710* (791–4589)
LysT (s)	4147 (1883–5779)	3220 (1941–4684)	2688 (1227–5929)	1951** (992–6918)
LR 10^{-4} (au/s)	0.514 (0.3–0.7)	0.601 (0.1–0.7)	0.346 (0.1–1.2)	0.457 (0.2–0.7)
AUC	585 (224–1004)	316* (229–547)	257* (42–1559)	129***/^{a} (23–1145)

All values are expressed as median (min–max)

LagC lag time of clotting taken at the very beginning of lateral aggregation of fibrin protofibrils, as revealed by the occurrence of an exponential increase of absorbance (expressed in s); *MaxabsC* maximum absorbance of clot (expressed in arbitrary units, au); *CRC* crude rate of clot formation, derived from LagC and MaxabsC (expressed as arbitrary units/s); $Lys50_{t0}$ time from initiation of clot formation to the time at which a 50% fall in absorbance from MaxAbsL occurred (expressed in s); *LysT* time to complete lysis calculated from MaxabsL to return of values to baseline (expressed in s); *LR*, crude lysis rate derived from time and absorbance values for MaxAbsL and the point at which absorbance values returned to baseline (expressed in arbitrary units/s); *AUC* area under the curve, which reflects the balance between clot formation and clot lysis

Mann–Whitney pts. Vs. HS *$p \le 0.05$; **$p \le 0.01$; ***$p \le 0.001$ pts. A vs. C or B: $^{a}p \le 0.05$; $^{aa}p \le 0.01$; $^{aaa}p \le 0.001$

median values of clotting formation, MaxAbsC (maximum absorbance of clotting) and area under curve (AUC) values (cumulative index of clot formation and lysis) were significantly different compared to HS ($p = 0.022$ and $p = 0.017$, respectively).

Figure 1a, b shows the curves derived from the median values of clotting (a) and lysis (b) assays. Curve shapes were clearly different in the three groups of patients compared to HS. CLT and CLR values (Table 3) were not significantly different between class B patients and HS, whereas statistically significant differences were observed between class C patients and HS ($p < 0.001$ and $p = 0.014$, respectively). PAI-1 values were higher both in B and C cirrhotics with respect to class A patients and HS (from $p < 0.05$ to $p < 0.01$). tPA values were higher in cirrhotic patients in respect to HS (from $p < 0.01$ to $p < 0.001$). α2-AP showed a significant reduction in cirrhotic patients in respect to HS (from $p < 0.01$ to $p < 0.001$). Plasminogen plasma levels showed a significant reduction in cirrhotics in respect to HS ($p < 0.001$). Moreover, Class B and C cirrhotics had very low levels of fibrinogen in respect to controls ($p = 0.003$ and $p < 0.001$, respectively), and markedly elevated plasma D-dimer levels both in class B and C cirrhotics in respect to HS and class A patients ($p < 0.001$).

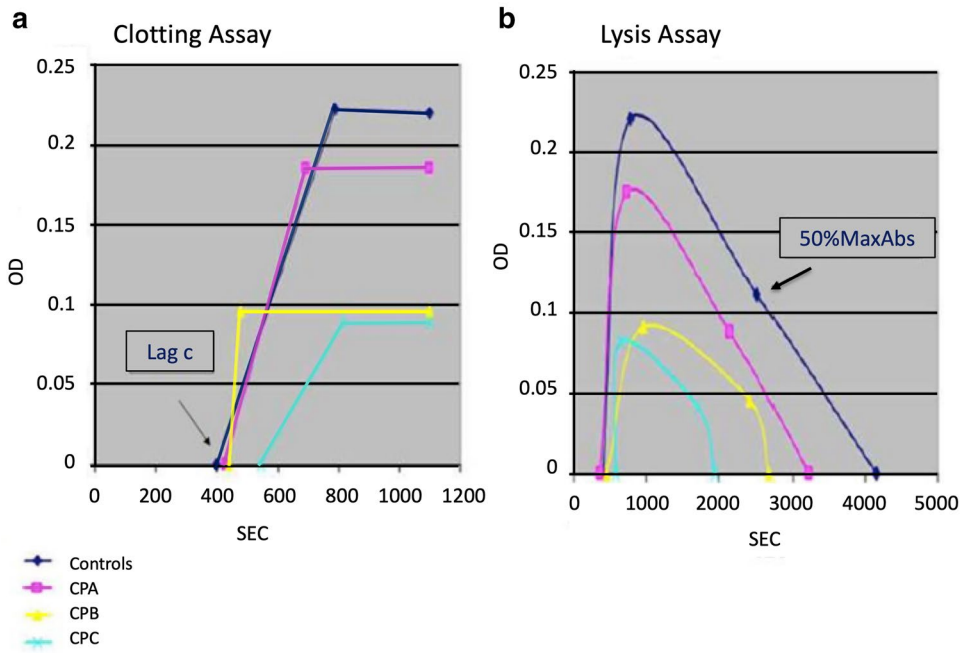

Fig. 1 Generated curves derived from the median values of clotting assay parameters (**a**). Generated curves derived from the median values of every lysis assay parameter regarding EuroCLOT analysis (**b**)

Table 3 Coagulation and fibrinolysis parameters in plasma according to the Child–Pugh class

	Healthy subjects[#] ($n = 21$)	Child–Pugh A[#] ($n = 11$)	Child–Pugh B[#] ($n = 9$)	Child–Pugh C[#] ($n = 13$)
CLT (min)	63 (52–78)	45*** (42–62)	65 (30–87)	42.5*** (22–66)
CLR (min)	1.35 (1.22–1.65)	1.21 (1.04–2.00)	1.30 (1.0–1.83)	1.13* (1.0–2.17)
TAFI (ag) (µg/mL)	8.4 (6.6–11.3)	8.32 (6.82–13.37)	4.09***/^{aa} (1.6–9)	3.6***/^{aaa} (1.96–5.6)
PAI-1 (ag) (ng/mL)	15.9 (8.0–36.3)	17.0 (6.0–31.0)	36**/^{aa} (10.9–84.6)	28*/^{a} (1.20–80)
tPA (ag) (ng/mL)	8.4 (3.0–15.8)	13.0** (7.8–17.6)	26*** (9.6–61)	33***/^{aa} (14.1–57.7)
α2AP act (%)	102 (89–124)	92** (68–97)	69***/a (51–108)	54***/^{aa} (37–97)
PLG act (%)	103 (67–131)	81*** (57–107)	60.5*** (38–92)	45***/^{aaa} (25–93)
Fb act (%)	332 (219–424)	358 (259–476)	167**/^{aa} (80–520)	175***/^{aaa} (91–325)
D-dimer (ng/mL)	103 (37–303)	102 (19–932)	1160***/^{aaa} (360–3025)	2360***/^{aaa} (138–3634)

CLT clot lysis time, *CLR* clot lysis ratio, *TAFI* thrombin-activatable fibrinolysis inhibitor, *PAI* plasminogen activator inhibitor, *tPA* tissue plasminogen activator, *act* activity, *α2AP* alpha-2-antiplasmin, *PLG* plasminogen, *Fb* fibrinogen

Mann–Withney pts. Vs. HS: *$p \le 0.05$; **$p \le 0.01$; ***$p \le 0.001$; pts. Vs. pts: A vs. C or B: $^{a}p \le 0.05$; $^{aa}p \le 0.01$; $^{aaa}p \le 0.001$

[#]Values are expressed as median (min/max)

Comparison between plasma and ascitic fluid in coagulation and fibrinolysis parameters

TAFI antigen, α2-AP, and PLG were higher in plasma than in ascites (Table 4). On the other hand, TAFI activity was significantly higher in ascites than plasma ($p < 0.001$) and fibrinogen was very low in the ascites itself. Ascitic fluid levels of D-dimer were extremely higher in both groups of cirrhotic patients ($p < 0.001$). No coagulability/fibrinolytic activity was found in ascitic fluid by EuroCLOT parameters and CLT. This was strictly correlated to the very low fibrinogen concentration found in ascitic fluid (data not shown). No differences were observed between B and C patients regarding all the analyzed parameters (Table 4, last column). No significant correlations were observed between EuroCLOT data and Child–Pugh score parameters, except AUC and bilirubin ($r = -0.515$, $p = 0.02$).

Plasminogen activators and gelatinases in ascitic fluids

Amiloride-dependent inhibition of caseinolysis (Fig. 2a) indicates that the observed lysis is dependent on uPA. Class C cirrhotic patients released a lower amount of uPA into ascites, as compared to class B patients ($p = 0.0037$). Gelatinolytic activity in ascites (Fig. 2b, left) was dependent on the pro- and active forms of MMP2 (gelatinase A), and MMP9 (gelatinase B). The difference in gelatinolytic

Table 4 Coagulation and fibrinolysis parameters in plasma and ascitic fluid

	CTP B ($n=9$) plasma	CTP B ($n=9$) ascites	CTP B plasma vs. ascites *p* value	CTP C ($n=13$) plasma	CTP C ($n=13$) ascites	CTP C plasma vs. ascites *p* value	CTP B vs. C ascites *p* value
TAFI (ag) (μg/mL)	4.09 (1.60–9.1)	0.98 (0.100–2.53)	<0.001	3.6 (1.96–5.6)	0.89 (0.128–1.7)	<0.001	0.508
TAFI act (%)	42.9 (30.8–154.2)	122.20 (33–374)	<0.001	32 (17.8–150)	84.06 (51.4–264)	<0.001	0.292
PAI-1 (ag) (ng/mL)	36 (10.9–84)	20 (7.9–136)	0.489	28 (1.20–80)	42.2 (4.11–194)	0.650	0.393
tPA (ag) (ng/mL)	26 (9.6–61)	38.5 (8.6–66)	0.436	33 (14.1–57.7)	30.4 (11.7–46.4)	0.295	0.431
α2AP act (%)	69 (51–108)	10.0 (1.56–17)	<0.001	54 (37–97)	4.64 (1.0–14.30)	<0.001	0.99
PLG act (%)	60.5 (38–92)	3.0 (1.0–13.5)	<0.001	45 (25–93)	2.6 (1–16.8)	<0.001	0.186
D-dimer (ng/mL)	1160 (360–3025)	40,898 (16,723–60,351)	<0.001	2360 (138–3634)	35,397 (3448–115,130)	<0.001	0.547

CTP Child–Turcotte–Pugh, *TAFI* thrombin-activatable fibrinolysis inhibitor, *PAI* plasminogen activator inhibitor, *tPA* tissue plasminogen activator, *act* activity, *α2AP* alpha-2-antiplasmin, *PLG* plasminogen

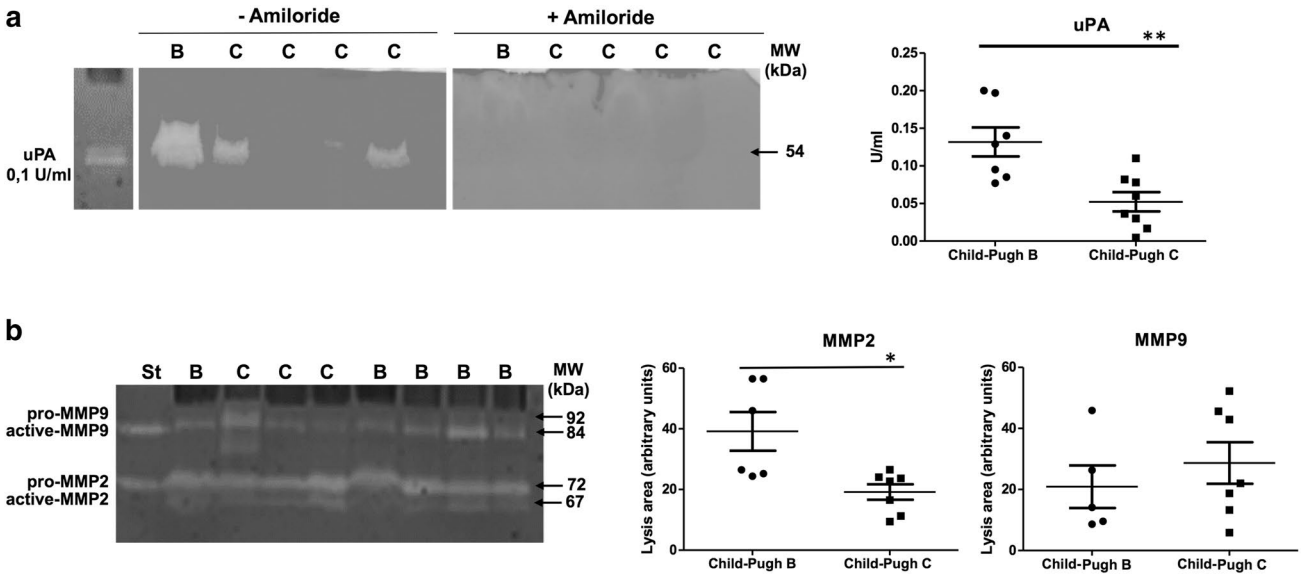

Fig. 2 Analysis of uPA (**a**), MMP2 and MMP9 (**b**) in ascitic fluid of Child–Pugh class B and C cirrhotics

activity in ascites between class B and C patients was not significant for MMP9 ($p = 0.45$), while it was highly significant for MMP2 ($p = 0.0104$), indicating that ascites of class C cirrhotic patients contains a lower MMP2-dependent collagenolytic activity. Overall, these data indicate that the uPA and MMP2 activities in ascites are inversely related to the worsening of liver function.

Discussion

The present study reports novel data about alterations of clot formation and lysis in cirrhotic patients obtained with the use of the EuroCLOT method. The advanced stages of cirrhosis were characterized by a prolonged latency time (LagC) in clot formation, a decrease in clotting absorbance (MaxabsC), and a reduced fibrin formation rate (CRC). Prolonged latency time (LagC) in clot formation showed a progressive trend to elongation from HS to cirrhotic patients, with significantly increased values in class C cirrhotic patients in respect to HS. The rise in LagC may be explained not only by the decreased plasma fibrinogen levels observed in the two groups of cirrhotics, but also by the abnormal sialic acid composition of the fibrinogen molecule, as previously described [16]. In fact, altered sialic acid composition enhances the negative charge of fibrinogen and delays fibrin polymerization [17]. Moreover, the decreased values of MaxabsC, AUC and plasma fibrinogen found in cirrhotic patients could be related to a less pronounced clot density. These data indicate that latency time in clot formation is prolonged, and once clot is formed, its absolute strength is reduced.

Data reported in this study are in agreement with findings reported in patients with acute-on-chronic liver failure [18], where defects in clot formation and a reduction of clot strength were described [18–20]. These data suggest that the alterations in advanced cirrhosis may indicate a tendency to bleeding. This is in contrast with everyday clinical practice, where venous thrombosis (especially portal vein thrombosis) is often observed in patients with cirrhosis. This apparent discrepancy may be explained by different lines of evidence. In fact, the altered clot structure and function observed in our study may be related to quantitative and qualitative alterations of the fibrinogen molecule, reported in many other studies [17, 21–23]. These alterations do not necessarily imply an increased bleeding tendency. Hugenholtz et al. [24] observed a decrease in clot permeability due to an increased carbonyl content of fibrinogen molecule for oxidative modifications of the protein. Importantly, a less permeable clot shows increased resistance to fibrinolysis, which may explain the potential pro-thrombotic nature of this condition. We recently confirmed that in cirrhotic patients there is a qualitative alteration of fibrinogen structure with a marked tendency toward thrombosis.

Interesting clinical data come from the study by Zanetto et al. [25]. Authors clinically confirmed the hypercoagulability of cirrhotics with the use of thromboelastometry. In particular, they analyzed data from patients with and without hepatocellular carcinoma showing that the presence of hepatocellular carcinoma itself and the increase of maximal clot firmness were independent predictors of portal vein thrombosis.

Thrombin-catalyzed fibrin formation and fibrin susceptibility to plasmin-induced lysis were considerably impaired in cirrhotic patients [26]. Coherently with the concept of a qualitative change of clot correlated to fibrinogen structure modification, Lisman et al. [27] indicated that hypersialylation of the fibrinogen molecule could contribute to a defective fibrinogen-to-fibrin conversion in patients with liver diseases. Moreover, these authors found that once the clot has formed, it has a thrombogenic nature as assessed by permeability assays. These thrombogenic properties of the fibrin clot in cirrhosis are associated with incompletely characterized intrinsic changes in the fibrinogen molecule, which may include oxidation and hypersialylation. Interestingly, the above-cited work by Lloyd-Donald et al. [18], demonstrated that patients with ACLF showed not only a decrease of clot strength but also a reduced tendency to clot lysis. In line with this consideration, Blasi et al. [19] reported that ACLF correlates with systemic inflammation and is associated with high mortality, but not with bleeding or transfusion requirements.

Fibrinogen plasma levels and TAFI were significantly reduced only in B and C patients. Moreover, TAFI activity, determined as CLR, was significantly decreased only in Child–Pugh class C patients. These reductions may be related to the impaired hepatic synthetic capacity [28, 29]. The role of TAFI deficiency on plasma fibrinolysis in cirrhotic patients is still debated [28]. Notably, TAFI level was shown to be a strong predictor of survival and was suggested to be useful as an additional model of prognosis for advanced liver disease [29]. PAI-1 and tPA plasma levels were significantly higher in both Child–Pugh class B and C cirrhotic patients. These findings are possibly related to either endothelial damage and/or reduced hepatic clearance [20]. In a recent study, Premkumar et al. [20] observed that tPA values > 20 ng/mL predicted mortality in patients with ACLF. As reported in previously published studies [30, 31], D-dimer plasma levels were markedly higher in both groups of cirrhotic patients, in respect to HS. It is still debated whether this is a primary or a secondary phenomenon, due to coagulation activation or delayed hepatic clearance.

There is some evidence that in liver cirrhosis there is clotting activation with secondary hyperfibrinolysis. Coleman et al. [21] observed that fibrinogen survival was improved by

low-dose heparin administration, suggesting that DIC can be a primary process in the defibrinating syndrome associated with cirrhosis. Emerging data indicate that inflammation and endothelial activation in severe liver disease might activate the coagulation cascade [8]. Moreover, enhanced thrombin generation has been observed in these patients [32].

We observed that ascites is characterized by a pattern consistent with activation of the fibrinolytic cascade within the peritoneal cavity. A marked increase (15–40 times) in D-dimer levels in ascitic fluid, when compared to plasma, was observed in association with increased tPA values, reduced concentration of plasminogen, α2-AP and TAFI antigen, suggesting increased plasmin production at this level. The growth of TAFI activity in ascites might be a compensatory response to this event. In addition, we observed plasminogen-activator induced caseinolytic activity. We also showed that the ascitic PA activity is completely inhibited by amiloride, and therefore should be ascribed to uPA. Fibrinogen was undetectable in ascites, a feature that made impossible to apply coagulation measurements to this fluid. Interestingly, several authors suggested that ascites, once reabsorbed, could trigger fibrinolysis into systemic circulation [33–35]. Furthermore, massive ascites usually occur in patients with advanced disease and severe portal hypertension with a high tendency to bacterial translocation, systemic spread of bacterial products, release of pro-inflammatory molecules, activation of coagulation cascade and fibrinolysis.

In our study, we observed that the levels of uPA and MMP2 in ascites are inversely related to the severity of liver impairment, while MMP9 did not show significant variations. Peritoneal mesothelial cells produce MMPs [36], therefore a reduced matrix clearance in the sub-mesothelial space could reduce ascites reabsorption through the peritoneal membrane. Interestingly, histologic changes consistent with non-specific chronic peritonitis were observed in cirrhotic patients with ascites [37]. It may be speculated that this condition could contribute to development of diuretic-resistant ascites in Child class C patients.

Further studies might investigate whether the inverse relationship between uPA and MMP2 in ascites and severity of liver disease might indicate an interaction among these substances and the liver fibrogenesis.

The findings obtained in this study and those available in the literature allow us to make a few clinical considerations: (a) the usual laboratory patterns are inadequate for an accurate evaluation of the coagulative status of cirrhotic patients; (b) quantitative and qualitative clot alterations can be correlated to the severity of cirrhosis, and may represent prognostic factors for the overall outcome; (c) considering our study and the amount of other lines of evidence, including some from our research group, we suggest that in patients with advanced cirrhosis the functionally altered clot, in presence of precipitating events (e.g., bacterial infection, systemic inflammatory response syndrome, renal failure), might favor a shift of a fragile and unstable hemostatic balance toward bleeding or thrombosis. Information about alterations in coagulation factors in the peritoneal cavity and ascites fluid expands our knowledge on the coagulation status in cirrhosis. In particular, determination of MMP2 and uPA may be useful to follow the progression of liver disease. Nonetheless, additional studies are needed to consolidate the prognostic significance of our findings. A limitation of this study is related to the small number of enrolled patients. Moreover, tests such as prothrombin fragment F1 + 2, PAP complexes and TAT were not performed.

In conclusion, patients with cirrhosis show alterations of clot formation and lysis due to quantitative and qualitative alterations of fibrinogen, modifications that become more pronounced with worsening of hepatic function. The described alterations can represent a prognostic factor for the overall patients' outcome.

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Compliance with ethical standards

Conflict of interest The author(s) declare that they have no conflict of interest.

Statement of human and animal rights All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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