Digestive and Liver Disease 56 (2024) 1327–1334

Contents lists available at ScienceDirect

Digestive and Liver Disease

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

Liver, Pancreas and Biliary Tract

Reticulated platelets are increased and hyper-activated in patients with cirrhosis, especially those with poor outcome

Alberto Zanetto[a,#], Serena Toffanin[b,#], Elena Campello[b,c], Claudia Maria Radu[b], Sabrina Gavasso[b], Patrizia Burra[a], Francesco Paolo Russo[a], Marco Senzolo[a], Paolo Simioni[b,c,*]

[a] Gastroenterology and Multivisceral Transplant Unit, Department of Surgery, Oncology and Gastroenterology, Padova University Hospital, Padova, Italy
[b] Thrombotic and Haemorrhagic Disease Unit and Haemophilia Center, Department of Medicine (DIMED), University of Padova, Italy
[c] General Internal Medicine Unit, Azienda Ospedale - Università Padova, Padova, Italy

ARTICLE INFO

Article history:
Received 15 January 2024
Revised 26 February 2024
Accepted 11 March 2024
Available online 28 March 2024

Keywords:
Coagulation
Hemostasis
Liver failure
Portal hypertension

ABSTRACT

Background: Reticulated platelets (RePLT) are emergency circulating platelets released to contrast peripheral platelet destruction.
Aim: We conducted a prospective study to [a] characterize RePLT in cirrhosis; [b] evaluate the association between RePLT and hepatic decompensation/death.
Methods: Cirrhosis patients without hepatocellular carcinoma were prospectively recruited and underwent assessment of RePLT and thrombopoietin (TPO). RePLT were evaluated by cytofluorimetry and immuno-fluorescence microscopy. Twenty healthy subjects were included as controls. Patients were followed for 6 months for hepatic decompensation and further decompensation/ACLF.
Results: Forty-five patients were included (Child-Pugh [CP] A/B/C 18/11/16). Compared to controls, RePLT in cirrhosis were significantly increased (0.82% vs. 0.05%; $p < 0.001$) and hyperactivated (4.35% vs. 0.17%; $p = 0.004$). No correlation was observed between RePLT and CP, platelet count, TPO, MELD score, and C-reactive protein. TPO was lower in cirrhosis than controls (28 pg/mL vs. 52 pg/mL; $p = 0.005$), decreasing significantly with CP stage. In CP B/C patients ($n = 27$), RePLT were significantly higher in those who progressed towards further decompensation/ACLF (2.11 [0.56–2.95] vs. 0.69 [0.02–1.22]; $p < 0.01$). A proportion of RePLT >2% accurately identified high-risk patients (AUROC 0.818; 95%CI: 0.639–0.997; sensitivity 94%, specificity 73%).
Conclusion: RePLT in cirrhosis are increased and hyper-activated. In decompensated patients, higher RePLT appear to be associated with worse outcomes.

1. Introduction

Reticulated platelets (RePLT) are immature platelets circulating in blood, which reflect bone marrow megakaryopoiesis [1]. RePLT have a larger volume, more dense granules, a higher content of RNA, and higher levels of surface activation markers than mature platelets [2–4]. In normal conditions, RePLT circulate for approximately 24–36 h, undergoing a progressive volume reduction and degradation of RNA [2–4]. Current theory posits that RePLT are "emergency" platelets, released to contrast peripheral platelet destruction [1], which is a major factor responsible for thrombocytopenia in cirrhosis [5].

Diabetes and coronary artery disease, which are associated with an increased platelet turnover and systemic inflammation such as cirrhosis ([6,7]), are associated with higher levels of RePLT ([8,9]). In cardiovascular diseases, RePLT have been associated with cardiovascular events and mortality [10]. In fact, the assessment of RePLT in these patients has been proposed to monitor the efficacy of antiplatelet therapy and guide patient management [2]. Previous studies assessing RePLT in cirrhosis show inconclusive results due to heterogenous cohorts including patients with and without hepatocellular carcinoma [11] and suboptimal comparison between cirrhosis with thrombocytopenia and healthy subjects with normal platelet count [12].

* Corresponding author at: General Internal Medicine and Thrombotic and Hemorrhagic Diseases Unit, Padova University Hospital, Padova, Italy.
E-mail address: paolo.simioni@unipd.it (P. Simioni).
# Equally contributed and joint first authors

https://doi.org/10.1016/j.dld.2024.03.007

RePLT appear to have greater procoagulant and prothrombotic potential than aged platelets, acting as seeds for the assembly of platelet aggregates at the site of vessel injury [13]. Although their primary role is in the prevention/control of bleeding, platelets contribute to multiple functions that extend beyond hemostasis and thrombosis [14]. Evidence from pre-clinical studies indicates that alterations in platelet functions may play a role in hepatic toxicity and progression of liver injury [15,16]. In a nationwide study of patients with chronic liver disease, low-dose aspirin was associated with a significantly lower risk of lower liver-related mortality than no use of aspirin [17]. A better understanding of RePLT in cirrhosis may lead to a more rational and individualized management of hemostasis in these patients [18,19], and it may help to identify new therapeutic targets [20].

In this study, our primary objective was to assess presence and characteristics of RePLT in a prospective cohort of patients with cirrhosis. Secondly, we evaluated the association between reticulated platelets and liver disease progression (secondary outcome) during 6-month follow-up.

2. Materials and methods

2.1. Patient selection

This is a single center cohort study wherein consecutive patients with cirrhosis, evaluated at the Gastroenterology and Multivisceral Transplant Unit of Padova University Hospital between January 1, 2023, and June 1, 2023, were prospectively screened for recruitment. Both inpatients and outpatients were eligible for inclusion. In the inpatients, blood samples were collected within 24 h after admission; in the outpatients, blood samples were collected during clinics.

Assessment of platelets was performed within an ongoing prospective study investigating biomarkers of chronic gastrointestinal diseases at Padova University Hospital (HIC protocol #0,034,435). The study was conducted in compliance with the Declaration of Helsinki and all patients signed consent to participate.

The diagnosis of cirrhosis was based on clinical, biochemical, imaging and/or histological findings. Compensated and decompensated cirrhosis were defined according to the Baveno VII consensus [21]. Exclusion criteria for inpatients were ACLF at time of screening [22]; admission to intensive care units; transfer from other hospitals. Patients with ACLF were excluded because ACLF is a distinct syndrome with specific coagulation features [23,24]. Additional exclusion criteria for both inpatients and outpatients were chronic kidney disease; presence and/or history of portal vein thrombosis and/or venous thromboembolism; presence of any intra or extra-hepatic tumor or known hematologic disease; recent major surgery (within 1 month); HIV-infection, history of organ transplantation; therapeutic anticoagulation and/or antiplatelet and/or anti-fibrinolytic therapy; recent (<7 days) transfusion of any blood product. Patients receiving anticoagulant prophylaxis were eligible for recruitment.

These exclusion criteria were chosen to mitigate the effect of potential confounding factors on the assessment of RePLT. Since previous data showed that acute kidney injury (AKI) in decompensated cirrhosis is associated with reversible platelet dysfunction [25], we did not exclude patients with AKI to assess its effects on RePLT.

Twenty healthy, adult individuals were tested as a control group for platelet parameters. This group consisted of 12 males (60%) and 8 females (40%) without history of any acute and/or chronic disease, and not taking any antithrombotic, antibiotic, and hormonal therapy. Healthy controls were recruited at our outpatient clinics; peripheral blood was collected and processed with the same modalities used in patients with cirrhosis (see below).

2.2. Sample collection and laboratory assays

2.2.1. Blood sampling

Peripheral blood was collected via venipuncture from an antecubital vein into BD Vacutainer® Citrate Tubes containing 0.109 M (3.2%) sodium citrate (9:1 blood to anticoagulant ratio) using a 21 g needle with light tourniquet. The blood was kept at room temperature; platelet rich plasma (PRP) was prepared within 2 h after collection by centrifugation at room temperature for 10 min at 120 g without brake [26]. Samples were then immediately processed for immunolabeling.

2.2.2. Assessment of RePLT (cytofluorimetry and immuno-fluorescence microscopy)

Assessment of platelet count was performed with the CELL-DYN Emerald22 hematology analyzer cytometer (Abbott Diagnostics). Ten µL of PRP were stained with 3 µL of Alexa Fluor 647-labeled Annexin V (Abcam, cod.ab214484), 3 µL of phycoerythrin (PE)-labeled anti-CD62P (P-selectin, clone CLB-Thromb/6, Beckman Coulter, cod.IM1759U), 3 µL of PE/Cyanine 7-labeled anti-CD41 antibody (clone P2, Beckman Coulter, cod.6607115), Acridine Orange AO (Sigma-Aldrich, cod.318337) (0,1 mg/ml final concentration). Upon staining, samples were incubated for 1 h at room temperature in complete dark [27]. The final concentration of the antibodies used, except for Acridine Orange (AO), was from 2 to 5 µg/ml. Prior to staining, the antibody mixtures were centrifuged at 20,000 g for 30 min to remove fluorescent particles. The dilution buffers used, PBS and Annexin V buffer (Abcam, ab214484), were sterilized through a 0.2 µm mesh filter to reduce background noise. Stained PRP was then diluted by adding 400 µL of Annexin V Buffer and analyzed within 1 h after immunolabeling. Unstained PRP incubated with filtered PBS was used as a blank for each sample. In addition, filtered PBS was incubated with single antibodies and the fluorescence measured was subtracted to avoid non-specific background signal.

Size gate of the flow cytometer was obtained by using a mix 1:1 of fluorescent polystyrene beads of known size including Megamix (BioCytex, cod.7801) and Flow-Count (Beckman Coulter, cod.7547053) for sizes 0.5, 0.9, 3 and 10 µm respectively. Violet side scatter (VSSC) and FL1 channel gain were set to visualize the beads. The side scatter (SSC) from the 405 nm violet laser (VSSC) was used as a trigger signal to exclude the noise. After turning the set in VSSC and forward scatter (FSC), a morphological gate was set between the 3 µm and 10 µm beads population diameter. CD41 and Acridine Orange positivity defines RePLT in morphological gate, identifying the upper edge of the platelets cluster with an area in the upper left corner of the dot plot of side scatter vs. Acridine Orange fluorescence obtained with a line starting at the origin and encompassing 1–1,5% of platelets. Size gate set from 0.5 to 3 µm and CD41 positivity define mature platelets [28].

Each analysis included 10.000 events and doublet events were excluded. Flow cytometer acquisition settings were maintained for all samples, including triggering threshold, voltages, and flow rate. Samples were analyzed by flow cytometer CytoFLEX SRT (Beckman Coulter); files were exported, and data evaluated by CytExpert (Software Version 2.3, Beckman Coulter).

Acridine orange is a fluorochrome specific for nucleic acids (DNA/RNA) [29]. Annexin-V is an index of apoptosis which binds phosphatidylserine, a membrane phospholipid whose presence on the external surface after activation of platelets promotes coagulation and thrombosis. CD62P encodes for P-selectin, a glycoprotein that is normally located in the platelet α-granules and is being exposed on the extracellular membrane following platelet activation [30]. Therefore, RePLT that are positive for both Annexin-V and CD62P (P-selectin) can be considered activated.

Table 1
Baseline characteristics in patients with cirrhosis.

	Patients with cirrhosis (n = 45)
Age, years	64 (55–68)
Male sex,%	70
Etiology of cirrhosis,%	
Alcohol	52
HCV[*]	28
HBV±HDV[*]	8
MASH	12
Child-Pugh class A/B/C,%	40/24/36
Decompensated,%	60
Decompensating event,%	
Ascites grade≥2	78
Variceal hemorrhage	13
Hepatic encephalopathy	9
MELD score	13 (9–17)
Type 2 diabetes,%	18
Arterial hypertension,%	22
Dyslipidemia,%	14
Leucocyte, x10^9/L	5.8 (3.9–6.7)
Hemoglobin, g/dL	11 (9–12)
Platelet count, 10^9/L	101 (76–185)
Thrombocytopenia, (%)	
Present	68
Mild >100≤150 × 10^9/L	31
Moderate 50≤100 × 10^9/L	56
Severe <50 × 10^9/L	13
Thromboprophylaxis,%	5
Total bilirubin, mg/dL	2 (1.2–5.1)
INR	1.4 (1.2–1.9)
Creatinine, mg/dL	0.7 (0.6–0.9)
Acute kidney injury^,%	6
Bacterial infections^,%	12
Albumin, g/dL	31 (27–37)
C reactive protein, mg/L	7 (3–18)
Presepsin, ng/L	403 (286–640)
Na, mmol/L	137 (134–141)

Median values reported with 25th and 75th percentile values in parenthesis. Abbreviations: MELD: Model for End-Stage Liver Disease; NASH: non-alcoholic steatohepatitis. ^in hospitalized patients.
[*] All patients were HCV-RNA and HBV-DNA negative at time of recruitment.

To confirm the presence of RePLT, we also performed immunofluorescence microscopy. Briefly, 20 µL of PRP, immunolabeled as described above, were loaded onto a sterile uncoated µ-Dish (Ibidi GmbH) covered with 10 µL of Mowiol antifade solution (Sigma-Aldrich). To assess non–specific binding, the corresponding isotype controls were used to stain the samples. We used TCS SP8 Confocal microscope (Leica Microsystem, Wetzlar, Germany); samples were analyzed with differential interference contrast (DIC) and fluorescence objectives. Images were acquired with 63x/1.4 oil immersion lens, using a DFC365FX camera and processed using the Leica Application Suite (LAS-AF) 3.1.1. software (Leica Microsystems).

2.2.3. Assessment of thrombopoietin (TPO)

TPO is the principal physiologic regulator of platelet production [31]. The plasma TPO level was measured using a commercially available enzyme-linked immunosorbent assay kit (abcam, cod. ab219632), according to the manufacturer's protocol. The sensitivity concentration of the ELISA kit was 11 pg/mL. All samples were measured in duplicate with the same assay, and the results were calculated as the mean of two values. All these laboratory tests were performed at the Thrombotic and Hemorrhagic Diseases Laboratory of Padova University Hospital by lab personnel with specific expertise in the assessment of platelets in cirrhosis. Lab personnel performing the tests was blind to the clinical characteristics of recruited patients.

2.2.4. Assessment of presepsin (PSP)

Peripheral blood was collected via venipuncture from an antecubital vein into a vacutainer tube (BD Vacutainer®, Becton, Dickinson and Company, UK) containing EDTA, using a 21 g needle with light tourniquet. The PSP concentration was determined using the PATHFASTTM analyzer (Mitsubishi Chemical Europe GmbH), with an analytical method based on the chemiluminescent enzyme immunoassay technique, as previously reported [32]. The measuring range was between 20 and 20.000 ng/L; the imprecision has been obtained by measuring for 20 non-consecutive days in duplicate 4 plasma samples that showed a mean value between 445 and 19.292 ng/L, with a coefficient of variation between 3.8 and 5.0%; the reference range was from 57 to 337 ng/L.

2.3. Data collection

Data collected from the medical records included demographics, laboratory data, Model for End-Stage Liver Disease (MELD) score, Child-Pugh stage, presence of bacterial infections and acute kidney injury at time of inclusion in hospitalized patients. Thrombocytopenia was defined as a platelet count <150 × 10^9/L, and sub-classified as mild (between 100 and <150 × 10^9/L), moderate (between 50 × 10^9/L and <100 × 10^9/L) or severe (<50 × 10^9/L) [33]. Acute kidney injury was defined according to the International Club of Ascites [34]. Patients with compensated cirrhosis were prospectively followed for up to 6 months for the development of first decompensating event; patients with decompensated cirrhosis were followed for up to 6 months for the development of further decompensation, ACLF, and liver transplantation [21]. All patients were followed at our center; clinical outcomes were registered by the same author responsible for patient's recruitment (AZ) during outpatient visits or in case of any new hospital admission.

2.4. Data analysis

See supporting information.

3. Results

3.1. Demographics

Of the 84 patients with cirrhosis screened for recruitment, 45 were included (70% male; median age 64 years). Reasons for exclusion are included in the Supporting Information.

As shown in Table 1, alcohol-related liver disease and HCV chronic infection were the most common etiologies of cirrhosis (47% and 30%, respectively). Eighteen (40%) patients were Child-Pugh A and 27 (60%) were Child-Pugh B/C. Child A patients all had compensated cirrhosis and were mostly outpatients, whereas 94% of Child-Pugh B/C patients were recruited while hospitalized due to difficult to treat/tense ascites, liver transplant evaluation, and hepatic encephalopathy. Among decompensated patients recruited during hospitalization, 9% had acute kidney injury and 16% had bacterial infection at time of recruitment.

Median platelet count was 101 × 10^9/L; 69% of patients were thrombocytopenic, with most having moderate thrombocytopenia. Both prevalence and severity of thrombocytopenia were greater in Child-Pugh C vs. B vs. A stage (prevalence: 88% vs. 67% vs. 53%; mild/moderate/severe in 21/64/15% vs. 0/67/33% vs. 0/64/36%, respectively). Median INR was 1.4 (1.2–1.9), and it was significantly higher in Child-Pugh C vs. B vs. A stage (1.9 [1.4–2.5] vs. 1.6 [1.5–1.8] vs. 1.2 [1.2–1.3]).

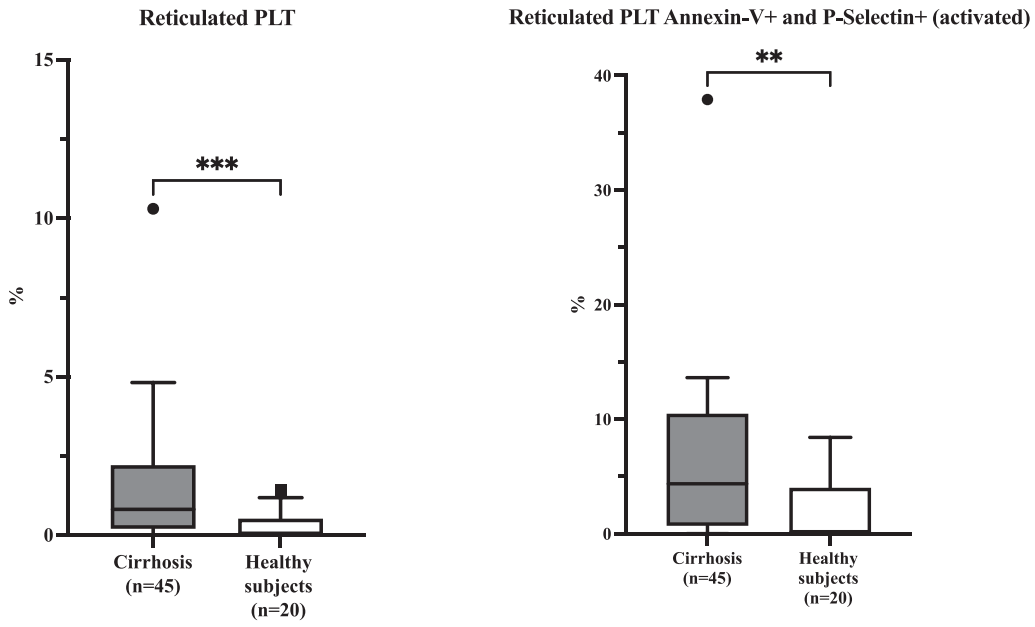

Fig. 1. Reticulated platelets are increased and hyper-activated in patients with cirrhosis vs. healthy subjects. Abbreviations: RePLT: reticulated platelets; AO: acridine orange.

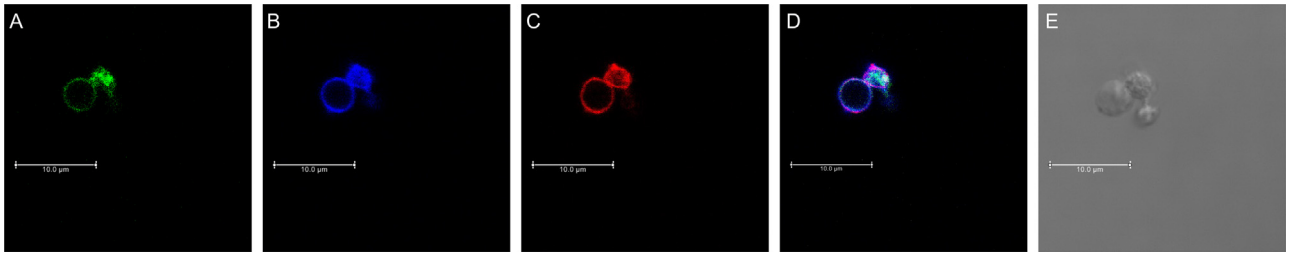

Fig. 2. Confocal microscopy of reticulated platelets. Confocal microscopy images (TCS SP8 Leica, 63x/1.4 oil immersion objective, zoom factor 6, scale bar 10 µm) of reticulated platelets. After immunolabeling as described above, PRP was covered with 10 µL of Mowiol antifade solution (Sigma-Aldrich) and images were acquired using a DFC365FX camera and processed using the Leica Application Suite (LAS-AF) 3.1.1. software (Leica Microsystems). A) Acridine Orange represents green fluorescence; B) Annexin V, blue fluorescence; C) P-Selectin, red fluorescence; D) Overlay of the three fluorescence; E) DIC image. Abbreviation: DIC: differential interference contrast.

3.2. Reticulated platelets are increased and hyper-activated in patients with cirrhosis, independently of etiology and disease severity

The proportion of RePLT was significantly higher in patients with cirrhosis than in healthy subjects (0.82% [0.21–2.21] vs. 0.05% [0.01–0.52]; $p < 0.001$) (Fig. 1A). The proportion of RePLT double positive for Annexin-V and P-selectin, indicating platelet activation, was significantly higher in patients with cirrhosis than in healthy subjects (4.35% [0.68–10.47] vs. 0.17% [0.00–4.01]; $p = 0.004$) (Fig. 1B). Fig. 2 shows confocal microscopy images of resting RePLT with increased expression of P-selectin and Annexin V.

In cirrhosis, the proportion of RePLT double positive for Annexin-V and P-selectin was significantly higher than the proportion of non-reticulated platelets double positive for the same markers (4.35% [0.68–10.47] vs. 0.93% [0.57–1.71]; $p < 0.001$).

We found no difference between patients with alcohol-related, HCV/HBV-related, and MASH-related cirrhosis regarding proportion of RePLT and activated RePLT. In patients with alcohol-related cirrhosis, no difference in the proportion of RePLT and activated RePLT was found between abstinent patients and those who reported active/recent drinking. Proportion of RePLT and activated RePLT were comparable between patients with and without thrombocytopenia (0.82% [0.12–2.55] vs. 1.29% [0.23–1.98]; $p = 0.7$ and 5.15% [0.68–9.92] vs. 2.32% [0.59–10.72]; $p = 0.6$, respectively). In cirrhosis with thrombocytopenia, no difference was found in the proportion of RePLT and activated RePLT between those with mild, moderate, and severe thrombocytopenia (data not shown). Both RePLT and activated RePLT were comparable between Child-Pugh A, B, and C patients (Supporting Fig. 1).

In decompensated patients, bacterial infections were associated with a significantly higher proportion of RePLT (2.21% [0.97–4.81] vs. 0.69% [0.14–1.68]; $p = 0.04$) and a significantly lower proportion of activated RePLT (0.54% [0.34–0.90] vs. 5.82% [4.06–11.69]; $p = 0.01$). Proportion of RePLT and their status of activation was comparable between patients with and without acute kidney injury (not shown).

3.3. Mature platelets are hyper-activated in patients with decompensated cirrhosis vs. healthy subjects

Overall, the proportion of non-reticulated platelets double positive for Annexin-V and P-selectin (i.e., activated) was comparable between patients with cirrhosis and healthy subjects (0.93% [0.57–1.71] vs. 0.40% [0.32–0.97]; $p = 0.1$). However, there was a significant increase in the proportion of mature, activated platelets from Child-Pugh A to C (0.32% [0.10–0.92] vs. 1.06% [0.78–2.49] vs. 1.25% [0.71–2.14], respectively; $p = 0.03$) (Supporting Fig. 2). In fact, Child-Pugh B/C patients had a significantly higher frequency of activated mature platelets than healthy subjects ($p < 0.001$).

Table 2
Correlations between reticulated/non-reticulated platelets and other clinical, hemostatic, and inflammatory factors.

	Child-Pugh A		
	Reticulated PLT	Activated, reticulated PLT (Annexin-*V*+ and P-selectin+)	Activated, non-reticulated PLT (Annexin-*V*+ and P-selectin+)
Platelet count	−0.4	−0.1	−0.5*
Hemoglobin	0.2	−0.1	−0.2
INR	0.4	−0.1	0.3
CRP	−0.1	−0.2	0.1
Presepsin	−0.1	−0.1	−0.1
TPO	0.5	−0.6*	−0.2
MELD	0.4	0.3	0.7*
	Child-Pugh B/C		
	Reticulated PLT	Activated, reticulated PLT (Annexin-*V*+ and P-selectin+)	Activated, non-reticulated platelets (Annexin-*V*+ and P-selectin+)
Platelet count	−0.2	−0.1	−0.5*
Hemoglobin	−0.1	0.4*	0.1
INR	0.5*	−0.2	0.2
CRP	0.1	0.1	−0.1
Presepsin	0.2	−0.6	0.7*
TPO	−0.1	0.1	0.1
MELD	0.3	−0.2	0.1

Note: Spearman correlation coefficients are shown.
Abbreviations: RePLT: reticulated platelets; AO: acridine orange; CRP: C-reactive protein; TPO: thrombopoietin; MELD: Model for End Stage Liver Disease.
* $p < 0.05$.

3.4. Correlation between platelet results and other clinical, hemostatic, and inflammatory factors

In Child-Pugh A patients, RePLT were not correlated with platelet count, TPO, severity of liver disease/portal hypertension, and markers of systemic inflammation and bacterial translocation such as CRP and PSP (Table 2). Interestingly, the proportion of non-reticulated (i.e., mature) platelets expressing Annexin-5 and P-selection was positively and negatively correlated, respectively, with MELD score (rho: 0.7) and platelet count (rho: −0.5), indicating that a more advanced chronic liver disease is associated with platelet activation even within the stage of compensated cirrhosis.

In Child-Pugh B/C patients, the proportion of RePLT was positively correlated with INR (rho: 0.5), whereas it was not correlated with platelet count, TPO, and other markers of disease severity including MELD score and C-reactive protein. Interestingly, the proportion of non-reticulated (i.e., mature) platelets in decompensated cirrhosis was strongly correlated not only with platelet count, but also with presepsin (rho: 0.7), which is a marker of bacterial translocation in patients with portal hypertension. A univariate linear regression analysis showed that albumin and INR were the only factors associated with frequency of RePLT (Supporting Table 1).

3.5. Thrombopoietin is significantly reduced in cirrhosis, especially in decompensated patients (Child-Pugh *c* < *b* < A)

Patients with cirrhosis had a significantly lower TPO than healthy subjects (28 pg/mL [12–50] vs. 52 pg/mL [44–65]; $p = 0.005$). We found a progressive, significant decreased in TPO levels from Child-Pugh A to B and C (45.5 pg/mL [25–59] vs. 39 pg/mL [11–50] vs. 14 pg/mL [7–29]; $p = 0.04$). No significant difference was found in TPO between patients with and without thrombocytopenia, nor according to the severity of thrombocytopenia in those with platelet count $<150 \times 10^9$/L. However, TPO was moderately correlated with platelet count (rho: 0.4; $p = 0.006$). As shown in Table 2, TPO was not correlated with any parameters of RePLT and mature platelets both in compensated and decompensated cirrhosis.

3.6. RePLT were associated with development of further decompensation and ACLF in decompensated cirrhosis

Among Child-Pugh A patients (all with compensated cirrhosis), no patient experienced the first episode of decompensation during 6-month follow-up. Among Child-Pugh B/C patients ($n = 27$), during a median follow-up of 138 days (43–180), 6 experienced further decompensation (2 hepatorenal syndrome with sepsis, 2 recurrent variceal hemorrhage, 1 refractory ascites, 1 sepsis due to spontaneous bacterial peritonitis), 3 ACLF (2 precipitated by bacterial infections and 1 by alcoholic hepatitis), and 2 underwent liver transplantation due to progression of decompensated cirrhosis (cumulative incidence of 40.7%) (Supporting Table 2). The 3 patients who developed ACLF died due to multiorgan failure. Decompensated patients who progressed towards further decompensation, ACLF/death, and liver transplantation had higher MELD score and C-reactive protein, and a higher frequency of RePLT than those who did not (Table 3). Bacterial infections at baseline were also more common in decompensated patients who progressed than in those who remained stable. However, a sub-analysis excluding patients with bacterial infections confirmed that frequency of RePLT was significantly higher in those who progressed towards the composite outcome.

Notably, of the 19 patients with RePLT ≤2%, only 3 (16%) progressed towards further decompensation/ACLF/liver transplantation, whereas of the 9 patients with RePLT >2%, 8 (89%) progressed towards the composite outcome ($p < 0.0001$) (Fig. 3A). Thus, a baseline value of RePLT >2% was associated with a 5-fold higher relative risk of progression during 6-month follow-up (relative risk: 5.25, 95%CI 1.80 −15.24; $p = 0.002$). Based on AUROC analysis (Fig. 3B), the cut-off value with the highest sensitivity and specificity (Youden index) in identifying patients at risk of progression was 2% (AUROC 0.818; 95% confidence interval [CI] 0.639–0.997; sensitivity 94%, specificity 73%). To further assess the prognostic value of RePLT, we divided the group of decompensated patients in terciles according to the baseline percentage of RePLT (≤0.29, between 0.30 and ≤1.71, and >1.72). Incidence of further decompensation/ACLF/liver transplantation was significantly different between groups: 18.2%, 18.2% and 63.6% in the three terciles, respectively ($p = 0.02$). Regarding thrombo-hemorrhagic

Table 3
Comparison between decompensated patients who progressed further decompensation, ACLF, death/liver transplantation vs. those who had not.

	Further decompensation, ACLF, death/LT (n = 11)	NO further decompensation, ACLF, death/LT (n = 16)	P value*
Age, years	67 (61–68)	66 (61–70)	0.8
MELD	**18 (14–26)**	**14 (11–19)**	**0.03**
Child-Pugh stage C,%	73	50	0.2
Bacterial infections, %	**80**	**18**	**<0.01**
Acute kidney injury,%	15	10	0.7
C-reactive protein, mg/L	**29 (10–57)**	**14 (6–18)**	**0.02**
Creatinine, mg/dL	0.7 (0.6–0.8)	0.7 (0.6–0.9)	0.4
Bilirubin, mg/dL	3.4 (2–5.3)	4 (2.1–5.1)	0.7
INR	**1.9 (1.8–3)**	**1.6 (1.4–2.1)**	**0.03**
Reticulated PLT, %	**2.21 (0.56–2.98)**	**0.69 (0.02–1.22)**	**<0.01**
Activated RePLT (Annexin-V+ and P-selectin+)	2.04 (0.42–11.82)	4.90 (2.31–10.63)	0.6

Median values reported with 25th and 75th percentile values in parenthesis.
* Mann-Whitney U test (quantitative variables); Chi-square test (qualitative variables). Legend: MELD: Model for End Stage Liver Disease; LT: liver transplantation.

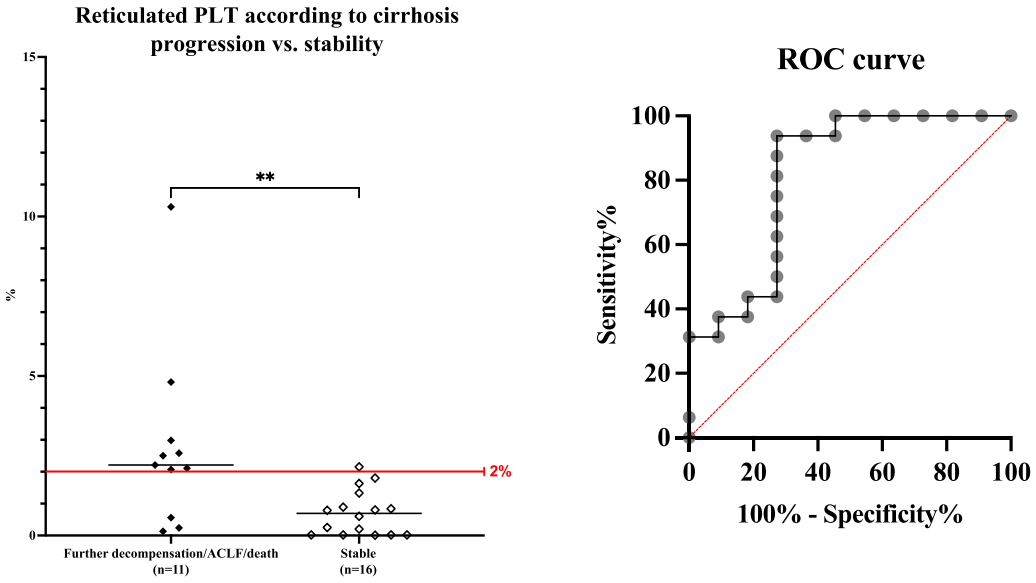

Fig. 3. Predictive value of baseline RePLT in patients with decompensated cirrhosis. A) A threshold of RePLT >2% identified two populations with a significantly different risk of further decompensation and liver-related death. B) AUROC curve.

complications, among patients with decompensated cirrhosis, 2 patients experienced complete portal vein thrombosis and 3 experienced major bleeding complications unrelated to portal hypertension during follow-up (2 procedure-related and 1 spontaneous) (Supporting Table 2). No difference in RePLT was found between patients who experienced thrombo-hemorrhagic complications and those who did not (2.21% [0.28–3.74] vs. 0.81% [0.13–2.07], p = 0.2).

4. Discussion

Characteristics of RePLT in patients with cirrhosis have not been thoroughly evaluated. Kajirara et al. showed that cirrhosis is associated with an increased proportion of RePLT [11]. However, 63% of patients had hepatocellular carcinoma, which may significantly affect platelet pathophysiology [35,36]. In an independent study including mostly compensated patients (i.e., those will less severe platelet alterations) [5], Pradella et al. found a comparable number of RePLT between cirrhosis and healthy subjects [12]. However, they evaluated absolute values of RePLT, thus hampering comparison between cirrhosis with thrombocytopenia and healthy subjects. Finally, none of these studies assessed RePLT according to cirrhosis severity nor evaluated their contribution to hemostasis and thrombosis.

Our study shows, in a prospective cohort of patients with cirrhosis, that the proportion of RePLT and activated RePLT are significantly higher than in healthy subjects. RePLT were not correlated with the severity of liver dysfunction/portal hypertension, as assessed by Child-Pugh stage, nor with plasmatic levels of C-reactive protein and presepsin (i.e., markers of systemic inflammation and bacterial translocation). Patients with decompensated cirrhosis and bacterial infections had a significantly higher proportion of RePLT that, however, were less activated than RePLT in patients without infections and a comparable severity of chronic liver disease.

These findings may seem at odds with those previously described by Raparelli et al. [37], which demonstrated that circulating lipopolysaccharide may be responsible for platelet activation in patients with cirrhosis and portal hypertension. However, it should be highlighted that the assessment of platelet function in the seminal study by Raparelli et al. was based on light transmission aggregometry, which limits comparability with our results. Moreover, we used an indirect marker of bacterial translocation such as presepsin, whereas they assessed plasmatic levels of LPS and its direct effects on platelet activatability [37]. On the other hand, it

could be also that RePLT constitute a distinct platelet population with specific response to cirrhosis progression and disease complications [38]. In fact, in agreement with Raparelli et al. [37], we found a positive, strong correlation between non-reticulated, activated platelets and plasmatic levels of presepsin, which reflects severity of bacterial translocation in cirrhosis with portal hypertension [32]. Moreover, we found that the proportion of activated, non-reticulated platelets increased significantly with liver dysfunction severity (comparable between controls and cirrhosis in Child-Pugh A, and significantly higher in Child-Pugh B/C). Further studies dissecting alterations of platelets and their subpopulations may improve our understanding of cirrhotic coagulopathy and settle the long-lasting debate on platelet activation in cirrhosis [39].

Thrombopoietin is synthetized by the hepatocytes and its production is impaired in chronic liver disease without hepatocellular carcinoma [12]. In fact, we found that TPO plasmatic levels significantly decreased from Child-Pugh A to Child-Pugh C stage, indicating that the insufficient production of TPO contributes to the development of thrombocytopenia in cirrhosis. However, 61% of Child-Pugh A patients were thrombocytopenic despite a normal level of TPO. Therefore, it could be that the pathogenesis of thrombocytopenia in cirrhosis partly varies according to disease stage with a relatively more important contribution for platelet sequestration in compensated patients with portal hypertension, and a combination of both reduced TPO and platelet sequestration in decompensated disease. Interestingly, the proportion of RePLT was not correlated with platelet count or plasmatic levels of TPO, suggesting that the dynamics of RePLT are relatively independent of central platelet production and peripheral sequestration/destruction.

Beyond hemostasis, recent studies suggest that alterations of platelets and von Willebrand factor may be used to improve prognostic stratification in cirrhosis [40]. Our group found that increased whole blood platelet aggregation – when adjusted for individual platelet count – is independently linked to the risk of portal vein thrombosis, further decompensation, and liver-related death [41,42]. Balcar et al. found no association between alteration of Platelet-Function-Analyzed 100 and cirrhosis progression in a retrospective cohort including patients undergoing HVPG [43]. In an overlapping cohort from the same group, Hofer et al. recently showed that a more pronounced platelet activation is linked to a lower risk of cirrhosis progression but a higher risk of portal vein thrombosis [44] though controversies remain due to different methodology used for assessment between different studies [45].

In patients without liver disease, RePLT have been independently associated with an increased risk of death and thrombosis [10,38,46]. We found that patients with decompensated cirrhosis and a higher frequency of circulating RePLT had a significantly higher short-term risk of progression towards further decompensation, ACLF, and liver-related death. Remarkably, patients with RePLT >2% had a 5-fold higher risk of composite outcome (AUROC 0.818; sensitivity 94%, specificity 73%). It should be highlighted that the number of patients and clinical events was low; therefore, we could not perform a comprehensive multivariate analysis including other potential predictors such as MELD score and C-reactive protein [47]. However, the proportion of RePLT was not correlated with any of these parameters. Based on our exploratory results, we suggest that larger studies should be performed to evaluate whether RePLT could become an independent prognostic biomarker to guide patient management [48].

Our study has significant limitations. Firstly, the sample size was calculated for primary outcome, that is comparison of RePLT between cirrhosis and healthy subjects. Therefore, our study was not powered to detect meaningful association between RePLT and clinical outcomes. Larger, prospective cohorts evaluating the association between RePLT and clinical outcomes in decompensated cirrhosis are required to validate our preliminary findings. These studies should include a comprehensive, multivariate model adjusting the association between RePLT and further decompensation/ACLF by severity of chronic liver disease and portal hypertension, systemic inflammation, and presence of disease complications that can lead per se' to ACLF (i.e., bacterial infections). Furthermore, a larger cohort would allow to evaluate whether there is a dose-effect association between increasing RePLT proportion and liver-related events. This notwithstanding, this is the first study thoroughly evaluating characteristics of RePLT in a well-selected, consecutive cohort of patients with cirrhosis of different stages, improving our understanding of platelet complexity in patients with cirrhosis. Moreover, platelets and TPO were evaluated once, at recruitment. It may be that a prospective assessment of RePLT could be more informative regarding patient's trajectories (ACLF vs. unstable decompensated vs. stable decompensated cirrhosis). Secondly, acridine orange bind ssDNA and RNA in a concentration-dependent manner, which depends on the chosen fluorescence emission channel. Therefore, further studies comparing the RNA content based on different staining including Syto-13 and thiazole orange are required to confirm our results [49]. Thirdly, we included mostly patients with alcohol and viral-related liver disease [5]. Whether these findings can be applied to patients with different etiologies of liver disease, such as MASH, is unknown. Finally, as in any study of hemostasis, drugs could have interfered with platelets, but this effect should have been mitigated by the exclusion of important confounders such as ACLF, hepatocellular carcinoma, and thrombosis.

In conclusion, we demonstrate that reticulated platelets are significantly increased and hyper-activated in patients with cirrhosis, independently of disease etiology and severity. In the subgroup of decompensated patients, we found that a proportion of RePLT >2% appeared to predict development of further decompensation and ACLF. Larger cohorts are now required to validate our findings and clarify whether the assessment of RePLT may become an additional biomarker to improve clinical management in patients with cirrhosis.

Financial support

Research grant from the Italian Ministry of Education, University and Research to PS. The funders had no role in study design, data collection and analysis, or preparation of the manuscript.

Authors' contribution

AZ: research design, performance of the research (patients' enrollment and follow-up), statistical analysis, interpretation of the data, writing of the manuscript.

ST: laboratory work.

EC: laboratory work, interpretation of the data, critical revision of the manuscript.

CMR: laboratory work.

SG: laboratory work.

PB: acquisition of the data.

FPR: acquisition of the data.

MS: acquisition of the data, critical revision of the manuscript.

PS: research design, funding of the research, organization of lab facilities and testing, interpretation of the data, critical revision, and final approval of the manuscript.

Trial registration number

NA

Data availability statement

Data are available from the first author (Dr. Alberto Zanetto: alberto.zanetto@unipd.it) upon reasonable request.

Conflict of interest

None declared.

Acknowledgments

The authors wish to thank the nurses of Gastroenterology and Thrombotic and Hemorrhagic Diseases Unit of Padova University Hospital for their assistance with collection of blood samples.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.dld.2024.03.007.

References

[1] Hoffmann JJ. Reticulated platelets: analytical aspects and clinical utility. Clin Chem Lab Med 2014;52(8):1107–17.
[2] Bongiovanni D, Han J, Klug M, Kirmes K, Viggiani G, von Scheidt M, et al. Role of reticulated platelets in cardiovascular disease. Arterioscler Thromb Vasc Biol 2022;42(5):527–39.
[3] Buttarello M, Mezzapelle G, Freguglia F, Plebani M. Reticulated platelets and immature platelet fraction: clinical applications and method limitations. Int J Lab Hematol 2020;42(4):363–70.
[4] Hille L, Cederqvist M, Hromek J, Stratz C, Trenk D, Nuhrenberg TG. Evaluation of an alternative staining method using syto 13 to determine reticulated platelets. Thromb Haemost. 2019;119(5):779–85.
[5] Zanetto A, Campello E, Senzolo M, Simioni P. The evolving knowledge on primary hemostasis in patients with cirrhosis: a comprehensive review. Hepatology 2023.
[6] Mandorfer M, Schwabl P, Paternostro R, Pomej K, Bauer D, Thaler J, et al. Von willebrand factor indicates bacterial translocation, inflammation, and procoagulant imbalance and predicts complications independently of portal hypertension severity. Aliment Pharmacol Ther 2018;47(7):980–8.
[7] Zanetto A, Pelizzaro F, Campello E, Bulato C, Balcar L, Gu W, et al. Severity of systemic inflammation is the main predictor of ACLF and bleeding in individuals with acutely decompensated cirrhosis. J Hepatol 2023;78(2):301–11.
[8] Dusse LM, Freitas LG. Clinical applicability of reticulated platelets. Clin Chim Acta 2015;439:143–7.
[9] Rauber P, Lammert F, Grotemeyer K, Appenrodt B. Immature platelet fraction and thrombopoietin in patients with liver cirrhosis: a cohort study. PLoS ONE 2018;13(2):e0192271.
[10] Bongiovanni D, Novelli L, Condello F, Kirmes K, Han J, Wein B, et al. Reticulated platelets predict cardiovascular death and adverse events in coronary artery disease: a systematic review and meta-analysis. Thromb Haemost 2023.
[11] Kajihara M, Okazaki Y, Kato S, Ishii H, Kawakami Y, Ikeda Y, Kuwana M. Evaluation of platelet kinetics in patients with liver cirrhosis: similarity to idiopathic thrombocytopenic purpura. J Gastroenterol Hepatol 2007;22(1):112–18.
[12] Pradella P, Bonetto S, Turchetto S, Uxa L, Comar C, Zorat F, et al. Platelet production and destruction in liver cirrhosis. J Hepatol 2011;54(5):894–900.
[13] Allan HE, Vadgama A, Armstrong PC, Warner TD. Platelet ageing: a review. Thromb Res 2023;231:214–22.
[14] Smyth SS, McEver RP, Weyrich AS, Morrell CN, Hoffman MR, Arepally GM, et al. Platelet functions beyond hemostasis. J Thromb Haemost 2009;7(11):1759–66.
[15] Kopec AK, Joshi N, Luyendyk JP. Role of hemostatic factors in hepatic injury and disease: animal models de-liver. J Thromb Haemost 2016;14(7):1337–49.
[16] Lisman T, Luyendyk JP. Platelets as modulators of liver diseases. Semin Thromb Hemost 2018;44(2):114–25.
[17] Simon TG, Duberg AS, Aleman S, Chung RT, Chan AT, Ludvigsson JF. Association of aspirin with hepatocellular carcinoma and liver-related mortality. N Engl J Med 2020;382(11):1018–28.
[18] Zanetto A, Campello E, Bulato C, Willems R, Konings J, Roest M, et al. Whole blood thrombin generation shows a significant hypocoagulable state in patients with decompensated cirrhosis. J Thromb Haemost 2023.
[19] Zanetto A, Northup P, Roberts L, Senzolo M. Haemostasis in cirrhosis: understanding destabilising factors during acute decompensation. J Hepatol 2023;78(5):1037–47.
[20] Zanetto A, Simioni P. PFA-100 in advanced chronic liver disease: end of the story? JHEP Rep 2024;6(1):100967.
[21] de Franchis R, Bosch J, Garcia-Tsao G, Reiberger T, Ripoll C, Baveno VIIF. Baveno vii - Renewing consensus in portal hypertension. J Hepatol 2022;76(4):959–74.
[22] Zanetto A, Shalaby S, Gambato M, Germani G, Senzolo M, Bizzaro D, et al. New indications for liver transplantation. J Clin Med 2021;10(17).
[23] Campello E, Zanetto A, Bulato C, Maggiolo S, Spiezia L, Russo FP, et al. Coagulopathy is not predictive of bleeding in patients with acute decompensation of cirrhosis and acute-on-chronic liver failure. Liver Int 2021;41(10):2455–66.
[24] Lisman T, Arefaine B, Adelmeijer J, Zamalloa A, Corcoran E, Smith JG, et al. Global hemostatic status in patients with acute-on-chronic liver failure and septics without underlying liver disease. J Thromb Haemost 2021;19(1):85–95.
[25] Zanetto A, Rinder HM, Campello E, Saggiorato G, Deng Y, Ciarleglio M, et al. Acute kidney injury in decompensated cirrhosis is associated with both hypo–coagulable and hyper-coagulable features. Hepatology 2020;72(4):1327–40.
[26] Hvas AM, Favaloro EJ. Platelet function analyzed by light transmission aggregometry. Methods Mol Biol 2017;1646:321–31.
[27] Kim HR, Park BR, Lee MK, Park AJ, Ahn JY. [Comparison of an immature platelet fraction and reticulated platelet in liver cirrhosis]. Korean J Lab Med 2007;27(1):7–12.
[28] Bodrova VV, Shustova ON, Khaspekova SG, Mazurov AV. Laboratory markers of platelet production and turnover. Biochemistry (Mosc) 2023;88(Suppl 1):S39–51.
[29] Harrison P, Robinson MS, Mackie IJ, Machin SJ. Reticulated platelets. Platelets 1997;8(6):379–83.
[30] Lador A, Leshem-Lev D, Spectre G, Abelow A, Kornowski R, Lev EI. Characterization of surface antigens of reticulated immature platelets. J Thromb Thrombolysis 2017;44(3):291–7.
[31] Khemichian S, Terrault NA. Thrombopoietin receptor agonists in patients with chronic liver disease. Semin Thromb Hemost 2020;46(6):682–92.
[32] Zanetto A, Pelizzaro F, Mion MM, Bucci M, Ferrarese A, Simioni P, et al. Toward a more precise prognostic stratification in acute decompensation of cirrhosis: the padua model 2.0. united. European Gastroenterol J 2023;11(9):815–24.
[33] European Association for the Study of the Liver. Electronic address eee, European Association for the Study of the LEASL clinical practice guidelines on prevention and management of bleeding and thrombosis in patients with cirrhosis. J Hepatol 2022;76(5):1151–84.
[34] Campello E, Zanetto A, Radu CM, Bulato C, Truma A, Spiezia L, et al. Acute kidney injury is associated with increased levels of circulating microvesicles in patients with decompensated cirrhosis. Dig Liver Dis 2021;53(7):879–88.
[35] Zanetto A, Campello E, Bulato C, Gavasso S, Saggiorato G, Shalaby S, et al. More pronounced hypercoagulable state and hypofibrinolysis in patients with cirrhosis with versus without hcc. Hepatol Commun 2021;5(12):1987–2000.
[36] Zanetto A, Senzolo M, Campello E, Bulato C, Gavasso S, Shalaby S, et al. Influence of hepatocellular carcinoma on platelet aggregation in cirrhosis. Cancers (Basel) 2021;13(5).
[37] Raparelli V, Basili S, Carnevale R, Napoleone L, Del Ben M, Nocella C, et al. Low-grade endotoxemia and platelet activation in cirrhosis. Hepatology 2017;65(2):571–81.
[38] Corpataux N, Franke K, Kille A, Valina CM, Neumann FJ, Nuhrenberg T, Hochholzer W. Reticulated platelets in medicine: current evidence and further perspectives. J Clin Med. 2020;9(11).
[39] Lisman T, Caldwell SH, Intagliata NM. Haemostatic alterations and management of haemostasis in patients with cirrhosis. J Hepatol 2022;76(6):1291–305.
[40] Semmler G, Binter T, Kozbial K, Schwabl P, Hametner-Schreil S, Zanetto A, et al. Noninvasive risk stratification after hcv eradication in patients with advanced chronic liver disease. Hepatology 2021;73(4):1275–89.
[41] Zanetto A, Campello E, Bulato C, Gavasso S, Farinati F, Russo FP, et al. Increased platelet aggregation in patients with decompensated cirrhosis indicates higher risk of further decompensation and death. J Hepatol 2022;77(3):660–9.
[42] Zanetto A, Campello E, Burra P, Senzolo M, Simioni P. Increased platelet ratio in patients with decompensated cirrhosis indicates a higher risk of portal vein thrombosis. Liver Int 2023;43(1):155–9.
[43] Balcar L, Simbrunner B, Paternostro R, Jachs M, Hartl L, Semmler G, et al. Platelet adhesion assessed by PFA-100 is not linked to progression of acld. JHEP Rep 2024;6(1):100934.
[44] Hofer BS, Brusilovskaya K, Simbrunner B, Balcar L, Eichelberger B, Lee S, et al. Decreased platelet activation predicts hepatic decompensation and mortality in patients with cirrhosis. Hepatology 2023.
[45] Zanetto A, Campello E, Senzolo M, Simioni P. Letter to the editor: are we comparing apples with oranges? Hepatology 2024.
[46] Schofield H, Rossetto A, Armstrong PC, Allan HE, Warner TD, Brohi K, Vulliamy P. Immature platelet dynamics are associated with clinical outcomes after major trauma. J Thromb Haemost 2023.
[47] Deltenre P, Zanetto A, Saltini D, Moreno C, Schepis F. The role of transjugular intrahepatic portosystemic shunt in patients with cirrhosis and ascites: recent evolution and open questions. Hepatology 2023;77(2):640–58.
[48] Giannini EG, Moscatelli A, Brunacci M, Zentilin P, Savarino V. Prognostic role of mean platelet volume in patients with cirrhosis. Dig Liver Dis 2016;48(4):409–13.
[49] Lee LG, Chen CH, Chiu LA. Thiazole orange: a new dye for reticulocyte analysis. Cytometry 1986;7(6):508–17.