Peripheral versus central venous blood sampling does not influence the assessment of platelet activation in cirrhosis

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Cirrhotic patients have an increased risk for bleeding and thromboembolic events, with platelets being involved as key players in both situations. The impact of peripheral versus central blood sampling on platelet activation remains unclear. In 33 cirrhotic patients, we thus analyzed platelet function in peripheral (P) and central (C) blood samples. Platelet surface expression of P-selectin, activated glycoprotein (GP)IIb/IIIa, and leukocyte-platelet aggregate formation were measured by flow cytometry in response to different agonists: thrombin receptor-activating peptide-6, adenosine diphosphate, collagen-related peptide (CrP), epinephrine, AYPGKF, Pam3CSK4, and lipopolysaccharide. Unstimulated platelet surface expression of P-selectin (p=0.850) and activated GPIIb/IIIa (p=0.625) were similar in peripheral and central blood samples. Stimulation with various agonists yielded similar results of platelet surface expression of P-selectin and activated GPIIb/IIIa in peripheral and central samples, except for CrP-inducible expression of activated GPIIb/IIIa (median fluorescence intensity, MFI in P: 7.61 [0.00-24.66] vs. C: 4.12 [0.00-19.04], p=0.001). The formation of leukocyte-platelet aggregate was similar in central and peripheral blood samples, both unstimulated and after stimulation with all above-mentioned agonists. In conclusion, peripheral vs. central venous blood sampling does not influence the assessment of platelet activation by flow cytometry in cirrhosis.

Keywords: liver disease, blood sampling, flow cytometry, platelet activation, platelet function analysis

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Abbreviations

ACLD advanced chronic liver disease

ADP adenosine diphosphate

ALD alcoholic liver disease

AYPGKF PAR-4 agonist AYPGKF

CrP collagen related protein

EPI epinephrine

FACS fluorescence-activated cell sorting

GP glycoprotein

HVPG hepatic venous pressure gradient

IQR interquartile range

LPS lipopolysaccharide

LSM liver stiffness measurement

MFI median fluorescence intensity

NAFLD non-alcoholic fatty liver disease

PAM lipopeptide Pam3CSK4

PAR protease-activated receptor

PBS phosphate-buffered saline

PH portal hypertension

TLR toll-like receptor

TRAP-6 thrombin receptor activator peptide 6

vWF von Willebrand factor

Introduction

Advanced chronic liver disease (ACLD) and portal hypertension (PH) cause significant morbidity and mortality worldwide.[1, 2] Besides impairing the synthesis of clotting factors, ACLD causes considerable abnormalities in platelet count and function.[3, 4] Moreover, patients with cirrhosis do not only show an increased incidence of bleeding but are also prone to develop thrombosis.[5-7]

Numerous studies revealed a considerable heterogeneity of platelet activation and reactivity in liver cirrhosis in the last decades.[8-11] While some studies described increased platelet reactivity as assessed by platelet surface expression of P-selectin and activated glycoprotein (GP)IIb/IIIa, platelet aggregation, and levels of von Willebrand factor (vWF),[11-13] others found decreased markers of platelet activation in patients with liver cirrhosis.[8, 14-16] The observed discrepancies may to some extent be due to differences in the studied patient populations and the used test systems. In detail, thrombocytopenia frequently impairs the results and validity of platelet aggregation tests in liver cirrhosis. In addition, pre-analytical variables, i.e., in particular differences in blood sampling, might play a role.[17, 18]

Flow cytometry is a well-established, sensitive, and reliable technique to evaluate platelet activation in a minimal amount of whole blood, largely independent of platelet count, and therefore ideal for the assessment of platelet function in ACLD/PH, which is often accompanied by thrombocytopenia.[19-21]

Measurement of the hepatic venous pressure gradient (HVPG) is the gold-standard technique to determine portal venous pressure and the severity of portal hypertension in patients with liver cirrhosis.[22] During HVPG measurement or transjugular intrahepatic portosystemic stent shunt (TIPS) placement,[22, 23] central venous blood samples can easily be collected in order to avoid the need for blood sampling from the cubital vein.[24] However, little is known about the effects of central vs. peripheral blood sampling on platelet activation in cirrhosis. One may speculate that differences in shear stress, which may be greater in peripheral blood withdrawal, [18, 25, 26] on the one hand, and platelet activation due to HVPG measurement or TIPS placement, which may be more present in the central veins, on the other hand, could influence the results obtained by flow cytometry.[26, 27] Finally, local platelet activation in the portal and hepatic veins in cirrhosis might be differently reflected in central and peripheral venous blood samples. In order to investigate if central and peripheral venous blood

sampling can be used interchangeably for the assessment of platelet activation in ACLD, we compared platelet activation as assessed by whole blood flow cytometry between central and peripheral venous blood samples from patients with cirrhosis.

Patients and methods

Study design and patient population

We screened adult patients with ACLD of known etiology and central venous access for eligibility in this study. ACLD was confirmed by either PH (i.e., HVPG of ≥ 6mmHg) or advanced fibrosis on transient elastography (i.e., liver stiffness, liver stiffness measure, LSM ≥ 10kPa). We excluded patients receiving antiplatelet drugs (n=3) or anticoagulants (vitamin K antagonists, direct oral anticoagulants, or low molecular weight heparin; n=1), and patients with hepatic or extrahepatic malignancies (n=7), previous liver transplantation (n=1), portal vein thrombosis (n=2), and acute decompensation/non-elective hospitalization (n=2). The final population comprised 33 patients with simultaneous venous blood sampling from cubital and internal jugular veins.

Sampling and processing

Two simultaneous blood samples were drawn from the cubital vein and the right internal jugular vein at the end of the HVPG procedure[22] into tubes containing 3.2% sodium citrate (#454332, Greiner Bio-one, Austria). Both samples were processed in parallel at room temperature within 30 minutes, and flow cytometry was performed within 1 hour. All pairs of samples were drawn by the same physician and analyzed by the same operator to minimize procedural deviations. All blood samples were kept at room temperature. Blood cell counts were determined by a Coulter counter analyzer (Beckman Coulter, Netherlands).

Platelet surface expression of P-selectin and activated glycoprotein (GP)IIb/IIIa

Platelet surface expression of P-selectin and binding of the monoclonal antibody PAC-1 to activated GPIIb/IIIa were determined in citrate-anticoagulated blood, as previously described.[28, 29] In brief, whole blood was diluted in phosphate-buffered saline to obtain $20x10^3$/µL platelets in 20µL and incubated for 10 minutes with the platelet-specific monoclonal antibody anti-CD42b (clone HIP1, allophycocyanin labeled; Becton Dickinson, USA). After in vitro exposure to suboptimal concentrations of thrombin receptor-activating peptide (TRAP)-6 (final concentration: 14.24µM, Bachem, Switzerland), the protease-activated receptor (PAR)-4 agonist AYPGKF (final concentration: 714µM, Roche Diagnostics GmbH, Germany), adenosine diphosphate (ADP; final concentration: 1µM, Roche Diagnostics GmbH, Germany), collagen-related peptide (CrP; final concentration: 0.04µg/mL, Department of Biochemistry, University of Cambridge, United Kingdom), epinephrine (EPI, final concentration: 10µM, MöLab, Germany), the toll-like receptor (TLR)-1/2 agonist Pam3CSK4 (final concentration: 8.9µg/mL; InvivoGen, USA), and the TLR-4 agonist lipopolysaccharide (LPS; final concentration: 1429µg/mL, InvivoGen, USA) each 10µL for 10 min in the dark, samples were incubated with a mixture of antibodies against P-selectin (anti-CD62p-phycoerythrin, clone CLB-Thromb6; Immuno-tech, Beckman Coulter, USA) and activated GPIIb/IIIa (monoclonal antibody PAC-1-fluorescein, Becton Dickinson, USA) for another 15 min. The concentrations of all agonists were determined in previous titration experiments with increasing dosages of each agonist in 10 healthy controls. The selected agonist concentrations induced about 60–70% of the maximally achievable increase in median fluorescence intensity (MFI) in healthy controls. Isotype-matched control antibodies (Becton Dickinson, USA) were used for the determination of non-specific binding. After 15 min of incubation in the dark, the reaction was stopped by adding 500µL PBS, and samples were immediately acquired on a FACS Canto II flow cytometer (Becton Dickinson, USA). The platelet population was identified by characteristics in the forward scatter versus side scatter plot. A total of 10,000 events were acquired within this gate. This population was further identified by platelets stained with the platelet-specific monoclonal antibody anti-CD42b versus side scatter. The binding of the antibodies against P-selectin and activated GPIIb/IIIa was determined in histograms for P-selectin and PAC-1, respectively. Cytometer setup and tracking beads (Becton Dickinson, USA), which consist of FITC, PE, PERCP-CY5.5, PE-CY7, APC, APC-H7, V450, and V500-C labeled beads, were used for daily

calibration of the cytometer applying the Diva software. The MFI based on all events was used for statistical calculations.

Determination of leukocyte-platelet aggregates

Monocyte-, neutrophil-, and lymphocyte-platelet aggregate formation were determined as previously described.[30, 31] In brief, suboptimal concentrations of the following agonists (each 10µL) were added to 5µL of citrate-anticoagulated blood diluted in 55µL of PBS: TRAP-6 (final concentration: 7.1µM, Bachem, Switzerland), AYPGKF (final concentration: 357µM, Roche Diagnostics GmbH, Germany), ADP (final concentration: 2µM, Roche Diagnostics GmbH, Germany), CrP (final concentration: 0.04µg/mL, Department of Biochemistry, University of Cambridge, United Kingdom), EPI (final concentration: 9.82µM, MöLab, Germany), Pam3CSK4 (final concentration: 4.17µg/mL, InvivoGen, USA), LPS (final concentration: 667µg/mL, InvivoGen, USA). After 10 min incubation, the monoclonal antibodies anti-CD14-allophycocyanin (clone MφP9, #345787, Becton Dickinson, USA), anti-CD45-peridinin chlorophyll protein (clone 2D1, #340665, Becton Dickinson, USA), and anti-CD41-phycoerythrin (clone P2; #1416, Immuno-tech, USA) or isotype-matched controls were added. The reaction was stopped after 15 minutes with 500µL FACS™ Lysing solution (#34920, Becton Dickinson, USA). Samples were acquired immediately on the FACS Canto II flow cytometer, and at least 10.000 CD45+ events were gated. Monocytes, neutrophils, and lymphocytes were identified within these events based on their CD14 versus side scatter characteristics. All the samples were analyzed within 30 minutes from blood withdrawal.

Statistical analysis

The GraphPad Prism version 9.1.1 (GraphPad Software, USA) was used for statistical analysis. Continuous variables are reported as median [interquartile range, IQR], dichotomous variables are reported as absolute (n) and relative (%) frequencies. The distribution of differences between peripheral and central venous values was analyzed using the Shapiro-Wilk test. Peripheral and central blood samples were compared using paired Student's t-test or Wilcoxon test, as appropriate. While the study setup included a paired assessment of peripheral vs. central samples, numerous tests with and without platelet agonists were performed. Thus, correction for multiple testing had to be applied.

Accordingly, the defined level of statistical significance at $p=0.05$ had to be adjusted for the number of respective tests by Bonferroni correction.

We tested each sample pair for platelet surface expression of P-selectin and GPIIb/IIIa activation (2 readouts) in an unstimulated condition and 7 agonist-stimulated conditions (i.e., in total 8 conditions). Thus, the final p-value for statistical significance of differences in platelet surface P-selectin and activated GPIIb/IIIa between peripheral and central venous blood samples was adjusted for 16 (2 x 8 conditions) multiple tests by Bonferroni correction and set at $p<0.003$.

Monocyte-, neutrophil- and lymphocyte-platelet aggregate formation (3 readouts) were assessed in an unstimulated condition and 7 agonist-stimulated conditions (i.e., in total 8 conditions). Thus, the threshold for statistical significance regarding differences in leukocyte-platelet aggregate formation between peripheral and central samples was adjusted for 24 (3 x 8 conditions) multiple testing using Bonferroni correction and set at $p<0.002$.

Ethics

All patients consented to peripheral and central venous blood withdrawal and gave written informed consent for study participation. The study protocol was approved by the Ethics Committee of the Medical University of Vienna (2317/2019) in accordance with the declaration of Helsinki and its later amendments.

Results

Overall, 33 patients were included in this study. Clinical and laboratory characteristics of the patient population are summarized in **Table-1.** Twenty-two patients were male (67%), with a median age of 54 [46-60] years. Underlying etiologies of liver disease were alcoholic liver disease (ALD; n=17, 52%), viral hepatitis (VIRAL; n=6, 18%), non-alcoholic fatty liver disease (NAFLD; n=3, 9%), cholestatic liver disease (CHOL; n=2, 6%), alcoholic liver disease with viral hepatitis (ALD & VIRAL; n=2, 6%), and other causes of liver disease (OTH; n=3, 9%). The median platelet count was 87 [67-110] G/L.

There were no significant differences in unstimulated platelet surface expression of P-selectin between peripheral and central venous blood samples (P: 0 [0.00-1.96] MFI vs. C: 0 [0.00-1.35] MFI, p=0.850). Moreover, agonist-inducible P-selectin expression did not differ significantly between central and peripheral venous blood samples. (**Figure-1A**, **Supplementary table-1**)

Platelet surface expression of activated GPIIb/IIIa without agonists was comparable between peripheral and central venous blood samples (P: 0 [0.00—0.00] MFI vs. C: 0 [0.00-0.00] MFI, p=0.625). Of all agonists, only CrP resulted in a significantly higher platelet surface expression of activated GPIIb/IIIa in peripheral compared to central venous blood samples (P: 7.61 [0.00-24.66] MFI vs. C: 4.12 [0.00-19.04] MFI, p=0.001). (**Figure-1B, Supplementary table-1)**

Monocyte-platelet aggregate formation in peripheral and central venous blood samples were similar without stimulation (P: 28.52 [18.16-37.36]% vs. C: 25.91 [16.64-38.49]%, p=0.916). Likewise, monocyte-platelet aggregate formation in peripheral and central blood samples did not differ significantly after stimulation with agonists. **(Figure-3A, Supplementary table-2)**

Furthermore, no significant differences of neutrophil-platelet aggregate formation were observed between peripheral and central blood samples without stimulation (P: 7.14 [4.02-11.96] % vs. C: 6.21 [4.41-9.72] %, p=0.414) and after the addition of platelet agonists. **(Figure-3B, Supplementary table-2)**

Finally, the formation of lymphocyte-platelet aggregate was similar in peripheral and central blood samples without (P: 11.48 [6.44-14.69]% vs. C: 9.48 [7.55-13.92]%, p=0.499) and with agonists. **(Figure-3C, Supplementary table-2)**

Discussion

Our study is the first to assess the impact of peripheral vs. central venous blood sampling on the assessment of platelet activation by flow cytometry in chronic liver disease. Importantly, we did not detect statistically significant differences in unstimulated platelet surface expression of P-selectin and activated GPIIb/IIIa between peripheral and central venous blood samples. Furthermore, only CrP resulted in a significantly higher platelet surface expression of activated GPIIb/IIIa in peripheral compared to central venous blood samples, while the addition of all other agonists resulted in comparable levels of platelet surface P-selectin and activated GPIIb/IIIa in peripheral and central venous blood samples. Finally, leukocyte-platelet aggregate formation without and with platelet agonists was similar following peripheral and central venous blood sampling.

Flow cytometry is a well-established technique that is commonly used for the characterization of platelet function.[19, 32] In contrast to light transmission aggregometry and impedance aggregometry, [33, 34] flow cytometric parameters of platelet activation are largely independent of platelet count and allow the assessment of platelet function even in thrombocytopenic patients. This is a major advantage, particularly for patients with ACLD, who frequently have low platelet counts.[20, 21] Previous studies investigated the impact of pre-analytical variables on platelet activation in order to minimize sample pre-activation and standardize protocols for sample preparation.[32, 35-37]

An adequate blood sampling technique and site remain of great importance for the reliability and comparability of results. Consequently, established protocols suggest sampling from a large peripheral vein to reduce artificial platelet activation through shear stress. [35, 37, 38] Moreover, previous publications on the standardization of blood sampling for platelet function testing recommended that blood should be drawn using a straight 19–21-gauge needle or butterfly cannula with a smooth lumen to avoid excessive platelet activation due to induced shear stress.[18, 25, 26] Additionally, they stated that the use of central venous catheters is not contraindicated but might increase artificial platelet activation and hemolysis.[26] In particular, blood withdrawal through long catheters may result in artificial platelet activation due to the local vessel trauma caused by catheter insertion and placement or the contact with thrombogenic foreign surfaces.[26, 27] On the other hand, Lance et al. compared platelet function in samples drawn from the arterial line, venous line, central venous line, and peripheral

venipuncture, using multiple electrode aggregometry and the platelet function analyzer-100, and found no significant differences in platelet function between different sampling sites and techniques.[39] However, the impact of central vs. peripheral venous blood withdrawal on platelet activation as assessed by flow cytometry is unknown. Since flow cytometry is less dependent on platelet count than aggregometry, it is supposed to provide the most valid assessment of platelet activation in ACLD, which is often accompanied by thrombocytopenia. Furthermore, for the assessment of platelet activation in patients with chronic liver disease, it would be advantageous to draw blood from central venous access if already established, e.g., for HVPG measurement, TIPS placement, or other reasons, and to avoid secondary sampling from a peripheral vein and the associated discomfort. Accordingly, we designed the current study to fill the above-described knowledge gap and investigate if central and peripheral venous blood sampling can be used interchangeably when assessing platelet function by flow cytometry in cirrhosis.

P-selectin is stored in the α-granules of resting platelets.[40] Platelet activation results in rapid translocation of P-selectin to the cell surface, where it acts as a receptor for P-selectin glycoprotein ligand-1 on leukocytes and facilitates the formation of leukocyte-platelet aggregates.[41] Upon platelet activation, GPIIb/IIIa undergoes conformational changes and is subsequently expressed on the platelet surface in its activated form as a high-affinity receptor for fibrinogen. Thereby, activated GPIIb/IIIa enables the interaction of platelets with other platelets and blood cells, resulting in platelet aggregation.[40, 42] Accordingly, both platelet surface P-selectin and activated GPIIb/IIIa, are sensitive flow cytometric markers of platelet activation.[32, 43] The aforementioned leukocyte-platelet aggregates have been shown to be elevated in several pathophysiological circumstances, including myocardial infarction,[44] and may represent an even more accurate marker of continuously ongoing platelet activation than P-selectin.[45] The latter may be due to their higher stability over time compared to platelet surface P-selectin and activated GPIIb/IIIa, which rather reflect a snapshot of platelet activation at the time of blood sampling.[41, 45, 46]

In order to comprehensively study platelet function by flow cytometry, we assessed all parameters of platelet activation without agonists and after the addition of TRAP-6, ADP, CrP, epinephrine, AYPGKF, Pam3CSK4, and LPS. The various agonists interact with specific receptors on the platelet surface, triggering downstream signaling that initiates platelet activation and aggregation.[40, 47] TRAP-6 and AYPGKF

bind to PAR-1 and PAR-4, respectively, thereby mimicking thrombin-inducible platelet activation. Recently, we have shown that PAR-1 mediated platelet activation is a strong predictor of adverse ischemic outcomes in patients undergoing infrainguinal angioplasty and stenting for peripheral artery disease.[43] Meanwhile, PAR-1 can be specifically targeted by the thrombin receptor antagonist vorapaxar. However, since the addition of vorapaxar to standard antiplatelet therapy resulted in a significantly increased risk of major bleeding, including intracranial hemorrhage,[48, 49] vorapaxar is still not routinely prescribed for platelet inhibition in atherosclerotic cardiovascular disease. ADP acts mainly via the $P2Y_1$ and $P2Y_{12}$ receptors on human platelets. Inhibition of $P2Y_{12}$ mediated platelet activation is an important component of current state-of-the-art dual antiplatelet therapy following acute coronary syndromes as well as percutaneous coronary and peripheral interventions with stent implantation. CrP and epinephrine were chosen as agonists to provide insight into physiological platelet activation by collagen and epinephrine. Finally, Pam3CSK4 and LPS were used to induce platelet activation via TLR.[29] By using various platelet agonists targeting different receptors, we evaluated a broad range of potential differences between peripheral and central venous blood samples. Interestingly, the only identified difference was a higher platelet surface expression of activated GPIIb/IIIa in response to CrP in peripheral compared to central venous blood samples.

Previously, Rubens et al. investigated the influence of arterial and venous (peripheral and central) blood sampling on platelet activation evaluated by flow cytometry in patients without liver disease.[35] Their results suggest increased platelet activation in arterial blood samples. However, in line with our findings, no significant differences were observed in platelet activation between peripheral and central venous blood samples.[35] Another study also suggests a similar level of platelet activation and reactivity in peripheral and central venous blood samples.[37] Moreover, in this study, arterial blood samples have shown comparable levels of platelet activation.[37]

Although data comparing platelet activation between blood samples obtained from different sampling sites in the specific setting or cirrhosis are not available, our results are consistent with previous observations in subjects without liver disease. Thus, our findings extend the knowledge about platelet activation in peripheral and central venous blood samples in patients with liver cirrhosis, which was not studied previously.

Our study has some limitations: First, the relatively small sample size might limit the statistical power of the analysis, and a type 2 error due to applied Bonferroni

correction may occur. However, the high number of performed tests requires correction for multiple testing as applied. Second, the observed statistical difference in GPIIb/IIIa activation in response to CrP between peripheral and central blood samples was not reflected by activation of P-selectin or leucocyte-platelet aggregate formation and thus, requires evaluation by further studies.

In conclusion, peripheral vs. central venous blood sampling does not significantly influence the assessment of platelet activation by flow cytometry in cirrhosis. Thus, both peripheral and central venous blood samples can be used to measure platelet activation by flow cytometry in chronic liver disease.

Author contributions

Data collection: KB, BS, BE, DB, PS, MM, TR, TG

Statistical analysis: KB, MM, TR, TG

Drafting of the manuscript: KB, TR, TG

Study supervision: TR, TG

Revision for important intellectual content and approval of the final version of the manuscript: All authors.

Declaration of Interest statement

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Table legends

Table-1. Patient characteristics.

Continuous data are shown as median [IQR; interquartile range]. Hepatic venous pressure gradient (HVPG); alcoholic liver disease (ALD); non-alcoholic liver disease (NAFLD); viral hepatitis (VIRAL); cholestatic liver disease (CHOL); alcoholic liver disease with viral hepatitis (ALD & VIRAL); other causes of liver disease (OTH).

Figure legends

Figure-1A. Platelet surface expression of P-selectin in response to thrombin receptor activator peptide (TRAP)-6, adenosine diphosphate (ADP), collagen- related peptide (CrP), epinephrine (EPI), PAR-4 agonist AYPGKF (AYPGKF), Pam3CSK4, and lipopolysaccharide (LPS) measured in peripheral (P) and central (C) venous blood samples. Results are shown as median fluorescence intensity (MFI). The significance level after Bonferroni correction for a total of 16 multiple tests was set at $p<0.003$.

Figure-1B. Platelet surface expression of activated glycoprotein (GP)IIb/IIIa in response to thrombin receptor activator peptide (TRAP)-6, adenosine diphosphate (ADP), collagen-related peptide (CrP), epinephrine (EPI), the PAR-4 agonist AYPGKF (AYPGKF), Pam3CSK4 and lipopolysaccharide (LPS) measured in peripheral (P) and central (C) venous blood samples. CrP-inducible activated GPIIb/IIIa was significantly higher in peripheral blood samples as compared to central samples ($p=0.001$). Results are shown as median fluorescence intensity (MFI). The significance level after Bonferroni correction for a total of 16 multiple tests was set at $p<0.003$.

Figure-2A. Monocyte-platelet aggregate formation in response to thrombin receptor activator peptide (TRAP)-6, adenosine diphosphate (ADP), collagen-related peptide (CrP), epinephrine (EPI), the PAR-4 agonist AYPGKF, Pam3CSK4, and lipopolysaccharide (LPS) measured in peripheral (P) and central (C) venous blood samples. Results are shown as a percentage of monocyte-platelet aggregates. The significance level after Bonferroni correction for a total of 24 multiple tests was set at $p<0.002$.

Figure-2B. Neutrophil-platelets aggregate formation in response to thrombin receptor activator peptide (TRAP)-6, adenosine diphosphate (ADP), collagen-related peptide (CrP), epinephrine (EPI), the PAR-4 agonist AYPGKF, Pam3CSK4, and lipopolysaccharide (LPS) measured in peripheral (P) and central (C) venous blood samples. Results are shown as a percentage of neutrophil-platelet aggregates. The significance level after Bonferroni correction for a total of 24 multiple tests was set at $p<0.002$.

Figure-2C. Lymphocyte-platelet aggregate formation in response to thrombin receptor activator peptide (TRAP)-6, adenosine diphosphate (ADP), collagen-related peptide (CrP), epinephrine (EPI), the PAR-4 agonist AYPGKF, Pam3CSK4, and lipopolysaccharide (LPS) measured in peripheral (P) and central (C) venous blood

samples. Results are shown as a percentage of lymphocyte-platelet aggregates. The significance level after Bonferroni correction for a total of 24 multiple tests was set at $p<0.002$.

Figure 1

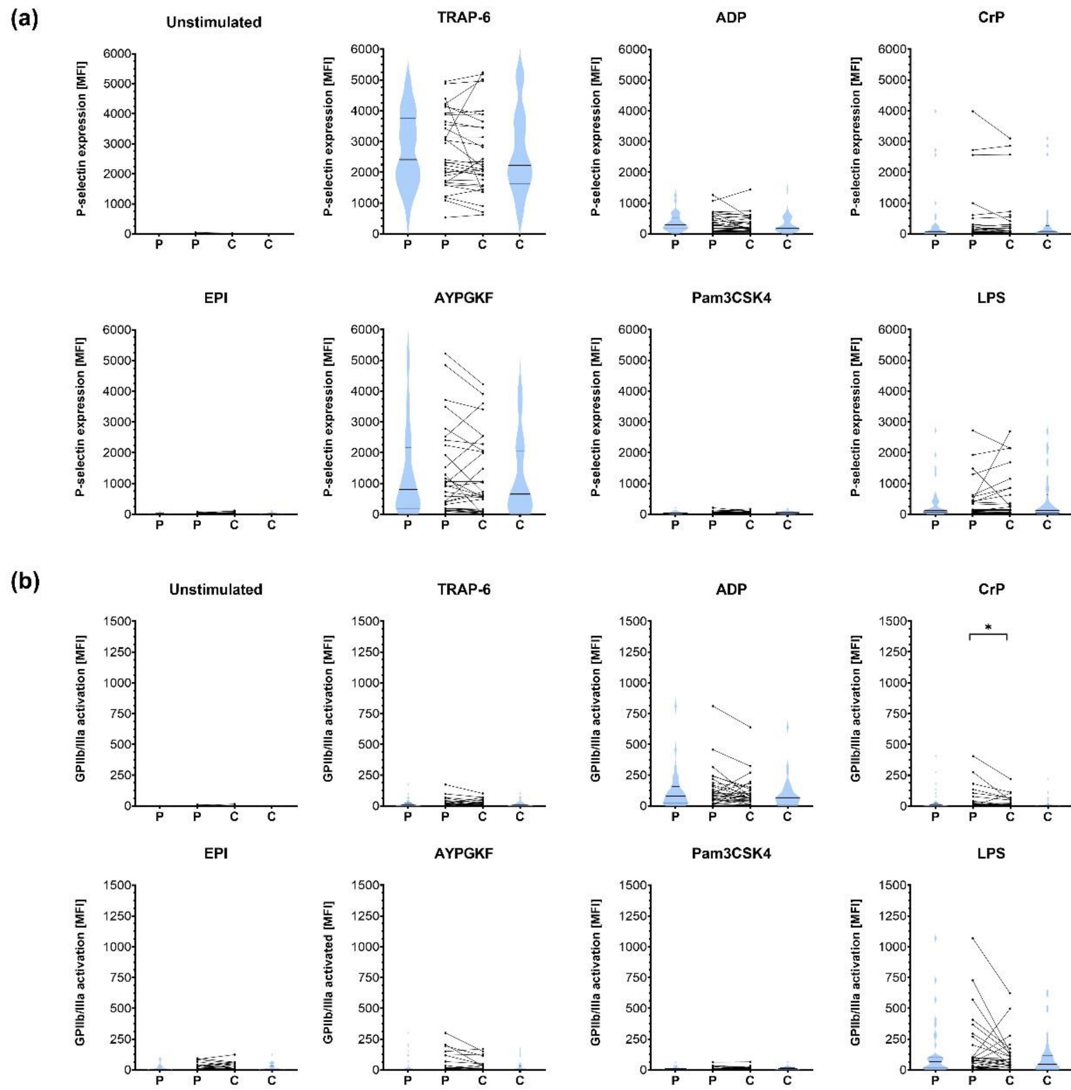

Figure 2

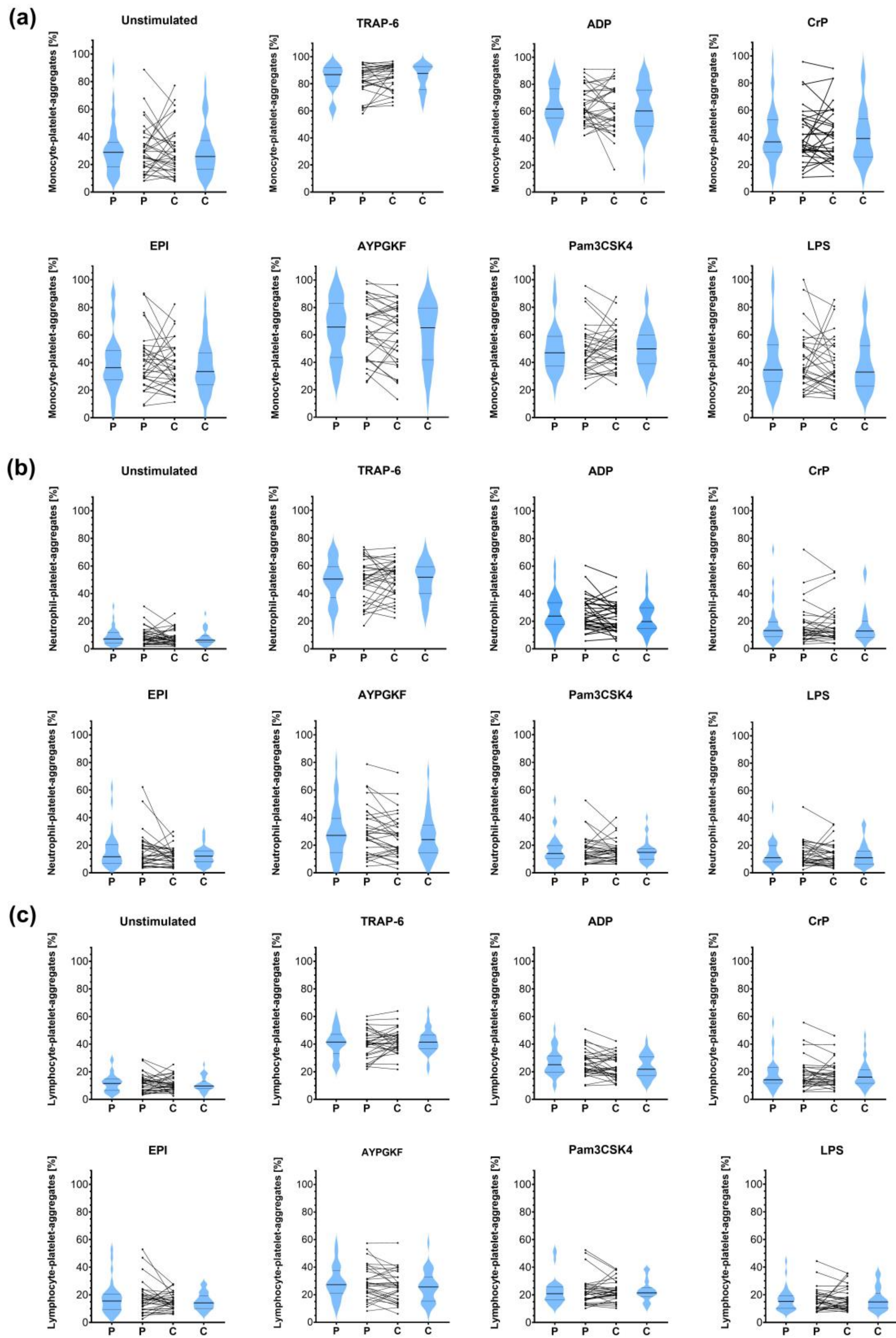

Table-1. Patient characteristics.

Gender, n (%)		
Male	22	67%
Female	11	33%
Age, yr.		
Median [IQR]	54 [46-60]	
Etiology, n (%)		
ALD	17	52%
VIRAL	6	18%
NAFLD	3	9%
CHOL	2	6%
ALD & VIRAL	2	6%
OTH	3	9%
Child-Turcotte-Pugh Stage (%)		
Child-A	15	45%
Child-B	15	45%
Child-C	3	9%
Platelets, G/L (%)		
PLT >150	3	9%
PLT 100-149	7	21%
PLT 50-99	20	61%
PLT <50	3	9%
Median [IQR]	87 [67-110]	

Continuous data are shown as median [IQR; interquartile range]. Hepatic venous pressure gradient (HVPG); alcoholic liver disease (ALD); non-alcoholic liver disease (NAFLD); viral hepatitis (VIRAL); cholestatic liver disease (CHOL); alcoholic liver disease with viral hepatitis (ALD & VIRAL); other causes of liver disease (OTH).