Platelet function in the context of global hemostasis: a comprehensive study in patients with cirrhosis.

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Abbreviations Table

2-Mes-ADP	2-Methylthioadenosine diphosphate
ADAM10	A disintegrin and metalloproteinase domain-containing protein 10
ADAMTS13	A disintegrin-like and metalloproteinase with a thrombospondin type 1 motif, member 13
ADP	Adenosine diphosphate
ALD	Alcoholic liver disease
ALT	Alanine aminotransferase
APTT	Activated partial thromboplastin time
AST	Aspartate aminotransferase
AT	Antithrombin
CAT	Calibrated automated thrombogram
CD	Cluster of differentiation
cfDNA	Cell-free DNA
CLD	Chronic liver disease
CLT	Clot lysis time
CRP-XL	Collagen-related peptide (cross-linked)
CTP	Child-Turcotte-Pugh
CV	Coefficient of variation
CVa	Analytical biological variation
CVi	Within-subject biological variation
CVg	Between-subject biological variation
DDAVP	Deamino D-arginine vasopressin
EFLM	European Federation of Clinical Chemistry and Laboratory Medicine
eGFR	estimated Glomerular filtration rate
ETP	Endogenous thrombin potential
FXIII	Factor XIII
GGT	Gamma-glutamyl transferase
GPO	Glycine-proline-hydroxyproline
GPRP	Gly-Pro-Arg-Pro
GPVI	Glycoprotein VI

HARI	Hepatic artery resistive index
Hb	Hemoglobin
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
II	Index of individuality
INR	International normalized ratio
ITAM	Immunoreceptor tyrosine-based activation motif
IQR	Interquartile range
LDH	Lactate dehydrogenase
LT	Lag time
LPS	Lipopolysaccharide
MELD	Model for end-stage liver disease
MFI	Mean fluorescence intensity
MPO	Myeloperoxidase
MPV	Mean platelet volume
NET	Neutrophil extracellular traps
OC	Oral contraceptives
PAI-1	Plasminogen activator inhibitor-1
PC	Protein C
PC1	Principal component 1
PC2	Principal component 2
PCA	Principal component analysis
PELD	Pediatric end-stage liver disease score
PFA	Platelet function Analyzer
PLT	Platelets
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PS	Phosphatidylserine
PT	Prothrombin time
PVT	Portal Vein Thrombosis
RCV	Reference change value
ROTEM	Rotational thromboelastometry

sGPVI	Soluble glycoprotein VI
TAFI	Thrombin activatable fibrinolysis inhibitor
TEG	Thromboelastography
TG	Thrombin generation
TGA	Thrombin generation assay
TIPS	Transjugular intrahepatic portosystemic shunt
TM	Thrombomodulin
tPA	Tissue-plasminogen activator
TPO	Thrombopoietin
TRAP-6	SFLLRN-NH2
TTP	Time to peak
VET	Viscoelastic test
VWF	von Willebrand factor
WBC	White blood cell count

1. SUMMARY

The hemostatic system in patients with cirrhosis is complex with alterations in pro-hemostatic and anti-hemostatic drivers leading to so-called rebalanced hemostasis. Literature on the hemostatic system in pediatric cirrhosis is limited, particularly regarding primary hemostasis. To address this gap, we conducted a proof-of-principle study to inform future, clinically focused research. We investigated the hemostatic system—including primary hemostasis, the coagulation cascade (secondary hemostasis), and fibrinolysis (tertiary hemostasis) — in a cohort of pediatric patients with cirrhosis, just before and 3 months after living donor liver transplantation and in a small adult cohort for comparison.

A total of 27 pediatric cirrhotic patients, most with cholestatic liver disease, and 15 controls were enrolled. In assessing primary hemostasis, a static flow cytometry assay, independent of platelet count and von Willebrand factor, revealed reduced platelet activatability in response to high agonist concentrations. In contrast, a microfluidic assay indicated high inter-patient variability, distinguishing two patient subgroups pre-transplant: one with preserved primary hemostasis and another with moderate impairment. This difference in platelet thrombus formation was likely related to liver disease severity, platelet count and von Willebrand factor levels.

Regarding the coagulation system, we confirmed a rebalanced coagulation profile in pediatric patients prior to liver transplantation evidenced by marked resistance to thrombomodulin. Interestingly, resistance to thrombomodulin persisted even 3 months post-transplant. Lastly, global fibrinolysis assays confirmed normal fibrinolysis in the majority of patients, although both hypo-and hyperfibrinolyis were demonstrated in patients with advanced cirrhosis.

Overall, pediatric cirrhotic patients displayed a relatively rebalanced hemostatic system before liver transplantation, with primary hemostasis varying according to platelet count, von Willebrand factor levels, and disease severity. Few hemostasis failure-related bleeding events occurred pre- and post-transplant and no excessive blood loss was observed during surgery. Hemostatic alterations improved three months post-transplant, although they were not fully reversed.

2. OUTLINE AND AIM OF THE THESIS

Each year, approximately 30 pediatric patients undergo liver transplantation at Cliniques universitaires Saint-Luc. Most of these transplants are living donor liver procedures, where a parent or other family member, if eligible, donates a portion of their liver. The majority of children undergoing liver transplantation have cirrhosis, often in an advanced stage. Cirrhosis is the end stage of chronic liver disease and is characterized by the development of fibrosis with destruction of the normal liver architecture and development of regenerative nodules. Chronic liver diseases, responsible for cirrhosis in children, include cholestatic disease, metabolic liver disease and chronic hepatitis. Biliary atresia, a cholestatic disease, is the most common indication for pediatric liver transplantation after failed hepatoportoenterostomy (Kasaï surgery).

The liver plays a central role in hemostasis, synthesizing most, though not all, of the proteins involved in coagulation. These include both pro-hemostatic and anti-hemostatic factors. In cirrhosis, liver synthetic function becomes compromised, resulting in complex alterations to the hemostatic system.

The hemostatic system is arranged into three phases: primary hemostasis, the coagulation cascade and fibrinolysis. Primary hemostasis involves the interaction between the blood vessel wall, platelets and von Willebrand factor. Upon vascular injury or endothelial breach, collagen in the underlying subendothelial matrix becomes exposed, activating both von Willebrand factor and platelets. Von Willebrand factor enhances platelet adhesion, further leading to platelet activation and aggregation at the injury site to form an initial unstable clot. The coagulation cascade follows, activating a series of clotting factors, ultimately converting fibrinogen into fibrin by thrombin. Fibrin then strengthens the platelet clot, stabilizing it. Once the blood vessel wall is healed, the clot is dissolved through fibrinolysis, breaking down fibrin and restoring normal blood flow.

In cirrhosis these 3 processes are altered leading to a so-called rebalanced hemostatic system with simultaneous changes in both pro-and anti-hemostatic drivers. This balance is however fragile and can tip towards bleeding or thrombosis.

The purpose of this doctoral thesis is an in-depth description of the hemostatic process, and in particular primary hemostasis, in children with cirrhosis before liver transplantation. Given the complexity of the assays and the challenges in recruiting pediatric cohorts, there is limited research on this topic. Therefore, we performed state-of-the-art platelet assays in pediatric patients before liver transplantation, evaluated a panel of plasma proteins, including von Willebrand factor, and markers of in vivo platelet activation. To address the

constraints of working with limited plasma volumes in pediatric subjects, we selected assays such as multicolor flow cytometry, which allow for the assessment of multiple platelet parameters simultaneously, with minimal blood volume requirements. Additionally, we performed a microfluidic assay under moderate to high shear rate, which, in contrast to flow cytometry, takes von Willebrand factor levels and platelet count into account. We hypothesized that primary hemostasis is relatively preserved in pediatric patients with cirrhosis before liver transplantation.

We also aimed to characterize secondary hemostasis or the coagulation cascade by performing a thrombin generation assay, and assessing levels of individual coagulation factors and natural anticoagulants. Thrombin generation in the presence of thrombomodulin provides a more accurate representation of rebalanced hemostasis compared to routine hemostasis assessments. Fibrinolysis was outlined by global plasma-based fibrinolytic assays. The evolution of hemostasis, including primary hemostasis, the coagulation cascade and fibrinolysis, was then reevaluated three months after liver transplantation. For comparison we also included a small cohort of adult cirrhotic patients.

The upcoming chapters will be structured as follows: introduction, methods, results, discussion, perspectives and conclusion. The first 3 chapters will each cover the three phases of hemostasis, along with a section on inflammation. A deep dive into the results of the adult cohort is presented separately.

3. INTRODUCTION

3.1. OVERVIEW OF INTRODUCTION CHAPTER

This chapter is structured around the 3 phases of coagulation: primary hemostasis, the coagulation cascade and fibrinolysis. A short introduction on the link between hemostasis and inflammation is also provided.

First, an overview of the existing knowledge on primary hemostasis in cirrhosis is presented as a review published in the International Journal of Molecular Sciences [1]. In this review the impact of chronic liver disease on platelet count and platelet function is outlined, as well as the role of von Willebrand factor, a protein with a key role in both primary and secondary hemostasis. The review is concluded with the role of global hemostasis assays in the study of primary hemostasis in patients with chronic liver disease. To finalize the part on primary hemostasis, the evaluation of primary hemostasis in the portal vein of adult patients with cirrhosis is discussed.

In the second part of this introduction, we focus on the coagulation cascade and explain the concept of rebalanced hemostasis. This paragraph is in part based on a paper published in Transfusion Medicine in which we outlined the role of prothrombin complex concentrates in patients with liver disease [2]. Global hemostasis assays, viscoelastic tests and thrombin generation, are also briefly discussed here. In our research paper submitted to *RPTH*, titled “Persisting thrombomodulin resistance at three months after liver transplantation in children with cirrhosis”, we also review the current literature on the coagulation cascade in pediatric patients with cirrhosis. This paper can be found in the results chapter.

In the third part of the introduction we introduce the fibrinolytic system in the context of cirrhosis. Studying fibrinolysis through laboratory assays remains challenging and has not progressed as much as research on the coagulation cascade. A brief introduction to fibrinolysis is also given in our research paper published in RPTH and titled “Fibrinolytic profile depends on disease severity in pediatric patients with cirrhosis: illustration by two different plasma-based fibrinolysis assays” [3]. This paper can also be found in the results section.

We also included a final section offering a brief discussion on the role of inflammation, particularly focusing on platelet-leukocyte interactions and their link to coagulation.

The data discussed in this chapter are mainly derived from studies conducted on adult populations with chronic liver disease or cirrhosis.

3.2. PRIMARY HEMOSTASIS

3.2.1. REVIEW PAPER: PRIMARY HEMOSTASIS IN CHRONIC LIVER DISEASE AND CIRRHOSIS: WHAT DID WE LEARN OVER THE PAST DECADE?

3.2.1.1. Abstract

Changes in primary hemostasis have been described in patients with chronic liver disease (CLD) and cirrhosis and are still subject to ongoing debate. Thrombocytopenia is common and multifactorial. Numerous studies also reported platelet dysfunction. In spite of these changes, primary hemostasis seems to be balanced. Patients with CLD and cirrhosis can suffer from both hemorrhagic and thrombotic complications. Variceal bleeding is the major hemorrhagic complication and is mainly determined by high portal pressure. Non portal hypertension-related bleeding due to hemostatic failure is uncommon. Thrombocytopenia can complicate management of invasive procedures in CLD patients. Recently, oral thrombopoietin agonists have been approved to raise platelets before invasive procedures. In this review we aim to bundle literature, published over the past decade, discussing primary hemostasis in CLD and cirrhosis including (1) platelet count and the role of thrombopoietin (TPO) agonists, (2) platelet function tests and markers of platelet activation, (3) von Willebrand factor and (4) global hemostasis tests.

KEYWORDS: chronic liver disease; cirrhosis; primary hemostasis; platelet; platelet function; von Willebrand factor.

3.2.1.2. Introduction

Chronic liver disease (CLD) was for a long time considered as a model of acquired hemorrhagic disease, reflected by, for example, prolonged prothrombin time. In the last 15 years, it has been acknowledged that CLD patients present complex alterations in the hemostatic system leading to so-called rebalanced hemostasis [4, 5]. Patients with CLD and cirrhosis can suffer from both hemorrhagic and thrombotic complications. Regarding hemorrhage, a distinction is made between portal hypertension-related bleeding, spontaneous bleeding and provoked bleeding. Gastrointestinal (variceal) bleeding is common in CLD and cirrhosis and is due to the elevation in portal pressure giving rise to shunts between the portal and caval system. The risk of variceal hemorrhage is mainly determined by portal hypertension (hepatic venous pressure gradient >12 mmHg), severity of liver disease, the size of varices and presence of red signs [6, 7]. Variceal hemorrhage is not related to true hemostatic failure. The fragile balance recognized in cirrhotic patients can also tip towards thrombosis. Thrombotic complications encompass portal vein thrombosis (PVT) and venous thromboembolism. PVT is mainly caused by reduced portal

flow (<15 cm/s) [8]. In a recent meta-analysis, an increased venous thromboembolic risk was confirmed in cirrhotic patients with a prevalence of 3.7% [9]. Cirrhotic subjects were more at risk of deep venous thrombosis and pulmonary embolism compared to controls. A lower incidence of PVT, hepatic decompensation and an improved survival were reported in patients with advanced cirrhosis, randomized to prophylaxis with low molecular weight heparin, compared to standard of care [10]. Multiple authors have described a hypercoagulable state in cirrhotic patients [11-15]. Furthermore, liver transplantation without the need for blood products is feasible in this patient group [16].

Although rebalanced hemostasis is substantially studied for the coagulation cascade, alterations in primary hemostasis are not well understood. Variable thrombocytopenia is present in the majority of CLD patients [17]. This is in part counterbalanced by elevated von Willebrand factor (VWF) supporting enhanced platelets' adhesion [18, 19]. By their complex phenotype, platelets play a diverse and key role in hemostasis. However, their role in rebalanced hemostasis is not clear. In this review we will highlight current knowledge of primary hemostasis in CLD.

3.2.1.3. Chronic liver disease and platelet count

3.2.1.3.1. Studies on CLD and platelet count

Thrombocytopenia is common in patients with CLD. The prevalence depends on disease severity and the criteria used: 6% in chronic hepatitis patients versus 64% in cirrhotic patients [17]. A low platelet count is an indicator of more advanced disease and is associated with poorer prognosis [20, 21]. In patients with chronic hepatitis C infections, immune thrombocytopenic purpura can also cause low platelet counts asymmetric to portal hypertension and hypersplenism. Platelets have been implicated in both CLD progression and liver regeneration. This topic is extensively reviewed elsewhere and will not be discussed here [22].

In a prospective study [20], the non-provoked bleeding rate was investigated in 280 patients with cirrhosis. Overall, 52 patients experienced a major or minor bleeding event over four years (5.45%/year). No association was observed between platelet count and bleeding. Two studies addressing variceal bleeding found opposite results regarding the association between platelet count and bleeding [23, 24]. It is, however, important to stress that platelet count may merely reflect the degree of portal hypertension responsible for variceal hemorrhage [25]. Low platelet count was identified as a risk factor for major bleeding in a retrospective study of cirrhotic patients admitted to intensive care [26].

By possibly increasing the risk of bleeding, severe thrombocytopenia can complicate the management of patients with CLD undergoing invasive procedures. A threshold of 50×10^9/L is often used for platelet transfusion to mitigate the risk of procedure-related bleeding [27-29]. This platelet transfusion cut-off is also based on a paper by Tripodi and colleagues [30]. They demonstrated that a platelet count of 56×10^9/L was necessary for normal thrombin generation in platelet-rich plasma. In an article by Giannini et al [31], bleeding related to invasive procedures was more frequent in cirrhotic patients with severe thrombopenia (PLT $<75\times10^9$/L, 10 out of 32 with procedure-related bleeding) compared to patients with moderate thrombocytopenia (75×10^9/L<PLT<150×10^9/L, 0 out of 19 with procedure-related bleeding). Similarly, platelet count was strongly correlated with serious bleeding in patients with the hepatitis C virus (HCV) related to advanced CLD undergoing a liver biopsy. The bleeding risk increased significantly with platelet counts $<60\times10^9$/L [29]. However, patients that experienced bleeding had more advanced liver disease, so it is not clear if platelet count caused the bleeding or was a manifestation of advanced liver disease (ie. bleeding caused by elevated liver stiffness, presence of ascites). The association of severe thrombocytopenia and post-procedural bleeding was also reported in a recent study on 874 cirrhotic patients [27]. In contrast to the previous findings, no post-procedural bleeding episode was reported in cirrhotic patients with a platelet count $<50\times10^9$/L scheduled for an invasive procedure [32]. In a report of 500 orthotopic liver transplantations, no blood products were transfused in 79.6% of patients [16]. A study addressing platelet transfusion did report a significant increase in platelet count, but did so without normalizing global hemostasis tests [33].

As an alternative to platelet transfusion, oral thrombopoietin (TPO) agonists, avatrombopag and lusutrombopag, were FDA approved in adult patients with CLD and a platelet count $<50\times10^9$/L before an invasive procedure [28].

Different authors already reviewed the mechanisms of thrombocytopenia in CLD: splenic sequestration, decreased platelet production and increased platelet breakdown are involved [21, 34-36]. Only TPO will be further discussed here due to the recent approval of TPO agonists. This does not imply that this is the most important mechanism for thrombocytopenia in CLD.

3.2.1.3.2. Thrombopoietin

Thrombopoietin (TPO), the humoral basis of thrombopoiesis, was revealed in 1994 [37]. TPO is a hematopoietic growth factor, produced primarily by the liver, which binds the c-MPL (myeloproliferative leukemia) receptor on megakaryocytes, platelets and most other hematopoietic precursor cells. In liver-healthy thrombocytopenic patients, TPO levels rise

due to decreased degradation of TPO by platelets. Stimulation of megakaryocytes and an increase in platelet production ensues. However, in cirrhosis-associated thrombocytopenia, TPO levels may not rise sufficiently. Controversial reports exist regarding circulating TPO levels in cirrhotic patients. Some studies report low levels [38-40] while others report normal levels of TPO [21, 41-43]. According to the prevailing theory of TPO regulation by "c-MPL mass", circulating TPO levels, being the net effect of both production and clearance, do not reflect TPO production alone. The measurement of TPO mRNA expression in the liver better reflects TPO production and is decreased in patients with cirrhosis in most reports [44, 45]. Currently, four TPO agonists exist: romiplostim (Nplate®; Amgen Inc., Thousand Oaks, CA, USA), eltrombopag (Revolade®; Promacta®; Novartis Pharma; Basel; Switzerland) avatrombopag (Doptelet®; Dova Pharmaceuticals; Durham, NC, USA) and lusutrombopag (Mulpleo®; Mulpleta®; Shionogi, Japan). Eltrombopag is not recommended as an alternative for platelet transfusion for patients with CLD due to thrombotic complications during the ELEVATE clinical trial [46]. Romiplostim is approved as second line therapy for chronic immune thrombocytopenic purpura, but not in CLD. Avatrombopag and lusutrombopag were approved during the ADAPT-1/2 and L-PLUS 1/2 trials, respectively [47-49]. Approximately 70% of patients responded to their TPO agonist treatment. This means that 30% of patients are non-responders. An important advantage of TPO agonists is the avoidance of platelet transfusion and thereby not exposing patients to possible immunological or infectious risks as well as transfusion reactions and volume overload. It is important to know that TPO agonists take about ten days to elevate platelet count.

3.2.1.4. Chronic liver disease and platelet function

A variety of tests exist to assess platelet function. Some of them are extensively used, whereas others are confined to specialized or research laboratories. In vivo bleeding time was developed first but has become obsolete due to its invasive nature. The platelet function analyzer (PFA-100/200, Siemens Healthineers, Erlangen, Germany), a widespread and easy to perform assay, reflects high shear adhesion and aggregation during formation of a platelet plug. Platelet aggregation can be evaluated by light transmission aggregometry or whole blood aggregometry. Platelet glycoproteins and activation status (e.g., alpha granule release) can be appreciated by flow cytometry. In research, microfluidic devices have been developed for real-time assessment of thrombus formation in whole blood in response to a range of agonists. Soluble platelet release markers (e.g., soluble P-selectin and soluble CD40 ligand), also mainly used in research, investigate platelet activation state. PlateletMapping TEG technology employs specific platelet agonists but is mainly used for monitoring antiplatelet therapy [50].

About a decade ago, platelet function in CLD and cirrhosis was very thoroughly reviewed by Witters et al and Violi et al [15, 36]. In our review, the focus will lie on more recent papers investigating this topic. **Table 1** displays a chronological overview of recent studies (last ten years) of platelet function in CLD patients.

3.2.1.4.1. Platelet aggregation tests

As mentioned above, platelet aggregation is mostly studied by light transmission aggregometry or whole blood aggregometry. The following studies reported decreased platelet aggregation in CLD patients. In patients with chronic HCV, platelet aggregation measured with whole blood aggregometry was lower in more advanced fibrosis compared to mild fibrosis and healthy controls [51]. However, when platelet aggregation was corrected for platelet count and expressed as "multiplate units", aggregation was higher in patients with advanced fibrosis. This corrected parameter is not validated and should be interpreted with caution as the significance and mechanisms are not clear. Preoperatively, Jüttner et al [52] demonstrated decreased platelet aggregation in cirrhotic patients of mixed etiology after adjustment for platelet count. Cholestatic patients had significantly higher maximal aggregation in comparison to non-cholestatic cirrhotic patients. They hypothesize a better preservation of parenchymal function in the former group. Wosiewicz et al [53] reported decreased platelet aggregation in cirrhotic patients with or without PVT awaiting liver transplantation. Curiously, patients with PVT had decreased platelet aggregation compared to patients without PVT (platelet count was similar between groups). In a pediatric cohort of decompensated cirrhotic children, we also highlighted decreased platelet aggregation [54]. Although not corrected for platelet count, aggregation was lower in children presenting with upper gastrointestinal bleeding. Eyraud et al [55] demonstrated improved platelet aggregation one month after liver transplantation compared to pre-transplant values. In a study by Vinholt et al [56], flow cytometry-based aggregation was significantly lower in cirrhotic patients after stimulation with platelet agonists. The independence of platelet count is an advantage of this technique. No platelet hyperaggregability was revealed after testing different agonist concentrations. In contrast to these findings, Raparelli et al [57] demonstrated enhanced platelet aggregation in cirrhotic patients after stimulation with subthreshold concentrations of platelet agonists. This difference in aggregation between patients and healthy controls was not detected with threshold concentrations of agonist.

Table 1. Published studies of platelet function in CLD patients over the past decade.

Reference	Population	Tests	results
Jüttner et al., 2009 [52]	Tumor (n = 9) and cirrhosis (n = 25, mixed etiology)	LTA, mPs	decreased LTA in cirrhosis (cholestatic > non-cholestatic), decreased mPs in all patients
Sayed et al., 2010 [58]	cirrhosis (n = 60, viral)	platelet-monocyte complexes, mPs	increased
Basili et al., 2011 [11]	cirrhosis (n = 51, mixed etiology)	sNOX2dp, sPs, sCD40L, isoprostanes (urinary and platelet)	increased
Ghozlan et al., 2013 [59]	cirrhosis (n = 50, viral)	PFA-100 (COL/EPI)	prolonged CT
Basili 2014 [60]	cirrhosis (n = 51, mixed etiology)	serum polyunsaturated fatty acids, urinary isoprostanes, sNOXdp	higher n-6/n-3 polyunsaturated fatty acids ratio, correlation with elevated sNOXdp and isoprostanes
Alkozai et al., 2014 [61]	HCC (n = 22) and cirrhosis (n = 16, viral)	mPs (basal and stimulation), sPs	normal basal mPs, decreased stimulated mPs, increased sPs
Potze et al., 2016 [62]	NAFLD (n = 68, steatosis, NASH, NASH cirrhosis), ASH cirrhosis (n = 15)	mPs (basal and stimulation), sPs, PF4	normal basal mPs and PF4, decreased stimulated mPs in cirrhosis (NS), increased sPs (NS)
Egan et al., 2016 [63]	compensated cirrhosis (n = 46, mixed etiology)	soluble GPVI	increased
Wosiewicz et al., 2016 [53]	cirrhosis (n = 49, mixed etiology)	multiplate, zonuline, LPS (comparison presence/absence PVT)	decreased PA, NS difference zonuline, LPS between groups
Nielsen et al., 2017 [51]	chronic HCV (advanced fibrosis, n = 39; mild or no fibrosis, n = 43)	multiplate, TEG	decreased PA and increased PA/platelet count in advanced fibrosis, no difference in TEG
Vinholt et al., 2017 [56]	cirrhosis (n = 27, ASH)	96-well LTA, mPs, CD63, activated GPIIb/IIIa, FC platelet aggregation	decreased platelet aggregation/activatability
Raparelli et al., 2017 [57]	cirrhosis (n = 69, mixed etiology)	LTA, sPs, sCD40L, LPS, zonulin, platelet-monocyte complexes	increased sPs, sCD40L, LPS, zonulin, platelet-monocyte complexes, increased PA with STC (PLT adjusted)
Eyraud et al., 2018 [55]	HCC (n = 24) and cirrhosis (n = 26, mixed etiology)	LTA, mPs, platelet-leukocyte complexes, PDEV, sPs, sCD40L	decreased LTA pre-graft versus post-graft (D28, PLT adjusted); mPs, sPs and sCD40L, platelet-leukocyte complexes unchanged, sPs slightly higher pre-graft
Stoy et al., 2018 [64]	Cirrhosis (n = 19, mixed etiology)	Platelet-leukocyte complexes	increased platelet-neutrophil complexes
Bonnet et al.,2019 [54]	decompensated cirrhotic children (n = 22, bililiary atresia, Alagille, other)	multiplate	decreased PA (adult RV, PLT not adjusted)
Queck et al., 2019 [65]	decompensated cirrhosis (n = 20)	sPs, soluble GPVI, LPS, sNOXdp	increased in portal vein
Queck et al., 2019 [66]	decompensated cirrhosis (n = 89)	TXB2 portal vein	association with PHPG increase after TIPS insertion
Rogalski et al., 2019 [24]	cirrhosis (n = 58, mixed etiology)	multiplate	comparable in patients with and without variceal bleeding

Abbreviations: CT = closure time, COL = collagen, EPI = epinephrine, FC = flow cytometry, HCC= hepatocellular carcinoma, LPS = lipopolysaccharide acid, LTA = light transmission aggregometry, LT = liver transplantation, mPs = membrane P-selectin, NS = not significant, PA = platelet aggregation, PF4 = platelet factor 4, PFA = platelet function analyser, PHPG = portal hepatic venous pressure gradient, PLT = platelet, PVT = portal vein thrombosis, RV = refence values, sNOX2dp = soluble NADPH oxidase derived peptide, sPs = soluble P-selectin, STC = subtreshold concentrations, TIPS = transjugular intrahepatic portosystemic shunt.

The addition of Toll-like receptor 4 binding protein, an inhibitor of the lipopolysaccharide acid (LPS) receptor, inhibited platelet aggregation only in cirrhotic patients. This study concluded that high circulating levels of LPS, translocated in the systemic circulation, may be the cause of enhanced platelet activation in cirrhotic patients.

3.2.1.4.2. Platelet activation state by flow cytometry

Decreased platelet activatability assessed with P-selectin (= CD62P, alpha granule release), by flow cytometry, was seen in viral, non-alcoholic steatohepatitis, alcoholic steatohepatitis and cholestatic cirrhosis compared to controls [52, 56, 61, 62]. At baseline, without stimulation, no difference in P-selectin expression between patients and controls was seen across different etiologies [56, 61, 62, 67]. In line with these results, no differences in platelet activatability were seen before and after liver transplantation in cirrhotic patients [55]. Alcoholic steatohepatitis cirrhosis patients had lower percentages of platelets positive for CD63 (lysosomal marker) and activated GPIIb/IIIa after agonist stimulation compared to healthy controls [56], although some patients demonstrated normal platelet activatability. Platelet activation impairment was associated with severe liver disease (Child-Turcotte-Pugh B/C) and platelet count [56]. A lower response to stimulus rather than deficient GPIIb/IIIa was suggested since CD41 and CD61, GPIIb/IIIa subunits, were normally expressed. In contrast to these results, Sayed et al [58] and an older study by Panasiuk et al [68] demonstrated higher basal platelet activation in viral cirrhosis patients (P-selectin). As in the study by Raparelli et al [57] et al, they also reported increased platelet–monocyte complexes. Elevated platelet–leukocyte complexes were also confirmed by Støy et al's study [64].

3.2.1.4.3. Soluble release markers

Increased platelet activation markers (soluble P selectin and/or soluble CD40 ligand), corrected or not for platelet count, in cirrhotic patients were demonstrated by several authors [57, 60-62]. These levels were even more increased in decompensated patients and correlated well with serum LPS levels [57]. High oxidative stress and production of platelet isoprostanes were demonstrated to be related to higher soluble P selectin and soluble CD40 ligand levels [11, 60]. In decompensated patients with a transjugular intrahepatic portosystemic shunt (TIPS) procedure soluble platelet activation markers, a soluble oxidative stress marker and LPS were elevated in the portal circulation. This suggested increased platelet activation with a possible contribution of oxidative stress and bacterial translocation [65]. The same authors found an association between elevated levels of thromboxane B2 in the portal circulation and portal hepatic venous pressure gradient. They concluded that thromboxane B2 is possibly associated with prothrombotic

potential [66]. In the paper by Eyraud et al [55], soluble P selectin decreased after liver transplantation, suggesting a higher soluble P selectin/platelet count ratio in cirrhotic patients before transplantation. In compensated cirrhotic patients of mixed etiology, soluble glycoprotein VI was higher compared to healthy age-matched controls [63]. The authors interpreted this as an early onset marker of platelet activation. The majority of papers on soluble platelet release markers conclude a higher platelet activation state in CLD patients. However, these markers should be carefully interpreted since their elevation could also be a consequence of decreased clearance by the liver [69, 70].

3.2.1.4.4. Platelet function analyzer and studies under flow conditions

Under high shear flow conditions (5000–6000/s) platelets are sent through an aperture covered with collagen and epinephrine or adenosine diphosphate. Platelet adhesion and aggregation ensue. The closure time is recorded. The PFA is impacted by platelet count, VWF and hematocrit. It is insensitive to milder platelet disorders [71]. In patients with viral (HCV) cirrhosis PFA-100 closure times were prolonged compared to normal controls suggesting platelet hypofunction [59]. Patients with platelet count $<100\times10^9$/L were excluded in this study. In a cohort of CLD patients awaiting liver transplantation, PFA-epinephrine was prolonged in 48.6% of patients [72]. As expected, platelet count and hematocrit predicted pathological PFA. VWF improved closure times partially. Another small study did not report added value of PFA in cirrhotic patients undergoing an invasive procedure. Only 16 patients were tested, all with thrombocytopenia (40×10^9/L ± 20.7×10^9/L). No details of PFA results were provided [73]. Lisman et al [18] studied platelet adhesion under flow conditions in a reconstituted blood model. Platelet count and hematocrit were adjusted in this experiment to 200×10^9/L and 40%, respectively. Collagen and fibrinogen were used as surface activators and blood was passed through the chamber at a shear rate of 800/s and 300/s respectively. The capacity of cirrhotic platelets to adhere to the surface was identical to that of healthy volunteers.

3.2.1.5. Chronic liver disease and von Willebrand factor

VWF exerts a pivotal role in primary hemostasis. Through its interaction with collagen and platelets, it localizes platelets to the site of vessel wall injury, allowing further adhesion. VWF plasma levels are known to be elevated in CLD [19, 61, 62, 67, 72, 74-80]. The mechanism is not completely understood. Normally, VWF is mostly synthetized by the endothelial cells of large vessels. With liver injuries, VWF immunostaining also becomes positive in liver sinusoidal endothelial cells [81]. High VWF propeptide levels were reported in cirrhotic patients, which suggests ongoing acute endothelial damage [19, 78, 82]. Palyu et al demonstrated higher VWF propeptide levels in acutely decompensated cirrhotic

patients. Decreased clearance by the liver should also be taken into account. VWF was demonstrated to be positively associated with more severe liver disease [19, 76]. Although VWF seems to demonstrate a less efficient binding to platelet glycoprotein Ib, demonstrated by a reduced VWF activity to antigen ratio, the capacity to adhere platelets to a surface was preserved [19]. Interestingly, when platelets from healthy volunteers were mixed with cirrhotic plasma, platelet adhesion improved [19, 78]. DDAVP (1-deamino, 8-D arginine-vasopressin) is known to increase VWF and factor VIII in hemophilia A and von Willebrand disease. In cirrhotic patients, no significant effect of an intravenous bolus of DDAVP on VWF plasma levels was seen. The authors hypothesized exhaustion of endothelial cells on the one hand and immediate intrahepatic consumption or endothelial attachment on the other hand [82]. These results were in contrast to the results of an earlier study reporting on the efficacy of intranasal DDAVP in cirrhotic patients undergoing dental extraction [83]. The design of the study was however questionable. A disintegrin-like and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13) cleaves ultra-large VWF and is synthesized by the hepatic stellate cells in the liver [84, 85]. Both reduced, normal and elevated ADAMTS13 levels have been reported [19, 61, 62, 67, 75, 78, 79, 82]. Similarly, both presence and absence of high molecular weight VWF multimers in CLD patients were demonstrated [19, 78, 79]. No differences in VWF and ADAMTS13 levels were seen between different CLD etiologies [67].

3.2.1.6. Chronic liver disease and global hemostasis tests

Global hemostasis tests like viscoelastic testing (VET) and the thrombin generation assay (platelet-rich plasma/whole blood) take into account the role of platelets and other blood components.

3.2.1.6.1. Use of viscoelastic assays and the role of platelets

Viscoelastic testing (VET) gives a more global picture of the hemostatic system compared to routine coagulation testing by better approaching in vivo clot formation. It is performed on whole blood taking into account platelets and other figurative elements. Two techniques are available: rotational thromboelastometry and thromboelastography. A limitation of VET is the lack of thrombomodulin, needed for full activation of protein C, and the insensitivity to VWF. VET parameters like maximal clot firmness (thromboelastometry) and maximal amplitude (thromboelastography) reflect platelet count but are largely independent of platelet function [86]. In stable cirrhosis, platelets are suggested to be the main determinant of clot formation [87]. VET is readily used in cardiothoracic surgery and bleeding trauma patients to guide transfusion. Similarly, in liver transplantation and invasive procedures in cirrhotic patients, VET can be used to guide blood product

administration [88-91], although maximal clot firmness in FIBTEM conditions (addition of platelet inhibitor; thromboelastometry) is possibly less accurate in very low levels of fibrinogen (<100 mg/dL) [92]. In CLD patients, outside the context of liver transplantation, VET parameters correlated well with platelet count, fibrinogen level and severity of liver disease [87, 93, 94]. Multiple authors reported both normal and hypocoagulable profiles in cirrhotic patients [87, 93-97]. In a subset of patients with cholestatic cirrhosis, a hypercoagulable profile was shown [95]. No difference was, however, seen in viscoelastic parameters between stable cirrhotic patients with and without histories of bleeding or thrombosis [87, 96]. Accordingly, thromboelastography parameters did not predict bleeding or thrombosis in cirrhotic patients with different etiologies [95]. A limitation of VET is the lack of a validated target level for cirrhotic patients in evaluation of their hemostatic system (for example in a pre-operative context) [28].

3.2.1.6.2. Thrombin generation assay

In the majority of centers, the thrombin generation assay is performed on platelet-poor plasma with or without thrombomodulin. Tripodi et al reported a minimum platelet count necessary for normal thrombin generation (56×10^9/L) in platelet-rich plasma [30]. Very recently, Wan et al compared thrombin generation in whole blood with platelet-poor plasma in patients with cirrhosis and controls [98]. The advantage of a whole blood assay is the interaction with platelets, but also with leukocytes and erythrocytes. They reported lower peak thrombin generation in whole blood but preserved endogenous thrombin potential compared to controls. The authors hypothesize that this discordance between peak and endogenous thrombin potential could be explained by the more important impact of platelet and erythrocyte count on peak thrombin generation [99, 100]. Interestingly, the anticoagulant effect of thrombomodulin was much less pronounced in the whole blood assay compared to platelet-poor plasma.

3.2.1.7. Conclusion

The study of primary hemostasis in CLD is complex and has not been extensively studied over the past decade. This is in part due to the technical difficulties met in studying platelets. Platelets are fragile and very easily activated in vitro. Platelet assays are not standardized: varying concentrations of agonists are, for example, used. Differences between studies can also be explained by the heterogeneity of the CLD population: different degrees of severity and different etiologies. Although no clear-cut platelet target is available, TPO agonists avatrombopag and lusutrombopag are a good alternative in CLD patients with severe thrombocytopenia before a high-risk invasive procedure. VWF plasma levels are increased in cirrhotic patients and support primary hemostasis in reconstituted

blood models. The recent studies that did report in vivo platelet activation mostly analyzed soluble markers. An exception is the key study by Raparelli et al [57]. Overall, there is no clear consensus between papers regarding primary hemostasis in CLD. Ideally, platelet function tests approach in vivo platelet activation with minimal manipulation. In our opinion, microfluidic assays with correction for platelet count and hematocrit could be very interesting in future studies [101, 102] and hopefully allow better management and risk assessment of these complicated patients to guide therapeutic decision making.

3.2.2. PORTAL VEIN ASSESSMENT

Patients with cirrhosis are at risk of thrombotic complications and most importantly portal vein thrombosis (PVT) [9, 103]. The risk of PVT increases with progressing liver disease [104]. The etiology of PVT is probably multifactorial and proposed risk factors comprise diminished portal blood flow, vessel wall damage, risk factors associated with disease severity and portal hypertension, inflammation and hypercoagulability. Since patients that develop PVT, often have a high factor VIII/protein C ratio, high levels of VWF or thrombomodulin resistance [76, 105], several authors suggested a role for hypercoagulability as a risk factor for PVT development. However, Turon et al did not identify these changes as independent risk factors in a prospective study [106]. Systemic hemostatic changes probably reflect severity of liver disease. They identified two main risk factors: low portal blood flow velocity (<15 cm/s) and severity of portal hypertension. Local portal vein hemostatic or inflammatory alterations could also possibly impact the development of PVT. Since the portal blood system drains blood from the intestines via the superior and inferior mesenteric veins, it is more exposed to endotoxins which are capable of coagulation activation [57]. Moreover, endothelial dysfunction could further contribute to hypercoagulability [107]. Elevated levels of thrombin-antithrombin complexes, D-dimers, and plasmin-antiplasmin complexes were detected in the portal system compared to the jugular and hepatic veins. However, this may also reflect differences in hepatic clearance of these coagulation activation markers [108]. Regarding primary hemostasis, both enhanced and similar platelet function in the portal circulation compared to the systemic circulation have been reported [65, 109, 110]. In light of these possible differences between the portal vein circulation and the systemic circulation, we decided to add a portal vein sample in adult patients and performed platelet activation assays on these samples.

3.3. COAGULATION CASCADE

Currently, the concept of rebalanced hemostasis is widely accepted for adult patients with chronic liver disease (CLD) and cirrhosis. Multiple procoagulant and anticoagulant perturbations give rise to a fragile balance, elevating both hemorrhagic and thrombotic risk. The three phases of hemostasis are affected : primary hemostasis, the coagulation cascade and fibrinolysis (**Figure 1**) [5]. Primary hemostasis was discussed in length in the previous section. Fibrinogen plays a role in both primary hemostasis (platelet aggregation) and coagulation (fibrin formation). Levels of fibrinogen can be elevated in early liver disease but hypofibrinogenemia is often seen in more advanced stages, leading to reduced clot formation potential [5]. Qualitative defects (hypersialated fibrinogen, high carbonyl content) have been described [13, 111]. The majority of coagulation factors is synthetized by hepatocytes except for factor VIII which is predominantly produced by liver sinusoidal endothelial cells [112]. A decrease in factor II, V, VII, IX, X, XI and XII but an increase in factor VIII is observed in patients with liver disease. The natural anticoagulants antithrombin, protein S and C decrease progressively with disease severity [113].

Routine hemostasis tests, for example prothrombin time (PT) and activated partial thromboplastin time, are merely sensitive for procoagulant proteins and only reflect 5% of the total amount of thrombin generated during coagulation [114]. Important information on the anticoagulant pathway is therefore not provided. Viscoelastic tests, like rotational thromboelastometry (ROTEM) and thromboelastography (TEG), better reflect this rebalanced hemostatic system than conventional assays. A normal viscoelastic test may be useful to select patients that do not need preprocedural pro-hemostatic treatment. However, an abnormal viscoelastic test may still not require correction, since existing viscoelastic tests underestimate coagulation capacity (no activation of protein C, insensitivity to von Willebrand factor) [115].

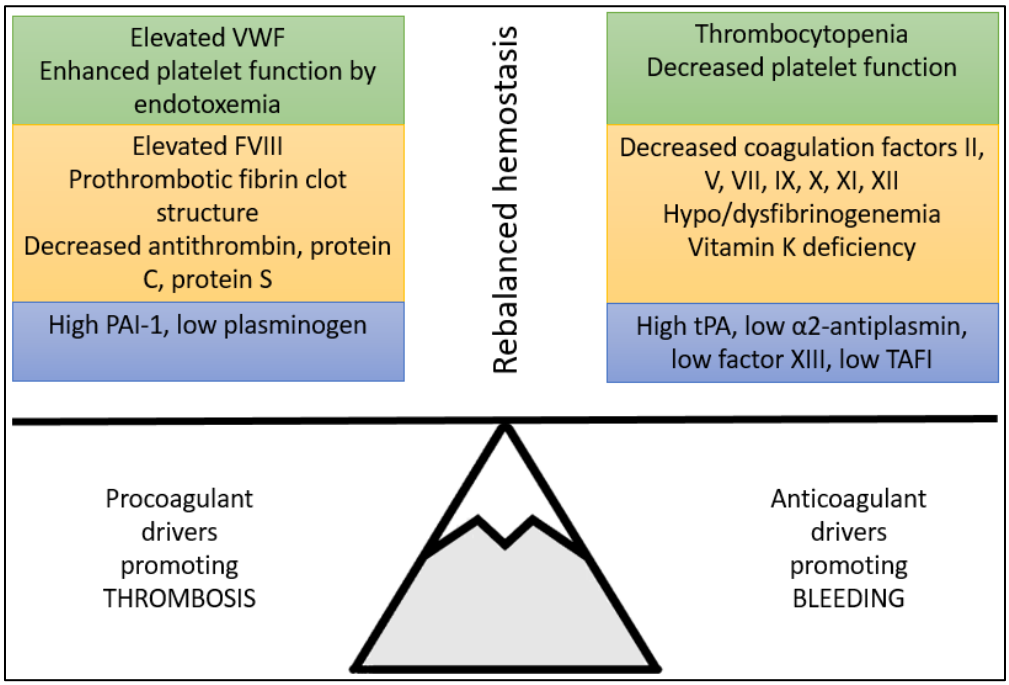


Fig.1. Rebalanced hemostatic system in liver disease patients. The three phases of hemostasis are concerned: primary hemostasis (green), coagulation cascade (orange), fibrinolysis (blue). tPA : tissue plasminogen activator, TAFI: thrombin activatable fibrinolysis inhibitor, PAI-1: plasminogen activator inhibitor-1, VWF: von Willebrand factor.

Plasma-based thrombomodulin-modified thrombin generation (**Figure 2**), in which exogenous thrombomodulin activates protein C, provides a good appreciation of rebalanced hemostasis [116]. Thrombin generation can also be performed in the presence of activated protein C to study acquired activated protein C resistance [117]. These assays are however not readily available in clinical laboratories. The predictive value for procedural or spontaneous bleeding of thrombin generation assays (TGA) is unknown. While hemostatic changes may contribute to bleeding or thrombosis in patients with cirrhosis, other critical factors also influence hemorrhagic and thrombotic risk in those with chronic liver disease. Variceal hemorrhage is primarily driven by portal hypertension, while reduced portal blood flow can lead to portal vein thrombosis. Additionally, renal disease, bacterial infection, and inflammatory changes in endothelial cells can further disrupt hemostasis, thereby increasing the bleeding risk.

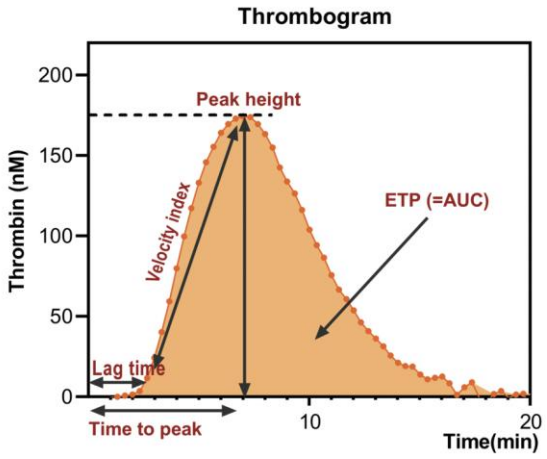


Fig. 2. A thrombogram is characterized by the following parameters: lag time, time to peak, thrombin peak, velocity index and endogenous thrombin potential (ETP) [118]

Although the literature is more limited, it appears that the hemostatic system in pediatric patients with cirrhosis is also rebalanced in a similar manner as is seen in adults [119, 120]. This is in line with the paucity of true hemostasis-related bleeds in these patients. Most bleeding events pre-transplant are variceal in nature and are mainly caused by high portal pressure. Similarly, thrombotic events are rarely seen in this patient group before liver transplantation. Werner et al [119] examined various hemostatic parameters before, during, and up to one month after liver transplantation in children with cirrhosis. The endogenous thrombin potential (ETP) was comparable to age-matched controls before liver transplantation. Magnusson et al [121] found lower ETP in children with prolonged routine coagulation assays, though they did not incorporate thrombomodulin in their assay.

3.4. FIBRINOLYSIS

3.4.1. REBALANCED FIBRINOLYSIS

Hyperfibrinolysis is often described in chronic liver disease and cirrhosis [122]. However, changes in both pro-and anti-fibrinolytic drivers are recognized that can lead to a rebalanced system: increased tissue plasminogen activator (tPA), decreased alpha 2-antiplasmin, factor XIII (FXIII) and thrombin activatable fibrinolysis inhibitor (TAFI) increase fibrinolytic potential whereas high plasminogen activator inhibitor type 1 (PAI-1) and decreased plasminogen levels decrease fibrinolytic capacity [5, 123]. These alterations result not only from impaired protein synthesis by the liver (i.e. reduced plasminogen) but also from endothelial activation leading to increased tPA and PAI-1 levels [124].

In comparison to the coagulation cascade, fibrinolysis assays have developed more slowly. Different approaches to evaluate fibrinolysis exist: measurement of individual antigen and activity levels, the euglobulin lysis time assay, viscoelastic testing, plasma-based and whole blood-based global fibrinolytic capacity assays [124, 125]. Unfortunately, well-validated tests are lacking. The euglobulin lysis time is for example not equipped to evaluate hypofibrinolysis since >90% of α2-antiplasmin and a substantial portion of PAI-1 and TAFI are eliminated. Classic viscoelastic assays are only sensitive to severe hyperfibrinolysis and individual antigen and activity levels reflect only one aspect of the fibrinolytic system [126]. Whereas fibrinolytic assays in whole blood may better represent physiology, plasma-based assays have been much more extensively used to estimate fibrinolytic potential in cirrhosis.

Lastly, products of fibrinolysis activation, such as D-dimer levels or plasmin-antiplasmin complexes, can be used as markers of hyperfibrinolysis. The presence of D-dimers indicates both stabilization of the fibrin clot by activated FXIII and fibrinolytic clot breakdown. D-dimer levels are however elevated in the majority of patients with cirrhosis. This can be attributed to ongoing coagulation activation, enhanced fibrinolysis but also to impaired clearance.

3.4.2. FACTOR XIII

FXIII, a transglutaminase, stabilizes fibrin by cross-linking the fibrin polymers. It is a tetrameric complex composed of 2 active catalytic A-subunits and 2 inhibitory B-subunits. The A-subunits are produced by hematopoietic cells and the B-subunits by hepatocytes. Both subunits are required for adequate FXIII concentration in plasma [127]. The A-subunits need activation by thrombin. In patients with cirrhosis acquired FXIII deficiency can be

observed, especially in more advanced stages. Low levels of FXIII are associated with decreased clot firmness in studies on viscoelastic testing [128].

3.5. INFLAMMATION

Cirrhosis is characterized by systemic inflammation due to the persistent activation of immune cells, resulting from tissue damage and bacterial translocation [129]. Systemic inflammation is illustrated by elevated serum pro-inflammatory cytokines and the presence of cellular activation markers. Platelets are, in addition to their role in primary hemostasis, recognized to play an important role in inflammatory processes. They express receptors (eg toll-like receptors) that can recognize pathogens (via pathogen associated molecular patters; PAMPS), promote the recruitment and interaction with immune cells and store a wide variety of cytokines, chemokines and adhesive molecules in their granules. Recently, the term immunothrombosis has been introduced [130]. Immunothrombosis consists of an interplay between innate immunity, platelet activation and coagulation activation to trap and kill pathogens and involves prothrombotic mechanisms [131].

Neutrophils play an important role in immunothrombosis through the release of neutrophil extracellular traps (NETs). NETs are fibrous structures, released by neutrophils, that are composed of DNA, histones, nuclear protein high mobility group box 1 and neutrophil enzymes, such as myeloperoxidase (MPO) and neutrophil elastase [132]. The release of NETs is triggered by various extrinsic stimuli, such as bacterial products and activated platelets. NETs are thought to increase the risk of thrombotic disease by providing a scaffold for thrombus components, including fibrinogen and platelets [132, 133]. They can bind tissue factor and factor XII, inactivate tissue factor pathway inhibitor, and activate platelets through NET-associated damage-associated molecular patterns (DAMPs), such as histones, S100A8/A9, and cathepsin G [134, 135]. This may enhance platelet aggregation, procoagulant platelet formation, and thrombin generation [136, 137]. In this way, NETs may contribute to prothrombotic status in patients with cirrhosis. NETs may also contribute to the pathogenesis of chronic inflammatory disease [133, 138]. In acute liver disease, NETs, highlighted with MPO-DNA complexes in plasma, were associated with poor outcome [139]. The authors suggested that NETs probably play a role in disease progression by formation of inflammation-induced microthrombi in organs, including the liver. A similar mechanism may also apply to patients with cirrhosis [140, 141].

Aberrant NET formation was already described in the pathogenesis of other inflammatory diseases and NET-targeting therapeutics have been put forward as potential treatments [142].

Different techniques can be used to highlight NET formation. Ideally, NETs are detected by colocalizing 3 components: extracellular DNA, histones and neutrophil elastase [133].

Cell-free DNA (cfDNA) is not a specific marker of NET formation as it originates from cell apoptosis and necrosis. MPO-DNA complexes are often used as markers of NETosis, although they could theoretically form as a result of neutrophil activation without NET formation [143]. Citrullinated histones are considered as more specific markers of NETosis [144].

4. METHODS

4.1. OVERVIEW OF METHOD CHAPTER

This chapter discusses the methods used to evaluate the three phases of hemostasis in pediatric patients: primary hemostasis, the coagulation cascade, and fibrinolysis. Detailed descriptions of the methods for the pediatric cohort, including recruitment, are provided in the papers included in the "Results and Discussion" chapter of this thesis. Readers are referred to those papers for further information. Here, we focus on the challenges and objectives encountered during method validation, addressed in two methodological papers. The first paper explores an issue with a fluorochrome-coupled antibody used in the platelet flow cytometry assay (primary hemostasis subsection). The second paper investigates biological variation in thrombin generation using an automated platform (coagulation cascade subsection). Finally, we outline the differences in methods applied to the adult patient cohort, including details on patient recruitment, assays, procedures, and statistical analyses.

4.2. PRIMARY HEMOSTASIS

4.2.1. INTRODUCTION

Details on the assays used for primary hemostasis are provided in our research paper titled “Primary hemostasis in children with cirrhosis: key roles of liver disease severity, von Willebrand factor, and platelet count” in the results chapter. The platelet flow cytometry setup involved the validation of three different panels. The primary panel was designed to identify procoagulant (Annexin V positive) and proaggregant (PAC-1 positive) platelets. A second panel focused on platelet receptors and activation markers, while a third panel was used to investigate platelet-leukocyte complexes. During assay validation, an issue arose with the fluorochrome-coupled anti-glycoprotein (GP) VI antibody (clone HY101), which activated platelets in vitro, leading to false positive results. As a result, this antibody was incorporated into a separate panel. This issue is discussed in detail in the paper below, published in Platelets [145].

4.2.2. METHOD PAPER: ANTI-GPVI (HY101) ACTIVATES PLATELETS IN A MULTI-COLOR FLOW CYTOMETRY PANEL

4.2.2.1. Abstract

The platelet transmembrane receptor GPVI can be assessed together with other platelet membrane markers in a whole blood multi-color flow cytometry panel. The advantage of combining multiple antibodies in a single tube is the possibility of distinguishing multiple platelet subgroups. In this short communication we describe an activation problem encountered with anti-GPVI, clone HY101. Activation of platelets was seen after the addition of anti-GPVI in a flow cytometry panel, highlighted by the expression of the activation markers CD62P, PAC-1, CD63 and CD107a. This was also confirmed by platelet aggregation studies.

4.2.2.2. Introduction

Glycoprotein (GP)-VI is a transmembrane protein, a member of the immunoglobin superfamily and one of the important platelet collagen receptors [86]. The expression of GPVI is restricted to platelets and megakaryocytes. GPVI is associated with dimeric Fc-receptor γ chain [146], which contains an immunoreceptor tyrosine-based activation motif (ITAM). Upon ligand binding, GPVI dimerization leads to phosphorylation of the tyrosine residues and further signal transduction ensues [147]. Finally, phospholipase Cγ2 becomes activated and calcium (Ca2+) level rises. Next to collagen, several other endogenous GPVI ligands exist, for example fibrin and adiponectin [148, 149]. The synthetic triple-helical collagen-related peptide (CRP), an exogenous ligand, consists of the glycine–proline–hydroxyproline (GPO)n tandem repeats that mediate binding of collagen to GPVI [150]. GPVI contains a cleavage site which enables shedding of the extracellular domain via the desintegrin and metalloproteinase 10 (ADAM10) upon activation [151].

GPVI on the platelet membrane can be assessed by flow cytometry. The availability of commercial anti-GPVI is still scarce. In the present work we would like to highlight an activation problem with anti-GPVI, clone HY101, directed against an epitope in the extracellular domain of GPVI. This antibody, coupled to a fluorochome, was used in a multi-color platelet flow cytometry protocol assessing both resting and activated state.

4.2.2.3. Methods

4.2.2.3.1. Flow cytometry

Peripheral blood was collected in a citrate tube (Monovette plastic 3.0 mL, 3.2% citrate, 106 mM (Sarstedt, Numbrecht, Germany); 9:1 blood:citrate ratio) after drawing a discard tube. Pre-analytical processing was performed according to Södergren et al [152]. In brief, blood samples were kept for 1 hour at room temperature before adding a mix of fluorochrome coupled Abs and HEPES buffer. Brilliant Stain Buffer (BD) was used in one experiment. For platelet activation experiments, agonists, Ca2+ and GPRP were added to the mix. Samples were incubated for 10 minutes at room temperature. Incubation was stopped by adding 1 mL of HEPES-Ca2+ buffer and analysed within 1 hour. The following platelet surface markers were included: CD41a phycoerythrin (PE, clone: HIP8, BD), CD42b BV711 (clone: HIP1, BD), CD42a APC-Vio® 770 (clone: REA209, Miltenyi Biotec, Bergisch Gladbach, Germany), PAC-1 fluorescein isothiocyanate (FITC, binds active conformation of αIIbβ3, BD), CD62P BV786 (anti-P-selectin, α-granule release, clone: AK-4, BD), CD107a PE-CF594 (anti-lysosomal-associated membrane protein 1 (LAMP-1), clone: H4A3, BD), CD63 BV510 (anti-LAMP-3, clone: H5C6, BD). This mix was tested both with and without anti-GPVI BV421 (clone: HY101, BD). In one analysis we also used anti-GPVI PE (HY101; BD) instead of anti-GPVI BV421. A BV421 Mouse IgG1, k isotype control was also included in the experiment (BD).

Anti-GPVI BV421 (0,2 mg/mL; 2 µL or 0,4 µg in a final volume of 60 µL; +-1.000.000 platelets) was tested at different dilutions: 1/10, 1/20, 1/30. An experiment in which paraformaldehyde (1% final concentration) was used to fix platelets before adding anti-GPVI BV421 was also executed. For platelet activation α-thrombin (T6884, Sigma-Aldrich, St. Louis, Missouri, US) and synthetic cross-linked collagen-related peptide (CRP-XL; CambCol Laboratories, Littleport, Cambridgeshire, UK) were used at final concentrations of 5 U/mL and 5 µg/mL respectively. Fibrin polymerisation was blocked with GPRP (G5779, Sigma-Aldrich, 2 mM final concentration). Analysis was done on the FACS ARIA II (Becton Dickinson (BD), Franklin Lakes, NJ, US). Compensation was executed with BD CompBeads (ref: 552843) and Miltenyi MACS CompBeads anti-REA (130-104-693). Acquisition was stopped after obtaining 10,000 platelets in a temporary gate. Platelets were selected on a forward/side scatter plot and, after removing doublets, the gate was set on the CD41 positive events.

4.2.2.3.2. Platelet aggregation

Platelet aggregation experiment was performed using light transmission aggregometry (Chrono-log 700, STAGO). Blood was drawn on a CPDA-tube (1/10; citrate, phosphate, dextrose, adenine) and centrifuged at 330 x g for 20 min. eptifibatide (4µg/mL) and apyrase (1 U/mL) were added to platelet rich plasma (PRP) and centrifuged 800 x g for 10min. Platelet pellets were washed once in modified Tyrode's buffer (135 mM NaCl, 12 mM NaHCO 3, 3 mM KCl, 0.3 mM Na 2HPO 4, 1 mM MgCl 2, 5 mM D-glucose, 10 mM Hepes, 0.35 % BSA, pH 7.4, 37 °C) containing eptifibatide (4 µg/mL) and apyrase (1 U/mL) at 1000 x g for 10 min. Platelets were re-suspended at a density of 2.5 x 10^5/µl in modified Tyrode's buffer. Light transmission of washed human platelets (52.2 x 10^6), stirred at 1200rpm, 37°C, was recorded after addition of 2mM $CaCl_2$ and GPVI Ab. A BV421 Mouse IgG1 isotype control was also tested.

4.2.2.4. Results

4.2.2.4.1. Flow cytometry

Results are depicted in **Figure 3**. In the flow cytometry panel without anti-GPVI BV421, no expression of platelet activation markers was seen in resting state. When anti-GPVI was added in resting state, activation was demonstrated with every platelet activation marker, reflecting α-granule release, lysosomal release and αIIbβ3 activated form. Compared to the positive control (addition of thrombin and CRP-XL), platelet activation marker expression was less pronounced in the resting panel with anti-GPVI. Activation diminished with lower concentrations of anti-GPVI (**Supplementary Figure 1**), although CD62P was still activated at 55.8% for a 1/30 dilution. Mean fluorescence intensity at different dilutions and in comparison with the isotype control is shown in **Supplementary Figure 2**. Replacing anti-GPVI BV421 with anti-GPVI PE also resulted in platelet activation

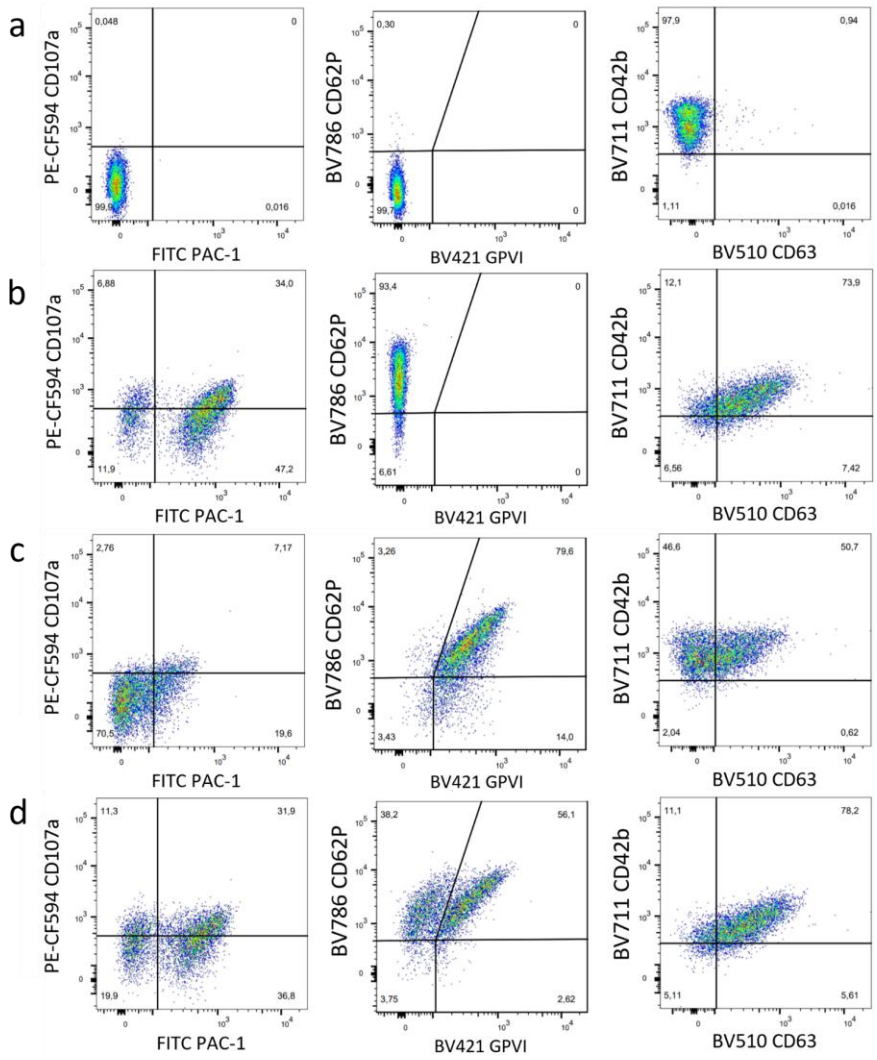


Fig. 3. Flow cytometry plots of different platelet surface markers in four different conditions (n = 4 biological replicates): without GPVI in resting state (**a**), without GPVI after stimulation by thrombin 5 U/mL and CRP-XL 5 µg/mL (**b**), with GPVI [6,67 µg/mL] in resting state (**c**), with GPVI [6,67 µg/mL] after stimulation by thrombin 5 U/mL and CRP-XL 5 µg/mL (d).

(**Supplementary Figure 3**). Adding the Brilliant Stain Buffer did not have an impact on results (**Supplementary Figure 4**). Fixation with paraformaldehyde 1% before addition of anti-GPVI abolished activation of platelets demonstrated by negative activation markers (**Supplementary Figure 5**).

4.2.2.4.2. Platelet aggregation

Results are depicted in **Figure 4**. Platelet aggregation was performed using a high (6.67µg/mL) and a low (0.2µg/mL) anti-GPVI Ab concentration. In agreement with the flow cytometry experiment, only high concentration of anti-GPVI Ab induced platelet aggregation.

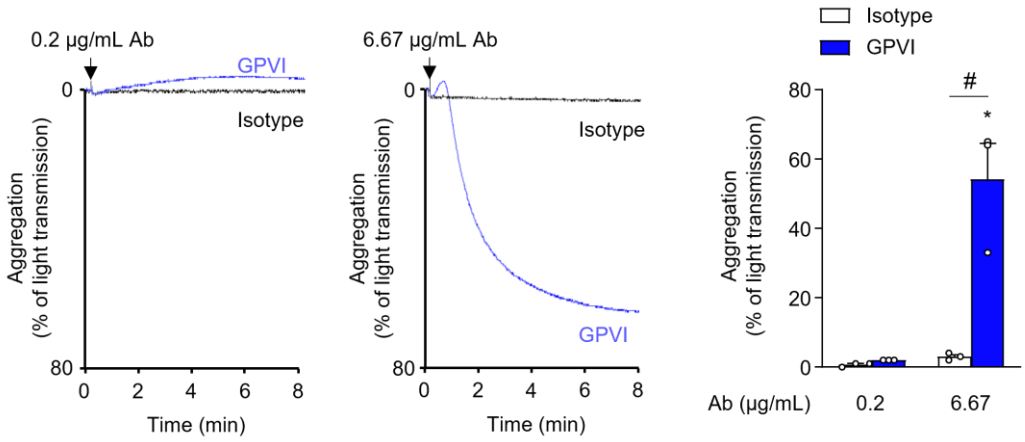


Fig. 4. Platelet aggregation stimulated with GPVI-BV421 (HY101) antibody or its isotype (BV421 Mouse IgG1k) at two different concentrations. Profiles are shown on the left. Data were expressed as means ± SEM (n = 3). * p-value ≤ 0.001 in relation to 0.2 µg/mL Ab concentration. # p-value ≤ 0.001 in relation to isotype Ab. Data were analyzed using 2–way ANOVA.

4.2.2.5. Discussions and conclusion

A whole blood multi-color platelet flow cytometry panel is a valuable tool to map platelet phenotype in a rather easy and rapid way. The advantage of combining multiple Abs in a single tube is the possibility of distinguishing multiple platelet subgroups. Platelets being easily activated, it is paramount to thoroughly validate the technique used beforehand to avoid any (pre-) analytical problems [153].

In this short communication we describe an activation problem encountered with anti-GPVI, clone HY101, in a whole blood platelet flow cytometry panel. This activation issue was confirmed with a platelet aggregation study. Neither replacing anti-GPVI BV421 (HY101) with anti-GPVI PE (HY101), nor the addition of Brilliant Stain Buffer, recommended when using >1 brilliant dye, had an impact on flow cytometry results. Fixating the platelets before addition of anti-GPVI, abolished the activation problem, but complicates the staining protocol, especially for agonist activation assays.

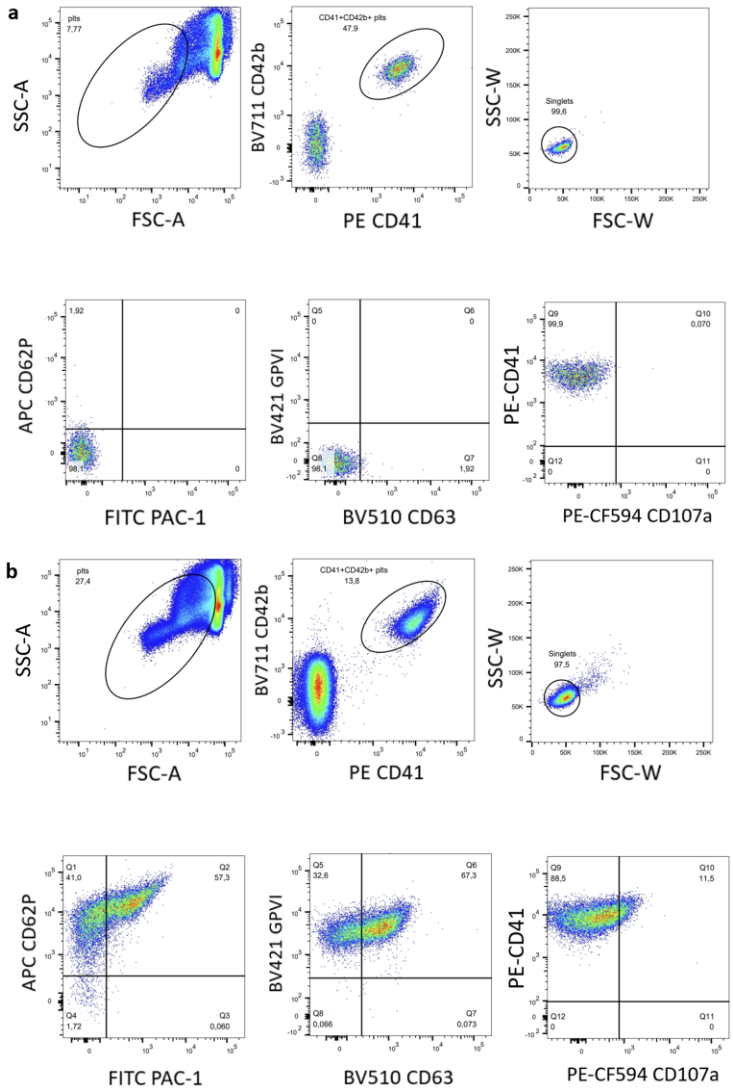
The anti-GPVI monoclonal antibody, recognizing the extracellular domain of GPVI, was generated for the first time by Chen et al [154]. The authors state that binding of HY101 on platelets does not interfere with collagen or convulxin signaling, suggesting a binding region distinct from the ligand binding site. However, the article did not mention experiments studying the ability of HY101 to aggregate nor activate platelets. Trifiro et al [155] state that "HY101 is a neutral monoclonal Ab that neither activates GPVI nor inhibits ligand binding to GPVI." It is however not clear if this was tested by the authors. In a paper looking at aggregation and shedding properties of different anti-GPVI Abs, HY101 was unfortunately not included [156]. In this article, the majority of Abs identified against the extracellular domain of GPVI (n = 8) were able to induce aggregation, except for the 11A7 clone. Fab fragments from the same Abs, except for 11A7, did not induce aggregation but were able to inhibit collagen- and CRP -induced aggregation. Another study, describing a GPVI deficient patient, used anti-GPVI HY101 to evaluate membrane GPVI expression. Other platelet membrane markers including the activation marker CD62P were analysed. Again, it is not clear if anti-GPVI was added to the cocktail of Abs. If not, platelet activation in resting state or inhibition of activation in collagen-stimulated conditions by anti-GPVI HY101 would not have been detected in the control samples. The majority of papers mentioned above did not test anti-GPVI HY101 induced aggregation nor activation, possibly explaining why the activating property of HY101 has not been described yet.

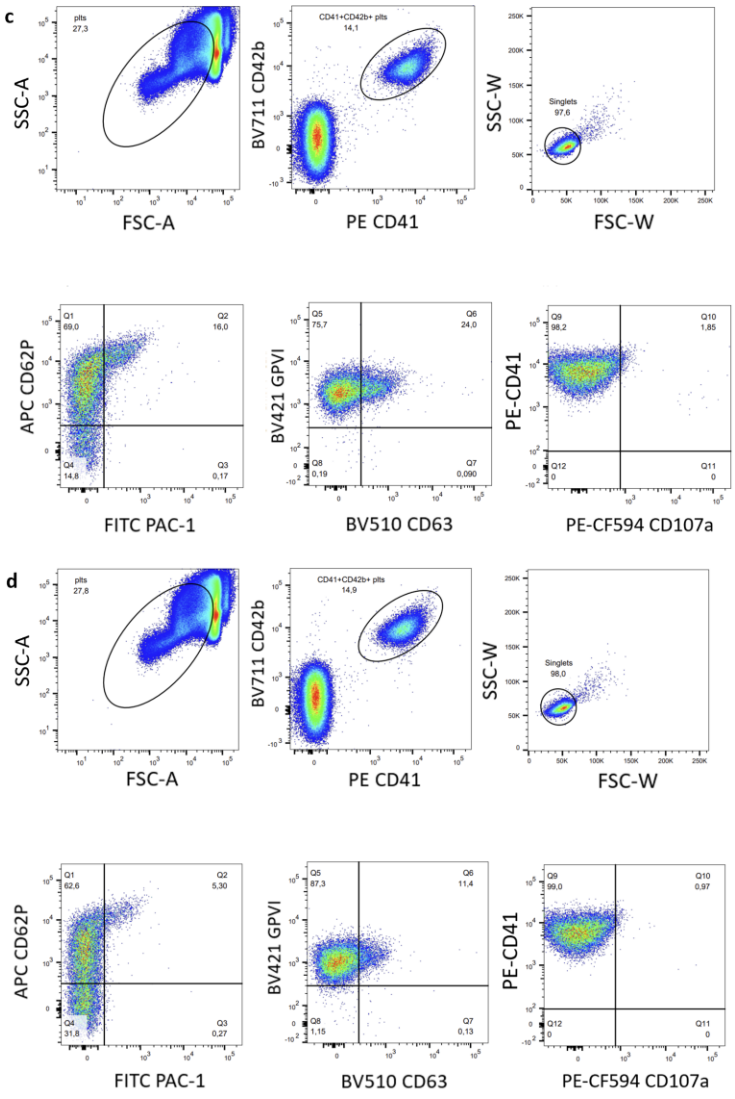
The mechanism of platelet activation by the monoclonal anti-GPVI Ab is not clear. Given that HY101 clone is described as neutral and activates platelets in a dose-dependent manner, it is very likely that platelets are activated by clustering FcγRIIa receptor. Why some monoclonal Abs cluster FcγRIIa receptor while others do not is not known [86]. This cluster effect diminishes with lower concentrations of Ab. By assessing aggregation in the presence of IV.3 (FcγRIIa blocker) the article by Al-Tamimi et al concluded a direct agonist role independent of the FcγRIIa receptor for the GPVI antibodies studied [156].

Recently, Biocytex (Stago, Asnières, France) developed a quantification assay using anti-GPVI 1G5 clone. It is believed that this assay should be used to establish international standards for GPVI expression at the platelet surface [157]. However the same clone (1G5)

has been described to induce aggregation and GPVI shedding [156]. In our opinion, this is not a suitable clone for multi-color platelet flow cytometry panels using platelet agonists. A non-activating GPVI antibody like anti-GPVI clone 11A7 could be a good alternative for utilization in multi-color platelet agonist experiments. To our knowledge, this clone is not commercially available. Also of interest, Jung et al [158] described GPVI dimer-specific antibodies, m-Fab-F and 204-11 Fab. They imply that GPVI dimer is the collagen-binding form of GPVI. These Fab fragments inhibit/block however collagen binding. Another alternative for avoiding anti-GPVI induced activation, is the "fix-first method" described by Spurgeon et al [159] where the antibody cocktail is added after formaldehyde fixation. The disadvantage of this technique is a possible decrease in staining intensity in for example anti-CD62P. PAC-1 staining may be abolished after formaldehyde fixation.

4.2.2.6. Supplementary data




c
plts
27,3
SSC-A
FSC-A
CD41+CD42b+ plts
14,1
BV711 CD42b
PE CD41
Singlets
97,6
SSC-W
FSC-W
Q1
69,0
Q2
16,0
Q4
14,8
Q3
0,17
APC CD62P
FITC PAC-1
Q5
75,7
Q6
24,0
Q8
0,19
Q7
0,090
BV421 GPVI
BV510 CD63
Q9
98,2
Q10
1,85
Q12
Q11
0
PE-CD41
PE-CF594 CD107a
d
plts
27,8
SSC-A
FSC-A
CD41+CD42b+ plts
14,9
BV711 CD42b
PE CD41
Singlets
98,0
SSC-W
FSC-W
Q1
62,6
Q2
5,30
Q4
31,8
Q3
0,27
APC CD62P
FITC PAC-1
Q5
87,3
Q6
11,4
Q8
1,15
Q7
0,13
BV421 GPVI
BV510 CD63
Q9
99,0
Q10
0,97
Q12
0
Q11
0
PE-CD41
PE-CF594 CD107a

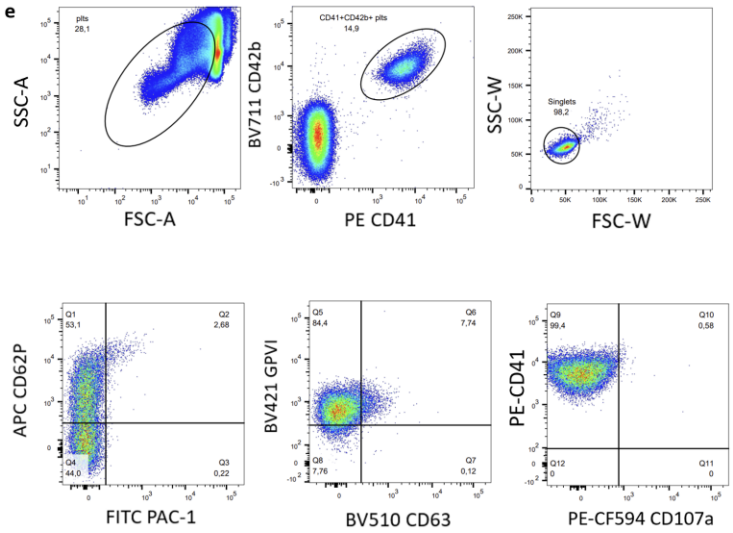


Fig. S1. Anti-GPVI at different dilutions (n = 1). Without anti-GPVI (**a**), not diluted (0.4 µg in a total volume of 60 µL; **b**), 1/10 dilution (0.04 µg in a total volume of 60 µL; **c**), 1/20 dilution (0.02 µg in a total volume of 60 µL; **d**), 1/30 dilution (0.013 µg in a total volume of 60 µL; **e**)

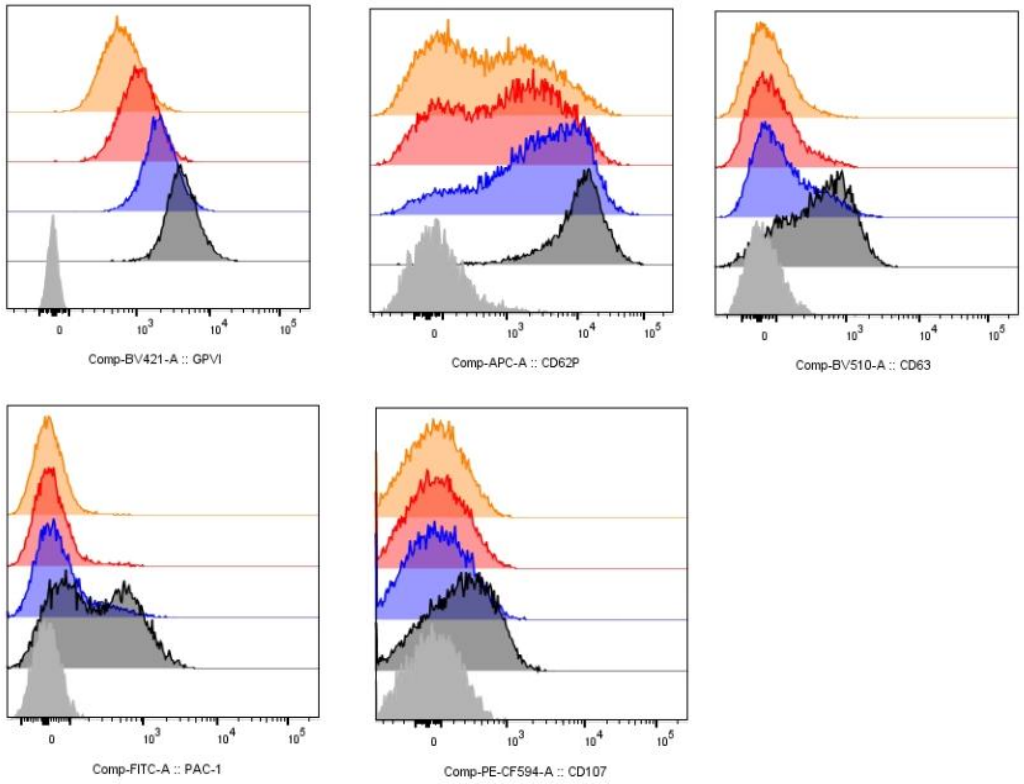


Fig. S2 (n = 1): **Mean fluorescence intensity of anti-GPVI BV421, anti-CD62P, anti-CD63, anti-PAC-1 and anti-CD107a at different dilutions**: not diluted (grey), 1/10 dilution (blue), 1/20 dilution (red), 1/30 dilution (orange). The BV421 k isotype control is depicted in light grey.

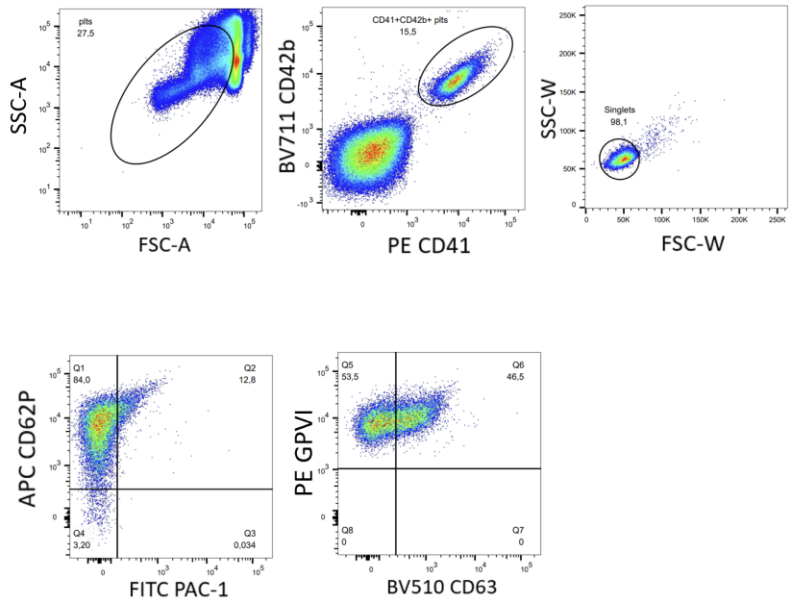


Fig. S3 (n = 1). **Anti-GPVI PE** (0.4 µg in a total volume of 60 µL)

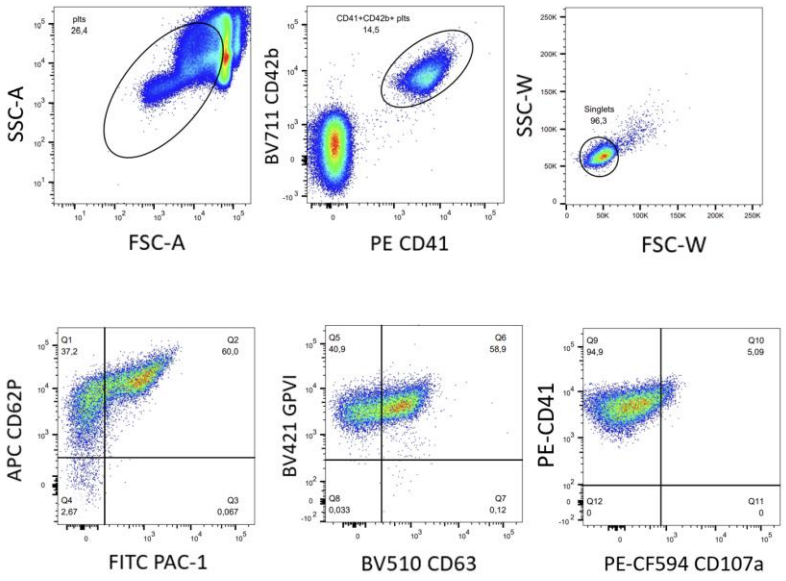


Fig. S4 (n = 1). **Addition of Horizon Brilliant stain buffer.**

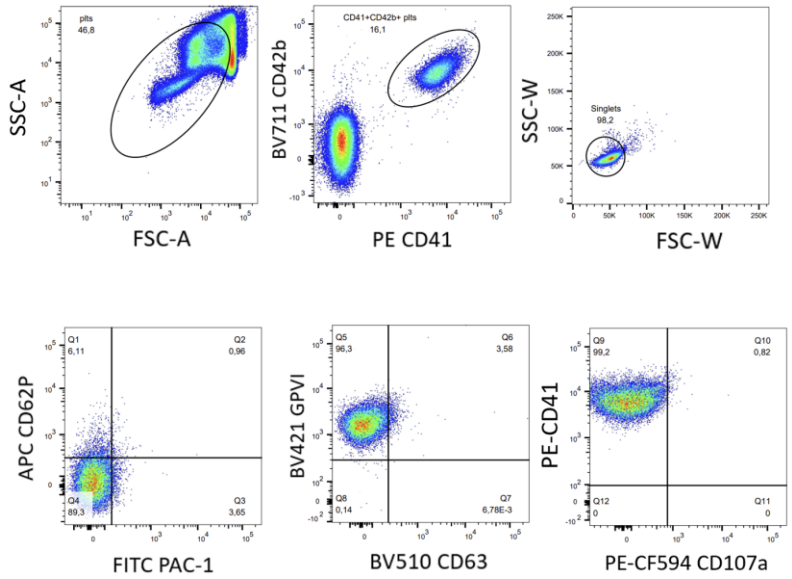


Fig. S5. Fixation with paraformaldehyde 1% before addition of anti-GPVI BV421 (n = 1).

4.3. COAGULATION CASCADE

4.3.1. INTRODUCTION

Details on the assays used for the evaluation of the coagulation cascade are provided in our research paper titled “Persisting thrombomodulin resistance at 3 months after liver transplantation in children with cirrhosis” in the results section.

In our adult cohort, we also performed a modified thrombin generation assay with the addition of activated protein C. In this assay resistance to activated protein C was studied independently of patient levels of protein C [160]. This was realized in collaboration with Prof. Jonathan Douxfils (University of Namur).

Thrombin generation was determined on platelet poor plasma using the recently released automated analyzer ST Genesia. In order to establish analytical goals for this new assay, biological variation was studied on previously collected plasma in healthy volunteers [161]. The findings from this study are presented in the paper included below [162].

4.3.2. METHOD PAPER: BIOLOGICAL VARIATION OF ST GENESIA

Dear Editors,

We previously reported on the biological variation of the thrombin generation assay (TGA) with and without thrombomodulin (TM) using a calibrated automated thrombogram (CAT) system [161]. Recently, the ST Genesia (Diagnostica Stago, Asnières sur Seine, France), a fully automated thrombin generation system, was launched with the aim of standardizing TGA by reducing inter-experiment and inter-laboratory variation. This study therefore aimed to determine the biological variation of TGA on this automated platform.

Important information on the whole coagulation process is not provided in routine hemostasis tests based on optical or mechanical detection of clot formation, (i.e., prothrombin time and activated partial thromboplastin time) but deserves to be investigated to fine-tune the identification of coagulopathies and hemorrhagic status [163]. Thrombin generation depicts thrombin generation and inactivation using a fluorogenic substrate. In adding soluble TM to the testing mixture, the endogenous protein C pathway is also investigated. Thrombin generation has been largely used in the research setting but has gained interest in a clinical context, like in hemophilia A, or to assess inherited or acquired coagulopathies [164, 165].

Three different determinants are responsible for the total variability of a parameter in an individual over time: (i) pre-analytical variation, (ii) analytical variation, and (iii) biological variation. Biological variation consists of both within-subject variation (CV_I; fluctuation of a value around a subject homeostatic setting point) and between-subject variation (CV_G; difference in homeostatic setting points between different subjects). Few studies report on the biological variation of thrombin generation. This type of study helps laboratories to define reference change values (RCV) which may support the interpretation of serial measurements at the individual level. In addition, biological variation of a parameter can aid in setting analytical specification goals, now recommended by the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) [166].

To study biological variation, 19 healthy adults were followed for 5–6 weeks with a once weekly blood draw. Samples were analyzed on the ST Genesia using the STG-ThromboScreen reagent. After outlier exclusion, components of variation were obtained by nested analysis of variance (ANOVA). Analytical variation (CV_A), within-individual variation (CV_I), between-individual variation (CV_G), index of individuality (II) and RCV were calculated. Informed consent was obtained from each participant, and the study protocol was approved by the local Ethics Committee (2019/29JUL/340).

Nineteen apparently healthy volunteers were included with a median age of 33.5 (range 22–60 years), 11 (61%) were female. **Figure 5** represents the mean and absolute range for each individual for all parameters without and with TM. Due to the known effect on hemostasis, results of healthy volunteers taking oral contraceptives (OC; n = 4) were compared with results of healthy volunteers excluding healthy volunteers on OC. A significant difference was seen between subjects on OC and the group of subjects not taking OC for the mean of all parameters. Both subgroups were therefore studied separately. From the remaining group of 15 healthy volunteers, 2 were excluded for all parameters and 1 for lag time (LT) and time-to-peak (TTP) only. No difference between sexes was found after exclusion of the OC group. For both TGA without and with TM, CV_A,CV_I, and CV_G are represented in **Table 2**. For the non-OC group, CV_A ranged from 1.6% to 8.1%, CV_I from 3.9% to 16.0% and CV_G from 8.1% to 30.6% for parameters without TM. For TGA in presence of TM, CV_A varied from 1.9% to 4.7%, CV_I from 3.3% to 16.6% and CV_G from 7.2% to 44.6%. Details on the OC group can be found in **Table 2**. II and RCV are shown in **Table 3** for parameters without and with TM. For all TGA parameters (±TM) II was low (<1) in the non-OC group. For the OC subgroup, the index was high for TTP, peak, ETP, and velocity index. In the non-OC group, RCV values ranged from 13.5% to 49.8% and from 10.4% to 48% without and with TM respectively. Similar RCV's were seen for the OC-group, except for peak and ETP with TM. For all parameters, no significant difference in mean was seen between men and women without OCs. This is in line with the article by Ninivaggi et al [167]. In this paper 117 healthy donors were analyzed by ST Genesia to establish reference values. Calvarazini et al. reported different normalized reference ranges according to sex and age with ST Genesia [168]. After exclusion of women on OC, these differences were eliminated except for the differences seen between women <50 years and women ≥50 years. They proposed to use four separate reference interval categories for routine laboratories: men, women aged <50 years on OC and women aged ≥50 years. Kristensen et al. only saw a minor effect of age [169]. For women aged ≥50 years, the use of hormonal replacement therapy may also lead to change in hemostasis which may require an additional category of subjects.

Like our previous study on TGA with CAT, CV_I and especially CV_G were higher in TGA with TM compared to TGA without TM for LT, peak, ETP and velocity index [161]. As already mentioned in the previous study, this could be due to a difference in resistance to the TM/protein C axis or a difference in protein C or protein S concentration. Between-individual variation (CV_G) was 9.1% for protein C and 24.1% for protein S in this patient group which could support our hypothesis [170]. Absolute values are also lower in TG with TM for peak, ETP and velocity index, which could explain a higher CV in percentage. When comparing groups not taking OC between these studies, CV_A was comparable for parameters without TM (peak and velocity index a little higher with ST Genesia) and CV_A was

lower on ST Genesia for all parameters with TM. This would be expected since the ST Genesia is more standardized and less operator dependent, although performance was very similar with CAT in the present study. Comparable imprecision between ST Genesia and CAT was also seen by Kristensen et al [169]. CV_I was comparable for most parameters (except for ETP without TM: 4.1% with CAT versus 7.0% with ST Genesia and LT without TM: 5.9% with CAT versus 3.9% with ST Genesia). Oddly, more important differences in CV_G were observed between the two methods.

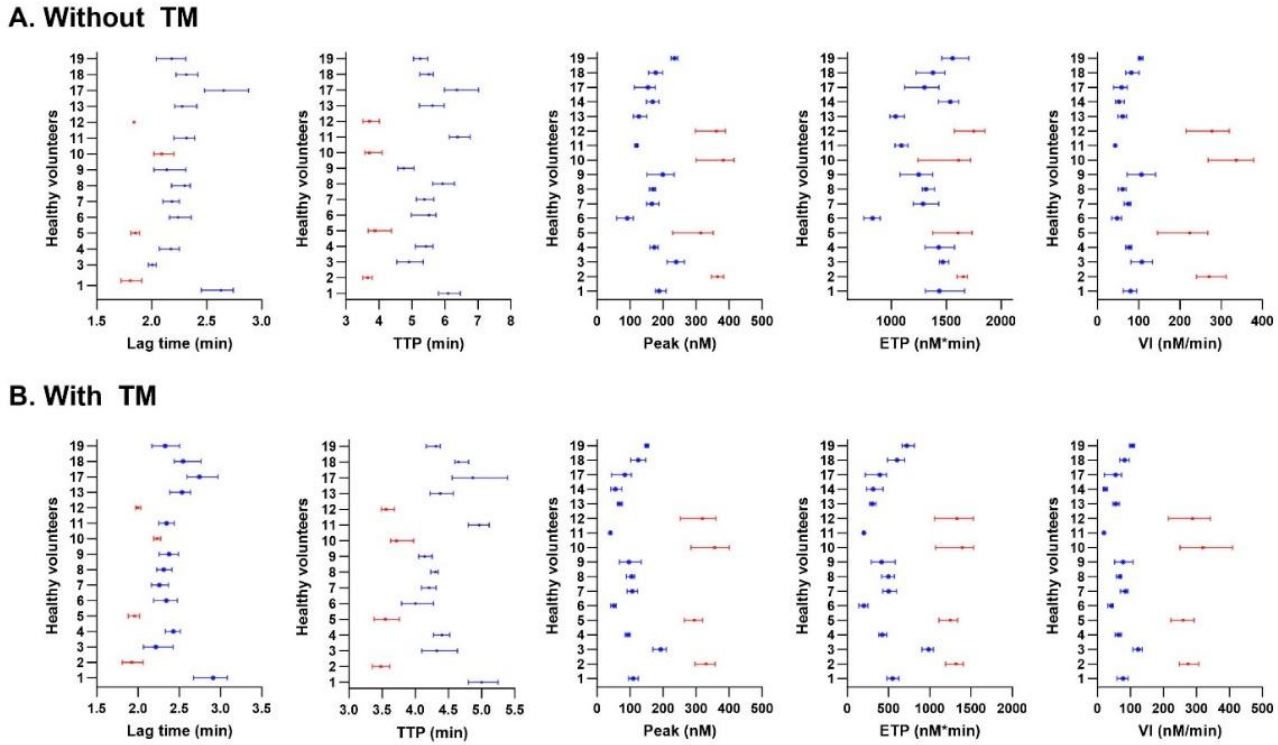


Fig. 5: **Representation of individual mean and absolute range (min-max) of values for each parameter without thrombomodulin (A ; Without TM) and with thrombomodulin (B, With TM)**. Blue: healthy volunteers not on oral contraceptives. Red: healthy volunteers on oral contraceptives. The outlying results are excluded from the Figure.

Table 2. Components of variation for ST Genesia with and without thrombomodulin.

Parameter	Group	Mean		CV_A (%)		CV_I(%)		CV_G (%)	
		w/o TM	TM	w/o TM	TM	w/o TM	TM	w/o TM	TM
LT	w/o OC group (n = 12)[a]	2,28	2,45	3,00	1,93	3,85	4,65	8,06	8,18
	OC (n = 4)[b]	1,91	2,04	2,38	1,78	2,67	3,20	6,65	7,13
TTP	w/o OC group (n = 12)[a]	5,59	4,47	1,64	1,86	4,67	3,26	9,07	7,24
	OC (n = 4)[b]	3,73	3,58	1,67	1,25	5,54	3,33	0,59	2,33
peak	w/o OC group (n = 13)[a]	170,76	98,25	3,08	3,74	9,94	14,23	24,10	41,38
	OC (n = 4)[b]	359,79	326,33	1,84	1,63	9,18	11,72	3,23	5,47
ETP	w/o OC group (n = 13)a	1316,89	494,90	2,54	4,52	7,01	14,11	15,29	44,63
	OC (n = 4)b	1664,17	1320,61	2,71	2,52	6,98	8,95	2,02	3,41
VI	w/o OC group (n = 13)[a]	72,81	68,83	8,13	4,73	16,03	16,66	30,57	41,97
	OC (n = 4)[b]	268,98	281,38	7,33	3,56	15,70	18,22	11,71	8,35

Abbreviations: CV_A, analytical coefficient of variation; CV_G, between-individual variation; ETP, endogenous thrombin potential; LT, lagtime; Peak,thrombin peak height; TM, CAT with thrombomodulin; TTP, time-to-peak; VI, velocity index; w/o, TM CAT without thrombomodulin.

[a] w/o OC group: healthy volunteers without subjects on oral contraceptive therapy.

[b] OC group: healthy volunteers on oral contraceptive therapy.

RCVs, based on CV_A and CV_I were very similar between the OC and non-OC subgroups, except for peak and ETP with TM. A low II, reflecting a larger CV_G compared to CV_I, was seen for all parameters in the subgroup without OC. This was already reported by Hemker et al. in 2003 [171]. Interestingly, II was high for all parameters, except for LT, in the OC subgroup. Although it is difficult to draw conclusions in this very small (n = 4) subgroup, this high index suggests an impact of the OC treatment on the individual's homeostatic setting point. OC is well known to induce a hypercoagulable TGA profile and resistance to inactivation by TM or activated protein C [172]. CV_G was a lot smaller in the OC group compared to the subgroup without OC. A fixed reference interval could be of value in this latter group. For the non-OC group, clinicians could better rely on sequential follow-up rather than reference intervals but this needs to be confirmed in a larger cohort.

Table 3. Index of individuality and reference change values for CAT without and with thrombomodulin.

Parameter	Group	II		RCV (%)	
		-TM	+TM	-TM	+TM
LT	w/o OC group (n = 12)[a]	0,61	0,62	13,53	13,96
	OC (n = 4)[b]	0,54	0,51	9,91	10,15
TTP	w/o OC group (n = 12)[a]	0,55	0,52	13,71	10,40
	OC (n = 4)[b]	9,84	1,52	16,04	9,85
Peak	w/o OC group (n = 13)[a]	0,43	0,36	28,84	40,78
	OC (n = 4)	2,90	2,16	25,95	32,80
ETP	w/o OC group (n = 13)[a]	0,49	0,33	20,67	41,07
	OC (n = 4)[b]	3,71	2,73	20,75	25,77
VI	w/o OC group (n = 13)[a]	0,59	0,41	49,82	48,00
	OC (n = 4)[b]	1,48	2,22	48,03	51,46

Abbreviations: ETP, endogenous thrombin potential; II, index of individuality; LT, lag time; RCV, reference change value; Peak, thrombin peak height; TM, thrombomodulin; TTP, time-to-peak; VI, velocity index.

[a]w/o OC group: healthy volunteers without females on oral contraceptive therapy.

[b]OC group: healthy volunteers on oral contraceptive therapy.

Some limitations can be pointed out in our work. One major limitation is the low subject size. As stated before, the subgroup on OC was very small. Although the subgroup not on OC is still within limits of the article by Ricos et al [173], subgroup analysis could be hampered by this low number of subjects. We did not evaluate normalized results. Kristensen et al [169] did not see an improvement of analytical variation after normalization of results, but normalization might improve within and between subject variation by lowering batch to-batch variation. However, as these samples were analyzed with the same batch of reagent, it is unlikely that normalization would improve the CVs. Also, since healthy volunteers were followed for 5 weeks, we can only comment on short term biological variation. A relatively young group of healthy volunteers was included (median age: 36 years) and the impact of age on biological variation requires further study.

In conclusion, we assessed the short-term biological variation of TGA parameters with STG-ThromboScreen on ST Genesia, a fully automated TGA platform. CV_I (%) and CV_G (%) for thrombin generation with TM were higher than for thrombin generation without TM for most

parameters. II and RCV were calculated for every TGA parameter. In general, parameters were highly individualized in healthy volunteers not on OC. The opposite was seen for healthy volunteers on OC for TTP, peak, ETP, and velocity index, suggesting a different approach for interpreting thrombin generation results in these subjects but this deserves further investigations in a larger cohort of subjects.

4.4. FIBRINOLYSIS

To evaluate fibrinolysis, two different plasma-based global fibrinolysis assays were used. Detailed information on both assays is provided in the paper titled "Fibrinolytic profile depends on disease severity in pediatric patients with cirrhosis: illustration by 2 different plasma-based fibrinolysis assays" in the results and discussion chapter [3]. In supplementary table 1, the differences between the 2 assays are highlighted.

Factor XIII was measured using an amine incorporation activity assay according to Ariëns et al [174]. In brief, fibrinogen was coated on a microtiter plate. After addition of diluted patient or control plasma (in duplicate), the cross-linking reaction was started by adding a mixture of α-thrombin, 5-(biotinamido)pentylamine, DTT and $CaCl_2$. Reaction was stopped with EDTA after 3 and 10 minutes incubation at room temperature. Streptavidin–alkaline phosphatase was added to the wells and incubated for 1 hour at 37°C. For color development *p*-nitrophenyl phosphate was added. The change in absorbance, measured at 3 and 10 minutes was proportional to FXIII activity and expressed as a percentage of pooled normal plasma. Between different steps, multiple wash steps were performed.

Plasminogen Activator Inhibitor 1 (PAI-1) antigen was measured using the Asserachrom ELISA (Stago, Asnières-sur-Seine, France).

D-dimer levels were assessed on the automated coagulation analyzer ACL-TOP 750 (Werfen, Barcelona, Spain) using the reagent HemosIL D-dimer HS 500.

4.5. INFLAMMATION

Both cfDNA and MPO-DNA complexes were measured in our study. This was realized by the University Medical Centre Groningen under the supervision of professor Ton Lisman. The method description below is cited from the paper by von Meijenfeldt et al [175].

“The concentration of cfDNA was measured using the Quant-iT PicoGreen double-stranded DNA assay kit (Fisher Scientific, Landsmeer, the Netherlands). In short, 10 µL of plasma was added to 90 µL of trishydroxyme thylaminomethane (Tris)–ethylene diamine tetraacetic acid (EDTA) buffer (10-mM Tris-HCl, 1-mM EDTA, pH 7.5) in a 96-well microtiter plate. Next, 100 µL of PicoGreen solution (diluted 1:200 in Tris-EDTA buffer) was added to the wells. After 10 minutes of incubation in the dark at room temperature, fluorescence was measured at 480-nm excitation and 520-nm emission using a Victor (PerkinElmer, Groningen, the Netherlands) photometer. Plasma levels of MPO-DNA complexes, which are a more selective marker for NETs, were quantified by ELISA as previously described [176], using a commercially available monoclonal antibody to MPO (Sanbio, Uden, the Netherlands) and the detection antibody derived from the cell death detection ELISA (Sigma Aldrich, Zwijndrecht, the Netherlands).”

4.6. ADULT CIRRHOTIC COHORT

4.6.1. STUDY PARTICIPANTS

From April 2022 to September 2023, adult cirrhotic patients were prospectively enrolled 1 day before liver transplantation. Written informed consent was obtained from the patient. Exclusion criteria were acute liver failure, re-transplantation, platelet transfusion before blood draw and combined organ transplantation. In a subset of patients measurement were also executed on portal blood, sampled directly during liver transplantation (**Figure 6**), and 3 months after transplantation. Upper gastrointestinal endoscopy and hepatic Doppler ultrasound were performed as standard of care. In addition, apparently healthy control subjects were included.

4.6.2. ASSAYS

Standard diagnostic testing was performed in all patients and included a complete blood cell count, routine hemostasis tests and biochemistry assays for C-reactive protein, liver enzymes, bilirubin, creatinine and albumin. Platelet functionality was assessed with flow cytometry, along with plasma proteins including von Willebrand factor and ADAMTS13. Platelet flow cytometry was assessed as outlined in the paper on the pediatric cohort titled "Primary hemostasis in children with cirrhosis: key roles of liver disease severity, von Willebrand factor, and platelet count" in chapter 5.2.

Due to practical considerations microfluidic measurements were not performed in adult patients and controls.

The evaluation of the coagulation cascade included measuring levels of coagulation factors and natural anticoagulants, as well as thrombomodulin-modified thrombin generation. Details on these assays are outlined in Chapter 5.4. As outlined in the methods chapter, we also conducted an activated protein C-modified thrombin generation assay.

Similar to pediatric patients, fibrinolysis was assessed with two plasma-based global fibrinolytic assays. Antigen levels of PAI-1 were also performed. Detailed descriptions of these assays can be found in Chapter 5.5.

4.6.3. STATISTICAL ANALYSIS

Statistical analysis was performed with GraphPad Prism, version 9.5.1 and R, version 3.4.0. Normal distribution was assessed with the Kolmogorov-Smirnov test. Comparisons between patient and control groups were made using an unpaired Student's t-test for

normally distributed data or a Mann-Whitney U test for non-parametric data. For comparisons between peripheral and portal blood, a paired Student's t-test was used for normally distributed data, or a Wilcoxon test for non-parametric data. RStudio (version 2023.12.1) was employed to correct for multiple comparisons using the Benjamini-Hochberg method. Clinical patient data were entered into a REDCap database.

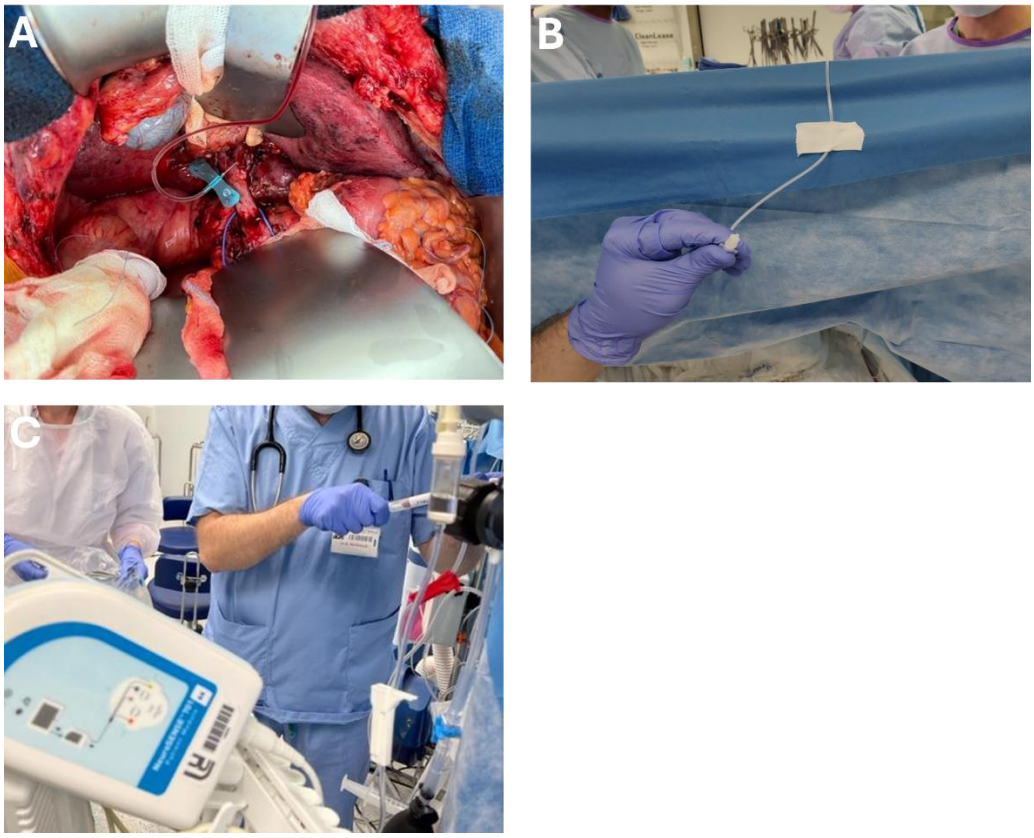


Fig.6. Direct sampling of the portal vein. A. Puncturing the portal vein with a 23G butterfly needle. (B) the butterfly needle tubing is connected with a Multi-Adapter. (C) Collection of portal blood directly in a 3.2% citrate tube (after collecting a discard tube).

5. RESULTS AND DISCUSSION

5.1. OVERVIEW OF RESULTS AND DISCUSSION SECTION

Similar to the method section, this section is structured into the 3 phases of hemostasis with a research paper presented for each coagulation phase. Following this, results related to inflammation (specifically neutrophil-leukocyte interactions) and the adult patient cohort are discussed and, where relevant, compared to the pediatric cohort.

The first paper covers the key findings of this doctoral thesis regarding primary hemostasis in children with cirrhosis. Pediatric cirrhotic patients demonstrated relatively preserved primary hemostasis. Two subgroups with differences in platelet thrombus formation were identified pre-transplantation, likely depending on severity of liver disease, platelet count and von Willebrand factor levels.

In our second research paper, we investigated thrombomodulin-modified thrombin generation in children with cirrhosis before and 3 months after liver transplantation. Interestingly, even 3 months after liver transplantation thrombomodulin resistance persisted in virtual all patients sampled.

In the third paper, the results obtained with two plasma-based fibrinolysis assays are presented. Here, we observed significant heterogeneity among patients. The majority of children with liver disease displayed a normal fibrinolytic profile. However, children with severe cirrhosis exhibited an abnormal profile, showing both hypo- and hyperfibrinolysis.

5.2. RESULTS PAPER: PRIMARY HEMOSTASIS IN CHILDREN WITH CIRRHOSIS: KEY ROLES OF LIVER DISEASE SEVERITY, VON WILLEBRAND FACTOR, AND PLATELET COUNT

5.2.1. ABSTRACT

Background & Aims

Thrombocytopenia is common in pediatric cirrhotic patients, but the extent and relevance of functional platelet defects remain unclear. This proof-of-principle study aimed to characterize platelet properties in cirrhotic children before living donor liver transplantation, using state-of-the-art platelet-based assays, hypothesizing that primary hemostasis in this group is relatively well preserved.

Methods

From January 2022 to July 2023, pediatric cirrhotic patients were prospectively enrolled 1 day before liver transplantation. An age-matched control group was included. Platelet functionality was assessed using flow cytometry and microfluidic assays, along with plasma proteins including von Willebrand factor, ADAMTS13, and soluble glycoprotein VI. A subset of patients was also re-evaluated 3 months post-transplant.

Results

Twenty-seven pediatric cirrhotic patients, primarily with cholestatic liver disease, and 15 controls were enrolled. Patients had pediatric end-stage liver disease scores ranging from -10 to 44. Flow cytometry revealed limited platelet activation without agonists and reduced activation with high agonist concentrations. Microfluidic assays showed moderately impaired thrombus formation with high inter-patient variability. Unsupervised clustering identified two subgroups: one with severe liver disease and higher platelet counts, showing preserved primary hemostasis and another with lower platelet count and moderate hemostatic impairment. Few bleeding events occurred pre- and post-transplant and no excessive blood loss was observed during surgery.

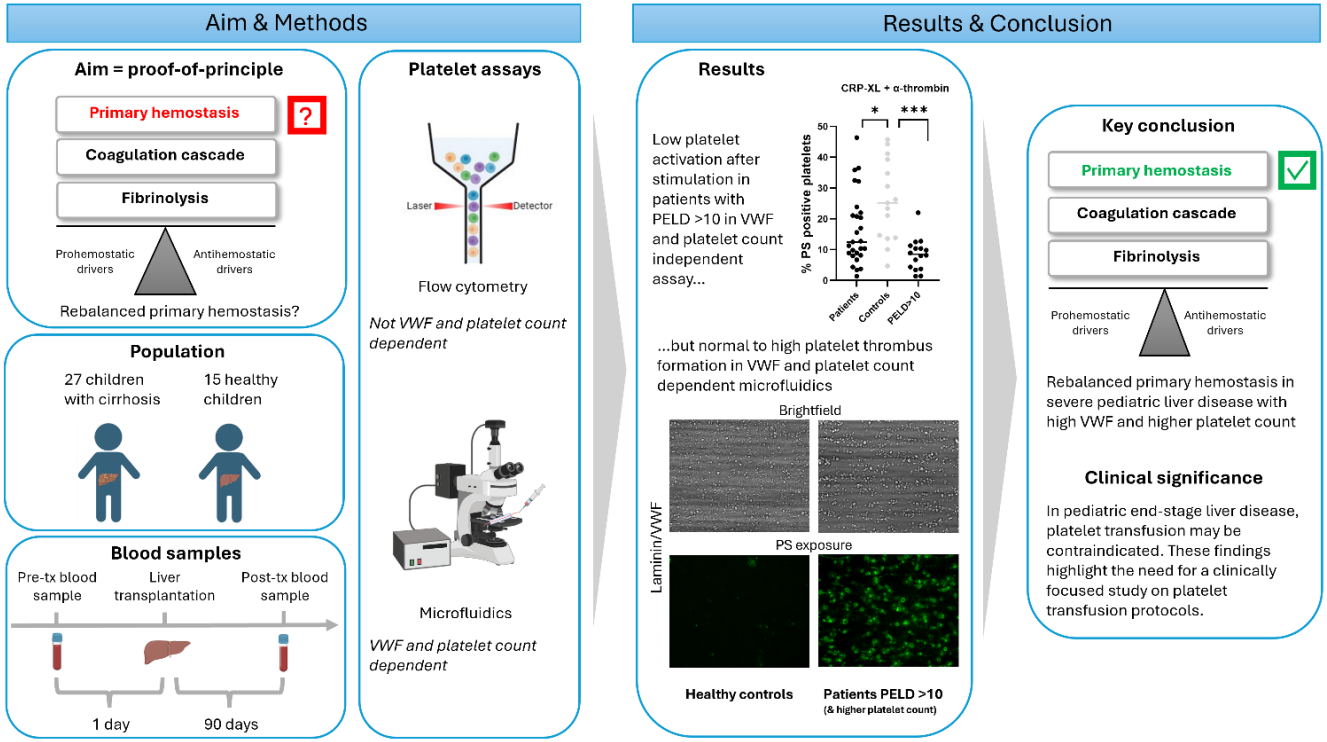
Conclusion

Pediatric cirrhotic patients demonstrated relatively preserved primary hemostasis. Two subgroups with differences in platelet thrombus formation were identified pre-transplantation, likely depending on severity of liver disease, platelet count and von Willebrand factor levels. Whether the need for platelet intervention is different between these subgroups, requires further clinical investigation.

5.2.2. IMPACT AND IMPLICATIONS

This proof-of-principle study investigated primary hemostasis in children with cirrhosis, a topic that is not well documented in literature. A deeper understanding of primary hemostasis in this patient group could enhance future hemostatic and transfusion management strategies. Our results support a relatively preserved primary hemostasis in pediatric patients undergoing liver transplantation and revealed two subgroups: one with severe liver disease and higher platelet counts, showing preserved primary hemostasis and another with lower platelet count and moderate hemostatic impairment. These results could be of use to investigate, in a more clinically oriented study, if the need for platelet intervention is different between these subgroups of patients.

Graphical abstract



5.2.3. INTRODUCTION

Pediatric patients with cirrhosis present a complex hemostatic balance with normal or increased thrombin generation capacity due to a reduction of both procoagulant and anticoagulant factors [119]. Primary hemostasis parameters in these patients are characterized by a low platelet count, which is considered to be compensated for by high plasma levels of platelet-adhesive von Willebrand factor (VWF). In patients with liver disease, an increase in plasmatic VWF can be due to endothelial cell activation, an increased synthesis in the cirrhotic liver, and possibly a reduced hepatic clearance [177, 178]. Platelet functionality is still poorly defined in patients with cirrhosis, which is in part due to the challenge of assessing platelet function in patients with thrombocytopenia. Published studies so far demonstrate either normal, decreased or increased platelet functionality in adult populations of cirrhotic patients [56, 57]. Literature on platelet activation in the pediatric population is incomplete.

The hemostatic system changes substantially during the first years of life. The system maturation after birth is referred to as developmental hemostasis [179], including post-natal changes in the coagulation system [180]. Although less well studied, there is also evidence that primary hemostasis develops over time. In children younger than 1 year, platelet aggregation, measured with light transmission aggregometry, appears to be lower compared to adults [181]. Platelet studies under flow conditions, however, show a normal to increased platelet function after birth [182], which is explained by relatively high VWF and hematocrit values in young children. A complexity in the research to hemostatic responses in pediatric patients is the requirement of age-matched control groups to account for hemostatic developmental changes in childhood.

For the evaluation of platelet activation and function in patients, several approaches can be used, providing complementary information. Flow cytometry to evaluate platelet activation requires only a small amount of blood and enables the simultaneous assessment of multiple platelet activation parameters. However, this type of assay is insensitive to VWF, a key player in primary hemostasis. Under moderate- to high-shear flow conditions though, endothelial-derived VWF is crucial for platelet adhesion to the damaged vasculature [183]. Ex vivo, this role of VWF has been approached by high-throughput microfluidic assays, which simultaneously evaluate platelet adhesion, aggregation, secretion and procoagulant activity at defined flow conditions [101]. At moderate to high wall-shear rates, VWF bound to immobilized collagens, captures platelets via the glycoprotein (GP) Ib-V-IX receptor. Stable platelet adhesion and activation is achieved by interaction with the collagen receptors, integrin $\alpha_2\beta_1$ and GPVI, and interaction with the fibrinogen receptor $\alpha_{IIb}\beta_3$ [184].

In the present study we evaluated the primary hemostasis process in children with cirrhosis before and after liver transplantation. For this purpose, we used a large panel of platelet

activation markers, whole-blood microfluidics, platelet-leukocyte complex formation, as well as VWF assays, including multimers, and ADAMTS13 levels. We hypothesized that primary hemostasis in the majority of children with cirrhosis is relatively preserved. This is the first comprehensive study on platelet properties in this patient population.

5.2.4. METHODS

5.2.4.1. Study participants

Pediatric patients with cirrhosis, listed for liver transplantation at Cliniques Universitaires Saint-Luc, Brussels, Belgium, were prospectively and consecutively enrolled between January 2022 and July 2023. Patients became eligible after their initial admission for a pre-transplant work-up. All therapeutic decisions were reviewed in multidisciplinary meetings. Once the transplant indication was validated and contraindications ruled out, patients were scheduled for living donor liver transplantation and at that moment patients were invited to participate. Written informed consent was obtained from the parents or legal guardian. A single blood draw was performed 1 day before liver transplantation in all patients and 3 months post-transplant in a subset of patients. Upper gastrointestinal endoscopy and hepatic Doppler ultrasound were performed as standard of care. Exclusion criteria were acute liver failure, re-transplantation, comorbidities affecting platelet activation/function, use of antiplatelet agents or nonsteroidal anti-inflammatory drugs 10 days prior to blood draw, platelet transfusion before blood draw and combined organ transplantation. In addition, age-matched control subjects were included comprising healthy children requiring minor surgery (such as circumcision, naevus removal, laser therapy, and adenotonsillectomy). Transfusion data were obtained by reviewing the anesthesia charts. Estimated blood volume was calculated depending on age: 75 mL/kg for infants ≤12 months and 70 mL/kg for children ≥12 months [185]. Patients were treated with acetylsalicylic acid for a duration of 6 weeks post-transplant, which was initiated when platelet counts were $>100x10^9$/L. Since this is a first proof-of-principle study reporting comprehensive characterization of platelet properties in pediatric cirrhotic patients, sample size calculation was based on platelet activation changes in the adult cirrhotic population [56]. Based on a 9% point difference in CD63 activation (TRAP-6 10μM) with a standard deviation of 13.9 in the patient population and a standard deviation of 6.7 in the control group using a non-parametric model, we obtained an sample size of 26 patients for 81% power. The study was conducted in accordance with the Declarations of Helsinki and Istanbul and was approved by the local ethical committee (2020/12MAR/157). Anonymized data were entered in a Redcap database.

5.2.4.2. Standard diagnostic testing

EDTA-anticoagulated whole blood was drawn for a complete blood cell count. Assays for C-reactive protein (CRP), total bilirubin, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transferase (GGT), lactate dehydrogenase (LDH), bile acids, creatinine and estimated glomerular filtration rate (eGFR) were performed using heparin-anticoagulated plasma. Routine coagulation measurements (prothrombin time (PT)/international normalized ratio (INR), activated partial thromboplastin time (APTT), fibrinogen) were performed on citrate (3.2%) anticoagulated plasma.

5.2.4.3. Platelet multiparametric flow cytometry assay

Flow cytometry with diluted whole blood was performed as described before [145]. In brief, blood samples were kept for 1 hour at room temperature before addition to a mix of fluorochrome-coupled antibodies, HEPES buffer (pH 7.4; 137 mM NaCl, 1 mM MgCl2, 2.7 mM KCl, 5.6 mM glucose, 1 g/L BSA, 20 mM HEPES) and agonists. Blood was diluted 1/12 and then analyzed on a FACS Canto II flow cytometer (10,000 events). Data were analysed using FlowJo software (BD Bioscience, San Jose, CA, USA). Platelets were gated using side scatter and CD41 as a platelet identifier. In analyses of GPVI expression, GPIbα was used as a platelet identifier. Results were expressed as % positive platelets for activation markers or as mean fluorescence intensity (MFI) for platelet receptor levels. Details on flow cytometry markers, gating strategy and dot plots are shown in Supplementary Materials & methods; **Table S1** and **Fig. S6.**

5.2.4.3.1. Platelet phosphatidylserine exposure

Platelets in diluted whole blood were supplemented with a mixture of $CaCl_2$ (Ca^{2+}, 2.5 mM) and Gly-Pro-Arg-Pro (GPRP, 2.0 mM), fluorochrome-coupled antibodies and HEPES buffer. Maximal stimulation was achieved with α-thrombin (5 U/mL) and cross-linked collagen related peptide (CRP-XL, CambCol Laboratories, Littleport, Cambridgeshire, UK; 5 µg/mL). Samples were incubated for 10 minutes at room temperature. Incubations were stopped by adding 1 mL of HEPES-Ca^{2+} buffer and samples were analyzed within 1 hour. For the assessment of platelet procoagulant activity, the following fluorescent markers were included: anti-CD41a (BD), PAC-1 (binds active conformation of αIIbβ3, BD) and annexin A5 (binds phosphatidylserine, BD).

5.2.4.3.2. Platelet receptors and activation markers

For multiparametric flow cytometry, two separate panels were used. Panel 1 contained anti-CD41a (BD), PAC-1 (BD) and anti-CD107a (BD). Panel 2 included anti-CD41a (BD), anti-CD42b (Miltenyi, Biotec, Bergisch Gladbach, Germany), anti-CD29 (Miltenyi), anti-

CD62P (Miltenyi) and anti-CD63 (BD). Agonists were used at low and high concentrations: TRAP-6 (SFLLRN-NH2, Sigma-Aldrich, 0.8 μM and 3.3 μM) and Me-S-ADP (Tocris Bioscience, Bristol, UK, 0.01 μM and 1 μM). Where indicated, platelets were activated with CRP-XL (150 ng/mL). Samples were incubated for 20 minutes at room temperature. Incubations were stopped and platelets were fixed with 1 mL of HEPES buffer containing paraformaldehyde 1%.

5.2.4.3.3. Platelet-leukocyte complexes

Freshly taken (<20 minutes) whole blood was mixed with HEPES-buffer plus anti-CD41 (BD) and anti-CD14 (Miltenyi) at a final dilution of 1/10. After a 10-minutes incubation period, 1 mL of VersaLyse Lysing Solution was added (Beckman Coulter, CA, US).

5.2.4.3.4. Platelet GPVI expression

Platelets in diluted whole blood were stained with anti-CD42b (Miltenyi) and anti-GPVI (BD). Samples were incubated for 20 minutes at room temperature. Incubation was stopped and platelets were fixed with 1 mL of HEPES buffer containing paraformaldehyde 1%.

5.2.4.3.5. Platelet-bound von Willebrand factor

For measurements of platelet-bound VWF by flow cytometry [15], platelets in diluted whole blood were stained with anti-CD41 (BD) and a monoclonal antibody to VWF (Abcam, Cambridge, UK). A positive control sample with addition of ristocetin (2 mg/mL) was included. Samples were incubated for 20 minutes at room temperature. Platelets were then fixed by the addition of 1 mL of HEPES buffer containing paraformaldehyde 1%.

5.2.4.3.6. Multiparameter whole-blood microfluidics

Whole blood thrombus formation under flow was performed using the Maastricht flow chamber, as described before [101]. In short, citrate anticoagulated blood was recalcified in the presence of the thrombin inhibitor PPACK (40 μM) and perfused at 1000 s-1 over three consecutive microspots consisting of laminin/VWF, collagen type III or collagen type I. After four minutes of blood perfusion, thrombi were stained to measure platelet activation. Brightfield and fluorescence images were recorded using an EVOS FL microscope equipped with a 60x oil objective to assess platelet deposition and aggregation, secretion (CD62P, P-selectin staining), fibrinogen binding and procoagulant activity (annexin A5 staining). Image analysis was performed using scripts in Fiji [186]. In blood from two selected patients with high PELD score (one with biliary atresia and one with progressive familial intrahepatic cholestasis) the contribution of VWF to thrombus formation and procoagulant activity under flow was assessed by pre-incubating citrated blood with the VWF A1 domain blocking antibody MCA4683 (Bio-Rad, Hercules US; [187]).

5.2.4.3.7. Assays using citrate plasma

After double centrifugation (2 x 2500g; 10 minutes) citrate blood was frozen at -80°C within one hour of sampling until analysis. Von Willebrand antigen and activity were measured with the HemosIL von Willebrand Factor Antigen and Ristocetin Cofactor kit (Werfen, Barcelona, Spain). ADAMTS13 was analyzed with the Technozym ADAMTS13 activity kit (Technoclone, Vienna, Austria) and soluble GPVI with the human GPVI ELISA kit (Thermofisher, Massachusetts, USA). For soluble GPVI, a maximum was set at the highest calibrator condition (6,000 pM, 10x dilution of plasma) at 60,000 pM. For the VWF multimer analysis the Kit Hydragel 11 von Willebrand multimers (Sebia, Lisses, France) was used.

5.2.4.4. Statistical analysis

Statistical analysis was performed with GraphPad Prism, version 9.5.1 and R, version 3.4.0. Normal distribution was checked with a Kolmogorov-Smirnov test. For comparison between patients and control subjects, an unpaired student t test (normal distribution) or Mann-Whitney-U test (non-parametric distribution) was used. R-Studio (2023.12.1) was used for correction for multiple comparisons with Benjamini-Hochberg, correlation matrix and principal component analysis as well as unsupervised clustering analysis. Sample size calculation was performed with the software PASS 14 (14.0.7).

5.2.5. RESULTS

5.2.5.1. Study participant characterization and conventional assays

For extensive hemostatic analysis, we enrolled 27 pediatric patients with cirrhosis and 15 pediatric healthy controls. Forty-four patients were screened, but 5 did not meet the inclusion criteria due to the absence of cirrhosis. In 4 cases, the parents did not provide informed consent. Six patients presented exclusion criteria: 1 patient was awaiting re-transplantation, 5 patients had comorbidities that could potentially affect platelet function, including cardiac conditions and hepatopulmonary syndrome and 2 patients were excluded due to organizational challenges. Blood sampling was performed with a median of 1 day (IQR: 1-7 days) before liver transplantation. Nine patients also provided a blood sample three months (IQR: 85-92 days) after liver transplantation.

Table 4. Characteristics of patients before and after liver transplantation and healthy controls.

	Patients before LT (n = 27)	Healthy volunteers (n = 15)	Patients after LT (n = 9)
Age, months	21 (12-48)	21 (12-47)	27 (15-56)
Gender, F/M	21/6	7/8	8/1
Etiology, n = 27			
Biliary cirrhosis			
Biliary atresia	18	NA	7
PFIC	2	NA	1
Unknown	3	NA	0
Cirrhosis			
Alpha-1 antitrypsine deficiency	1	NA	0
Drug related	1	NA	0
Heterozygous MDR3/ABCB4	2	NA	1
PELD	11 (5-20)	NA	NA
PELD ≤10	12	NA	NA
PELD >10	15	NA	NA
Hemoglobin, g/dL	9,7 (8,7-10,5)	11,2 (9,9-11,8)	10,7 (10,4-11,8)
Hematocrit (%)	29,0 (26,6-31,0)	32,0 (29,2-35,3)	30 (28-34)
Platelets ,10^9/L	133 (88-203)	280 (215-418)	193 (151-204)
MPV (fL)	11,7 (10,4-12,5)	10,0 (9,1-10,8)	10,1 (9,7-11,1)
WBC, (*10^9/L)	7,1 (3,8-9,8)	9,8 (6,6-12,0)	6,83 (5,86-9,01)
INR	1,33 (1,18-1,68)	1,08 (1,06-1,14)	1,2 (1,19-1,32)
Fibrinogen, mg/dL	302 (128-412)	320 (313-387)	355 (281-385)
D-dimers, ng/mL	1377 (410-4880)	319 (250-520)	289 (250-385)
Total bilirubin mg/dL,	16,3 (9,2-21,9)	<0,2 (<0,2-0,3)	0,3 (0,3-0,4)
AST (U/L)	168 (132-276)	34 (31-41)	35 (29-40)
ALT (U/L)	98 (55-155)	12 (10-16)	33 (26-79)
γ-GT (U/L)	122 (62-205)	8 (6-11)	24 (18-33)
LDH (U/L)	346 (263-373)	267 (227-300)	244 (211-247)
Albumin, g/L	35 (28-39)	ND	43 (42-45)
CRP, mg/L	6,3 (4,2-15,3)	1,9 (<1-2,9)	1,0 (1,0-1,4)

HARI	0,84 (0,78-0,91)	NA	NA
Esophageal varices			
absent	3	NA	NA
grade 1	14	NA	NA
grade 2	7	NA	NA
grade 3	2	NA	NA
unknown	1	NA	NA
Ascites			
absent	16	NA	NA
small /moderate	7	NA	NA
significant	4	NA	NA
Previous hemorrhage			
absent	20	NA	NA
variceal bleeding	5	NA	NA
spontaneous (epistaxis, hematuria)	2	NA	NA
Previous thrombosis (yes/no)	0/27	NA	NA
Previous Kasai (yes/no)	9/18	NA	NA

Values are represented as median (IQR). Where indicated, p-values between patients and healthy controls are shown. ALT: alanine aminotransferase, AST: aspartate aminotransferase, CRP: C-reactive protein, γ-GT: gamma glutamyl transferase, HARI: hepatic artery resistive index, INR: international normalized ratio, LDH: lactate dehydrogenase, LT: liver transplantation, MPV: mean platelet volume, NA: not applicable, PELD: pediatric end stage liver disease score, WBC: white blood cell count.

Median age was 21 months (IQR: 12-48) for patients and 21 months (IQR: 12-47; p = 0.83) for controls. Indication for minor surgery in controls comprised circumcision, naevus removal, laser therapy, tympanoplasty and adenotonsillectomy. Seventy-seven percent of patients were female, compared to 47% of the controls. Two distinct indications for transplantation were present. The first indication was biliary cirrhosis with jaundice and portal hypertension. This group consisted mainly of patients with biliary atresia with or without failed Kasaï surgery. A second indication comprised patients with cirrhosis and portal hypertension with non-biliary etiology. Details on medical condition in both groups are shown in **Table 4**. The hepatic arterial resistive index, assessed by ultrasound, varied between 0.74 and 1.00. The pediatric end-stage liver disease (PELD) score varied between -10 and 44 and was lower in the 4 patients with non-biliary cirrhosis and portal hypertension

("clinical phenotype 2"; PELD < 4). We did not include patients with acute-on-chronic liver failure.

Cirrhosis was histologically confirmed in all explanted livers. In 3 explanted livers hepatocarcinoma, related to the cirrhotic condition, was found.

Regarding coagulation, the patients had a higher international normalized ratio (INR) when compared to the healthy controls ($p < 0.0001$). Values for APTT and fibrinogen were not significantly different ($p = 0.06$ and 0.36 respectively). The patient blood samples showed a significantly lower platelet count with higher mean platelet volume (MPV) and lower red blood cell parameters (i.e., hemoglobin and hematocrit). Plasma levels of AST, ALT, bilirubin, γGT, LDH and CRP were higher in patients than in the controls (**Table 4**). Normal values were seen for creatinine and glomerular filtration rate. None of the patients were on dialysis.

In the 9 patients examined three months after transplantation, hemoglobin levels were nearly identical to those of the control group, with the exception of one patient with anemia (Hb 7.8 g/dL). Platelet counts were higher than pre-transplant but still lower compared to controls while MPV was normalized. INR remained slightly elevated, while D-dimer levels had also normalized. Most other biochemical parameters were comparable to those of the controls.

5.2.5.2. Normal expression of platelet collagen receptors GPVI and integrin α2β1 and higher levels of GPIbα and soluble GPVI in patients

Monitoring of the platelets by flow cytometry indicated similar expression of the collagen receptors GPVI ($p = 0.77$) and β1 integrin (CD29; $p = 0.77$) between the patients and control group (**Table 5**). In contrast, the expression of GPIbα (CD42b) was significantly higher compared to controls ($p < 0.05$), which is in concordance with a higher mean platelet volume ($r = 0{,}56$; $p < 0.01$). In plasma samples, we observed a higher circulating soluble GPVI in patients (median 21.1 pM) in comparison to controls (median of 8.3 pM; $p < 0.05$). After liver transplantation, GPVI expression was significantly lower compared to both patients pre-transplant and controls. No significant difference was observed in the expression of α2β1. GPIbα expression was also reduced post-transplant, aligning with levels seen in controls and in line with normalized MPV following transplantation. Soluble GPVI was not measured in post-transplant samples.

Table 5. Platelet flow cytometry results in patients and in pediatric healthy controls.

Agonist	Activation marker	Patients		Healthy volunteers		p-value
		n	%positive platelets	n	%positive platelets	
Resting	CD62P	27	5.1 (2.2-7.9)	15	3.5 (1.7-5.3)	0.086
	activated $\alpha_{IIb}\beta_3$	15	3.3 (0.6-6.0)	9	3.1 (0.8-5.5)	0.907
	CD63	27	3.0 (0.7-5.3)	15	1.7 (0.4-3.0)	0.086
	CD107a	25	1.8 (0.6-3.0)	15	1.1 (0.5-1.8)	0.086
ADP 0.01	CD62P	26	37.1 (25.8-48.3)	15	34.7 (21.8-47.7)	0.808
	activated $\alpha_{IIb}\beta_3$	14	89.6 (83.7-92.3)	9	91.4 (88.3-96.3)	0.282
	CD63	26	5.9 (1.8-10.0)	15	4.3 (1.3-7.3)	0.349
	CD107a	24	18.8 (15.0-24.7)	15	24.5 (18.4-28.9)	0.282
ADP 1	CD62P	26	50.0 (38.1-61.9)	15	50.3 (37.1-63.6)	0.932
	activated $\alpha_{IIb}\beta_3$	14	92.5 (90.8-95.6)	9	95.9 (95.0-97.7)	**0.029**
	CD63	26	7.9 (3.0-12.8)	15	5.8 (2.4-9.3)	0.310
	CD107a	24	29.0 (17.5-40.6)	15	37.4 (25.8-48.9)	0.071
TRAP 0.8	CD62P	27	28.3 (10.9-43.7)	15	22.4 (12.4-49.0)	0.588
	activated $\alpha_{IIb}\beta_3$	15	18.1 (10.4-63.1)	9	51.9 (18.0-67.4)	0.588
	CD63	27	5.3 (4.0-10.7)	15	4.9 (1.6-5.8)	0.588
	CD107a	25	5.5 (3.4-9.6)	15	4.3 (2.8-10.8)	0.588
TRAP 3.3	CD62P	27	77.0 (59.2-94.7)	15	86.9 (82.2-91.6)	**0.030**
	activated $\alpha_{IIb}\beta_3$	15	76.6 (55.8-97.4)	9	93.9 (88.8-99.0)	**0.013**
	CD63	27	30.8 (18.9-42.7)	15	35.3 (20.9-49.8)	0.277
	CD107a	25	37.1 (23.0-51.3)	15	50.9 (39.9-61.9)	**0.013**
CRP 150	CD62P	10	29.6 (15.3-65.5)	10	79.9 (45.5-92.7)	**0.031**
	activated $\alpha_{IIb}\beta_3$	10	40.5 (6.1-59.2)	8	88.6 (47.9-98.4)	**0.031**
	CD63	10	6.4 (3.3-10.4)	10	13.9 (6.4-25.1)	**0.042**
	CD107a	10	9.7 (3.3-28.3)	9	32.9 (9.6-65.3)	0.062
Agonist	**Platelet receptor**	**Patients**		**Healthy volunteers**		**p-value**
		n	**MFI**	**n**	**MFI**	
Resting	CD42b	24	14563 (12783-16344)	15	13055 (12058-14052)	**0.015**
	CD29	24	2720 (2291-3150)	15	2671 (2383-2959)	0.774
	GPVI	18	6940 (5670-8210)	15	6823 (5825-7821)	0.774

Platelet activation markers are expressed as % positive platelets and platelet receptors as Mean Fluorescence Intensity (MFI). Results are expressed as median with IQR. The p-value comparing patients and healthy controls is also shown. Bold values are statistically significant. An unpaired Student's t-test was performed for normally distributed parameters, while the Mann-Whitney test was used for non-normally distributed parameters. Multiple comparisons were corrected using the Benjamini-Hochberg method.

5.2.5.3. Impaired agonist-induced platelet activation in patients under stasis

Additional flow cytometry measurements with diluted blood indicated that the patient platelets had decreased α-granule release (CD62P expression); αIIbβ3 activation (PAC-1 mAb) and lysosomal release (CD107a expression) in comparison to control platelets, when stimulated with a high concentration of the thrombin receptor agonist TRAP-6 or with the GPVI agonist CRP-XL (**Table 5**). With the exception of αIIbβ3 activation, platelet activation in response to Me-S-ADP or with TRAP-6 at low concentration, was not significantly different between patients and the control group (**Table 5**). Likewise, circulating levels of platelet-monocyte (28.2% in patients versus 34.3% in controls; $p = 0.20$) and platelet-neutrophil (19.3% in patients versus 20.4% in controls; $p = 0.71$) complexes were comparable.

In unstimulated platelets, the proportion of procoagulant, PS-exposing platelets was higher in the patients compared to controls (**Figure 7A**, $p < 0.001$). However, after maximal stimulation with α-thrombin and CRP-XL, this proportion was lower in patients when compared to the healthy controls (**Figure 7B**, $p < 0.05$). The reduced PS exposure was most pronounced in the patient group with more severe liver disease (PELD >10; $p < 0.001$). Accordingly, low PS exposure was correlated with higher severity of liver disease (PELD score; $r = -0.68$; $p < 0.001$) (**Figure 7C-H**). When looking at PELD components, PS exposure was correlated to the level of albumin ($r = 0.54$), the INR ($r = -0.50$) and bilirubin levels ($r = -0.31$) but not to patient age ($r = 0.07$). The PS exposure was not related to the platelet count ($r = 0.05$) or the hepatic artery resistive index ($r = -0.12$). Most changes in platelet activation parameters after transplantation were not significant except for CD63 (platelet dense granule and lysosomal release) where a lower fraction of activated platelets was seen. Detailed results are shown in Supplementary Materials & methods, **Fig.S7**.

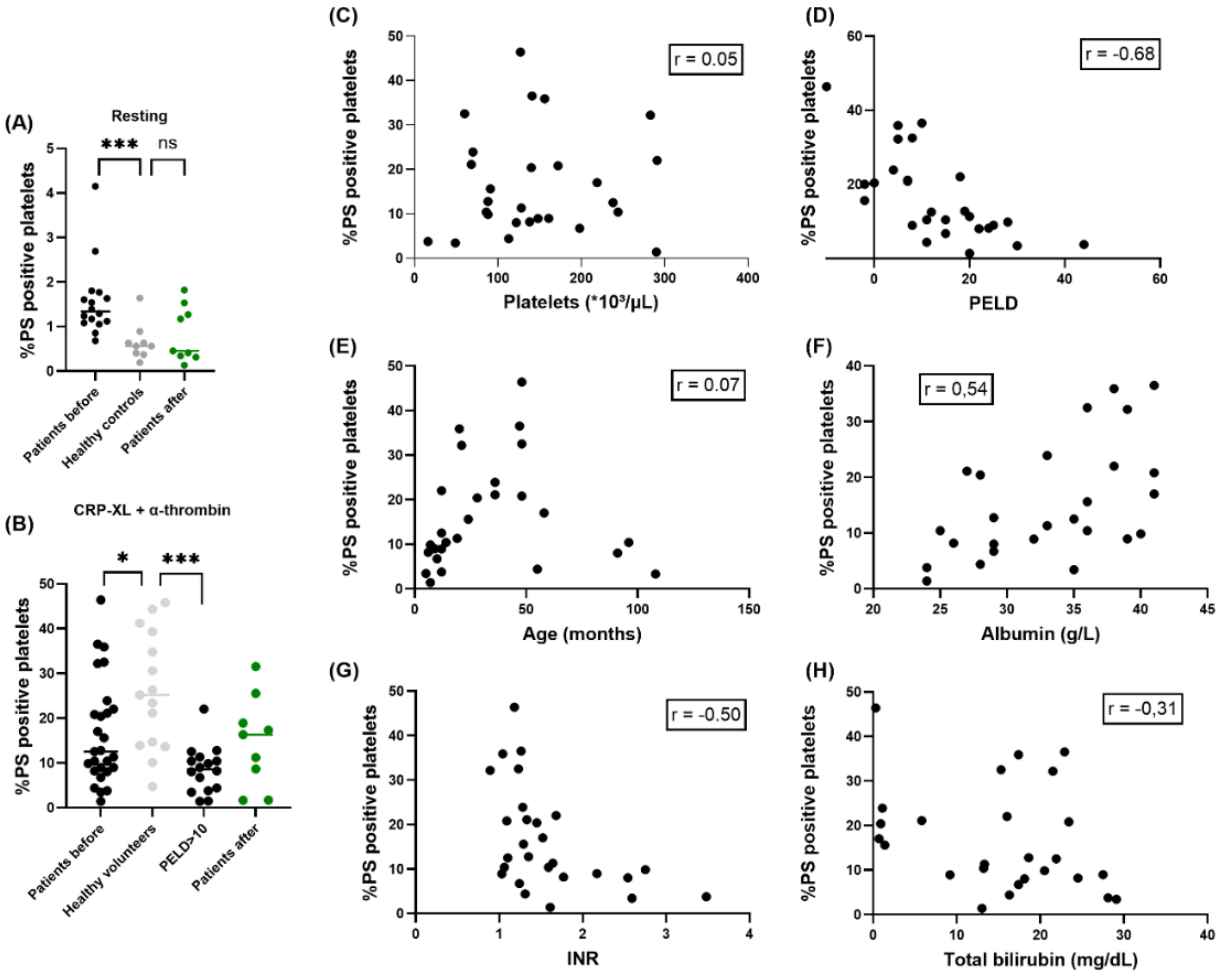
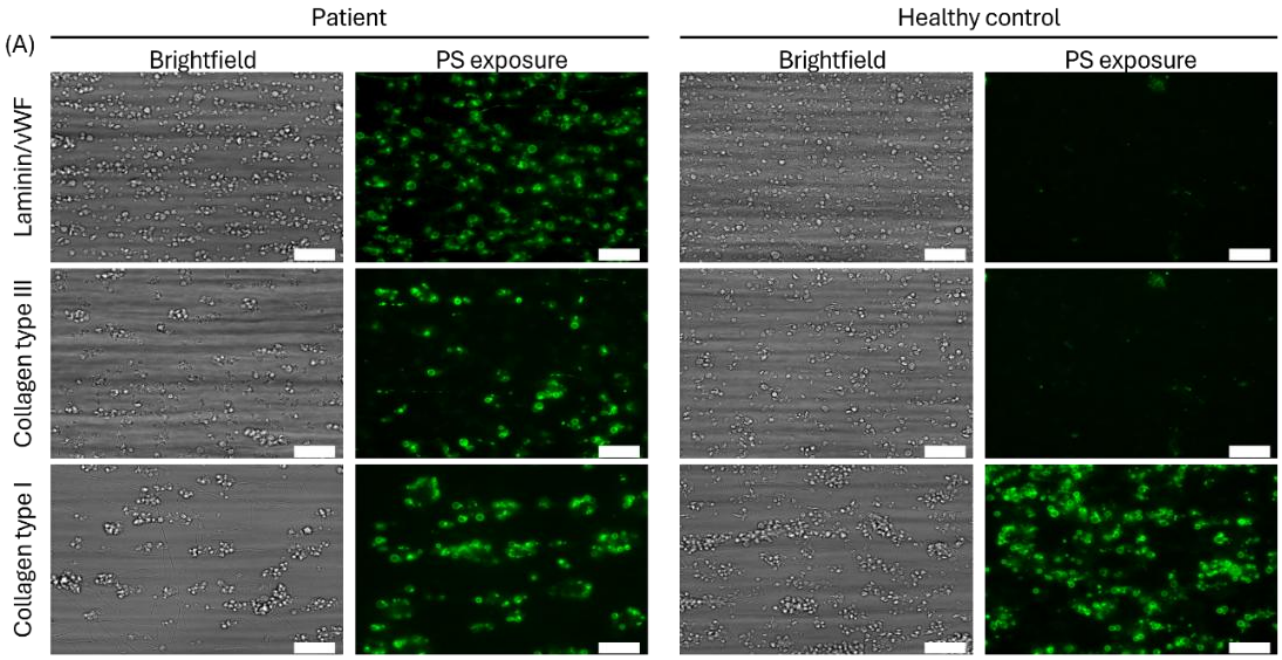

(A)
Resting
***
ns
%PS positive platelets
Patients before
Healthy controls
Patients after
(B)
CRP-XL + α-thrombin
*
***
%PS positive platelets
Patients before
Healthy volunteers
PELD>10
Patients after
(C)
r = 0.05
%PS positive platelets
Platelets (*10³/µL)
(D)
r = -0.68
%PS positive platelets
PELD
(E)
r = 0.07
%PS positive platelets
Age (months)
(F)
r = 0,54
%PS positive platelets
Albumin (g/L)
(G)
r = -0.50
%PS positive platelets
INR
(H)
r = -0,31
%PS positive platelets
Total bilirubin (mg/dL)

Fig. 7. Flow cytometric assessment of platelet phosphatidylserine (PS) exposure in patient blood before and after liver transplantation compared to healthy controls. (A) PS exposure of unstimulated platelets from patients (before and after liver transplantation) and healthy controls. (B) PS exposure of platelets stimulated with CRP-XL and α-thrombin (maximally effective concentrations) from cirrhotic patients, healthy controls, and patients with PELD >10 or PELD <10. Further panels indicate regression analysis of percentual PS exposure versus platelet count (C), PELD (D), patient age (E), albumin (F), INR (G), and total bilirubin (H). Abbreviations: PELD, pediatric end-stage liver disease score; PS, phosphatidylserine. Levels of significance *p < 0.05; ***p < 0.001 (Unpaired Student's t-test).

5.2.5.4. Moderately impaired thrombus formation under flow in blood samples with inter-patient variability

We also measured thrombus formation under flow conditions with blood samples from the majority of pediatric patients (n = 17) and healthy controls (n = 8) using an established microfluidic assay [101]. On highly reactive collagen I-coated microspots, cirrhotic patients exhibited lower platelet adhesion, aggregation, fibrinogen binding, and alpha-granule secretion compared to controls (**Figure 8**). Examination per individual patient showed a consistent variability in adhesion and aggregation parameters (Supplementary Materials & methods**, Fig. S8**). This variability was also seen when blood was perfused over microspots of collagen III and laminin/VWF. However, on these less reactive surfaces, aggregation, fibrinogen binding, and alpha granule secretion were more comparable between patients and controls. Exceptions were a strikingly high platelet aggregation (thrombus multilayer, multilayer score and morphological score) and procoagulant activity (PS exposure) on collagen III and laminin/VWF surfaces in some of the patients. After liver transplantation, median platelet thrombus formation on collagen I improved but high inter-individual variation remained. The higher platelet aggregation and procoagulant activity on less reactive surfaces, was still present in some patients, when analyzed at 3 months after liver transplantation.


(A)
Patient
Healthy control
Brightfield
PS exposure
Brightfield
PS exposure
Laminin/vWF
Collagen type III
Collagen type I

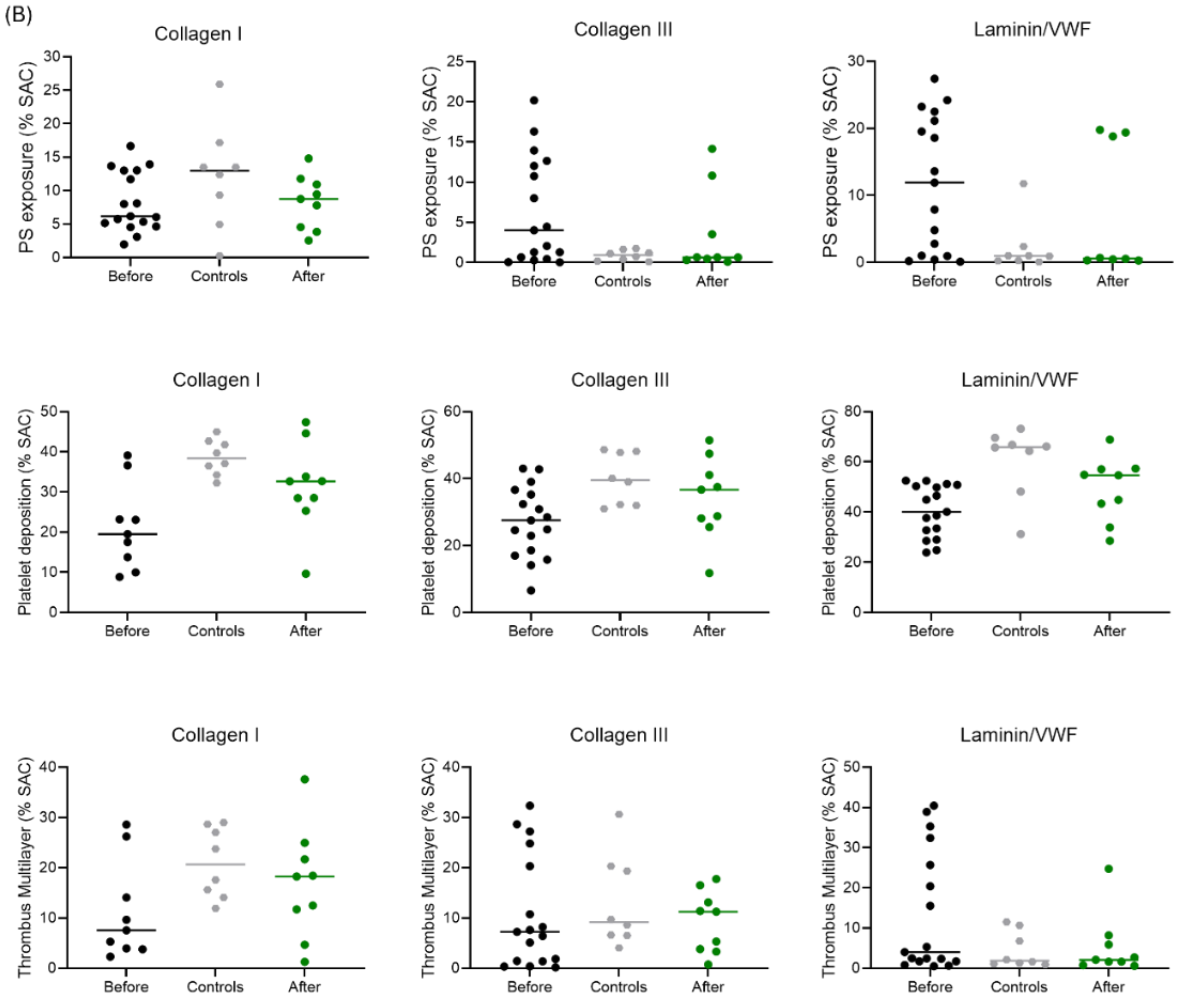

(B)
Collagen I
PS exposure (% SAC)
Before
Controls
After
Collagen III
PS exposure (% SAC)
Before
Controls
After
Laminin/VWF
PS exposure (% SAC)
Before
Controls
After
Collagen I
Platelet deposition (% SAC)
Before
Controls
After
Collagen III
Platelet deposition (% SAC)
Before
Controls
After
Laminin/VWF
Platelet deposition (% SAC)
Before
Controls
After
Collagen I
Thrombus Multilayer (% SAC)
Before
Controls
After
Collagen III
Thrombus Multilayer (% SAC)
Before
Controls
After
Laminin/VWF
Thrombus Multilayer (% SAC)
Before
Controls
After

Fig. 8. Whole-blood thrombus formation in pediatric patients before and after liver transplantation and in healthy controls. Whole blood from patients and age-matched healthy controls was flowed for 4 minutes over three microspotted surfaces; thrombi were post-perfused with FITC-annexin A5. (A) Brightfield and fluorescence images of platelet deposition and PS exposure on collagen I, collagen III and laminin/VWF. Scale bar = 20 μm (B) Graphical representation of PS exposure on indicated surfaces, plus thrombus parameters on collagen I, collagen III and laminin/VWF. Horizontal lines represent median values.

5.2.5.5. Increased VWF activity in the pediatric patients

Significantly higher VWF antigen and activity levels were measured in patient plasma samples when compared to the control group (**Figure 9**, $p < 0.0001$). The levels of ADAMTS13 (processing VWF multimers) did not differ between groups, except for those patients with a PELD score >10 ($p < 0.05$). The median VWF antigen to ADAMTS13 ratio was 6.1 for patients compared to 1.1 for healthy controls. However, no ultra-large VWF multimers were detected in the patient samples, indicating physiological VWF processing. Flow cytometric measurements did not show spontaneous attachment of VWF to platelets. The proportion of low molecular weight multimers was higher ($p < 0.0001$) and the proportion of high molecular weight multimers was lower ($p < 0.0001$) in patients compared to controls. Post-transplantation, VWF activity decreased notably and was not significantly different from controls. ADAMTS13 returned to normal (**Figure 9**).

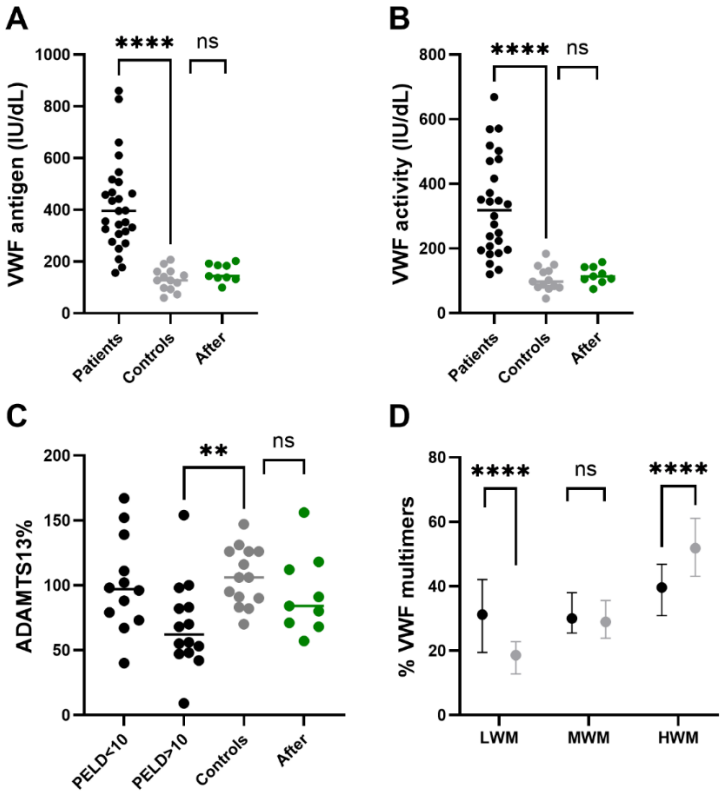


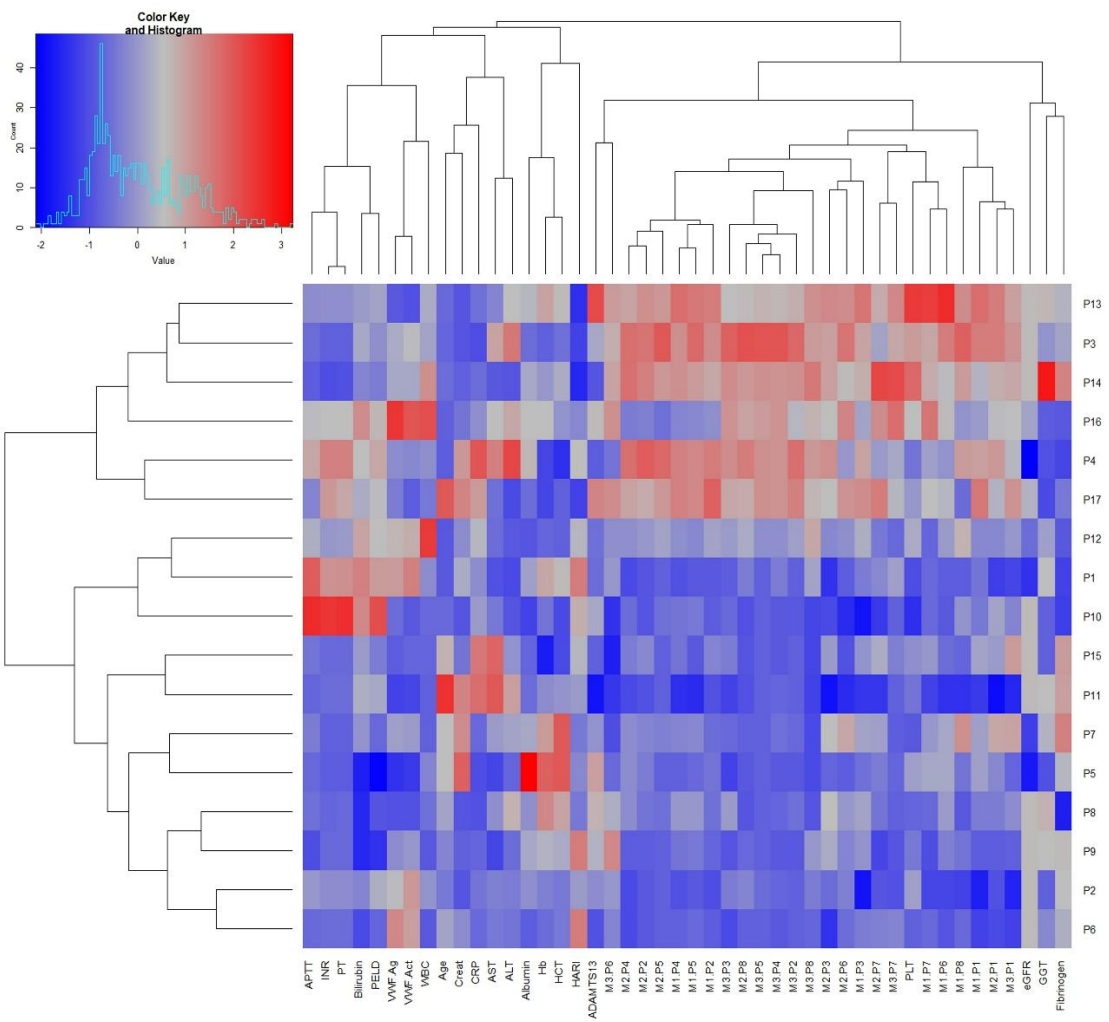
Fig. 9. Levels of von Willebrand factor and ADAMTS13 in patients before and after liver transplantation and healthy controls. (A) VWF antigen, (B) VWF activity and (C) ADAMTS13 levels in plasma from patients (before and after liver transplantation) compared to healthy controls. Note the difference in ADAMTS13 levels between PELD > 10 and PELD ≤ 10 (D) VWF multimers in patients compared to healthy controls. *Abbreviations*: LMW, low molecular weight multimers; MWM, medium molecular weight multimers; HWM, high molecular weight multimers; PELD, pediatric end-stage liver disease score. Levels of significance ****p < 0.0001; **p < 0.01; ns, not significant (Unpaired Student's t-test).

5.2.5.6. Cluster analysis highlights a subgroup of patients with normal to high platelet aggregation, granule secretion and procoagulant activity

To systemically compare the patients' clinical, laboratory and platelet functional data, we performed an unsupervised hierarchical clustering analysis in 17 patients. This revealed a primary cluster of two groups of patients (**Figure 10A**). To better understand the difference between the subgroups, we performed a principal component analysis (PCA), also adding the corresponding data from healthy controls (**Figure 10B**). This indicated a first component (PC1, 36.6% of variance) that was mainly determined by the microfluidic parameters and the counts of platelets and white blood cells. This first dimension separated the patient groups similarly as in the cluster analysis. The second component (PC2, 22.3% of variance) was mostly defined by VWF, inflammatory parameters (C-reactive protein), liver parameters (ALT, bilirubin) and inversely by hematocrit and levels of fibrinogen. The second dimension also separated individual patients from healthy controls. Patient age contributed to both dimensions. This multiparameter analysis prompted us to better determine the underlying factors for the two subgroups of patients.

Patients within the first group (**Figure 10A**, n = 7) had normal to high values for microfluidic parameters compared to the rest of the patients (n = 10). Platelet count clustered together with the microfluidic thrombus formation parameters. Interestingly, the hepatic artery resistive index correlated inversely with both microfluidic data (Supplementary Materials & methods **, Fig. S9**, $r = -0.46$ to -0.66, $p < 0.05$, except for M3P6 and M3P8) and the platelet count ($r = -0.72$, $p < 0.01$). The patients in subgroup 1 had higher platelet count ($p < 0.01$) and a lower hepatic arterial resistive index ($p < 0.05$). Although the PELD score was not significantly different between the groups, all patients in group 1 had severe liver disease with PELD >10. None of the patients in group 1 had previous variceal or spontaneous bleeding, compared to 4 patients with variceal bleeding and 1 patient with epistaxis in group 2. Group 2 was more heterogeneous in nature with both severe liver disease patients and better compensated patients. Patients with non-biliary etiologies were part of this latter subgroup.

A


Color Key
and Histogram
Count
Value
P13
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P7
P5
P8
P9
P2
P6
APTT
INR
PT
Bilirubin
PELD
VWF.Ag
VWF.Act
WBC
Age
Creat
CRP
AST
ALT
Albumin
Hb
HCT
HARI
ADAMTS13
M3.P6
M2.P4
M2.P2
M2.P5
M1.P4
M1.P5
M1.P2
M3.P3
M2.P8
M3.P5
M3.P4
M3.P2
M3.P8
M2.P3
M2.P6
M1.P3
M2.P7
M3.P7
PLT
M1.P7
M1.P6
M1.P8
M1.P1
M2.P1
M3.P1
eGFR
GGT
Fibrinogen

B

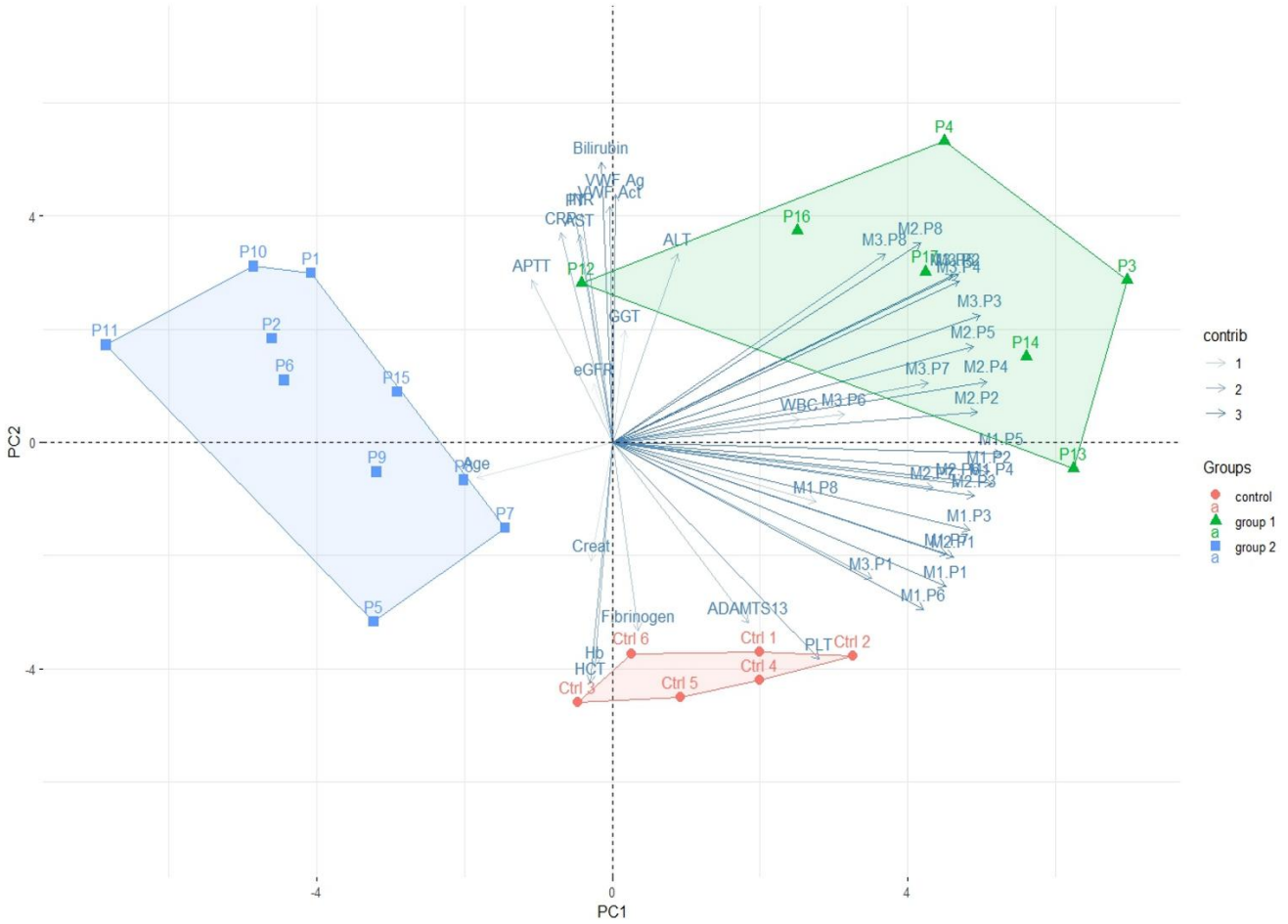

PC2
PC1
4
0
-4
-4
0
4
Bilirubin
VWF.Ag
VWF.Act
INR
CRP
AST
ALT
APTT
P12
GGT
eGFR
WBC
Age
Creat
Fibrinogen
ADAMTS13
Hb
HCT
PLT
P1
P2
P3
P4
P5
P6
P7
P9
P10
P11
P13
P14
P15
P16
Ctrl 1
Ctrl 2
Ctrl 3
Ctrl 4
Ctrl 5
Ctrl 6
M1.P1
M1.P2
M1.P3
M1.P5
M1.P6
M1.P8
M2.P2
M2.P3
M2.P4
M2.P5
M2.P8
M3.P1
M3.P3
M3.P6
M3.P7
M3.P8
contrib
1
2
3
Groups
control
group 1
group 2

Fig. 10. Unsupervised clustering and principal component analysis of relevant patient, plasma, and microfluidic parameters. Patient data and indicated plasma parameters as in Table 1 were compared with MP parameters for thrombus formation for 17 patients and 6 healthy controls. (A) Unsupervised clustered heatmap of scaled thrombus parameters (P1-P8; P1 = platelet adhesion, P2 = thrombus multilayer, P3 = morphological score, P4 = multilayer score, P5 = contraction score, P6 = fibrinogen binding, P7 = P-selectin expression, P8: phosphatidylserine expression) per microspot (M1-M3; M1 = collagen 1, M2 = collagen III, M3 = laminin/VWF), hematological parameters (Hb, HCT, PLT, MCV, WBC), coagulation parameters (PT, INR, APTT, fibrinogen, VWF, ADAMTS13), biochemical parameters (AST, ALT, Creat, eGFR, albumin, bilirubin, GGT), inflammatory parameters (CRP) and clinical parameters (PELD, hepatic artery resistive index (HARI), age). Color coding indicates a decrease (blue) or an increase (red) compared to means of control subjects. (B) Principal component analysis (biplot of PC1 and PC2) of overall parameters per patient (P1-P16) and healthy control (Ctrl1-Ctrl6). Included were scaled thrombus parameters (P1-P8) per microspot (M1-M3), hematological parameters (HCT, PLT), coagulation parameters (INR, fibrinogen, VWF, ADAMTS13), biochemical parameters (ALT, bilirubin), inflammatory parameters (CRP) and patient age.

Together, this pointed to a preserved hemostatic system in a subgroup of patients with severe liver disease, lower hepatic arterial resistive index and higher platelet count. The other patients presented with a moderate hemostatic impairment with lower platelet count and reduced thrombus formation.

5.2.5.7. Role of VWF in patients' platelet aggregation and procoagulant activity

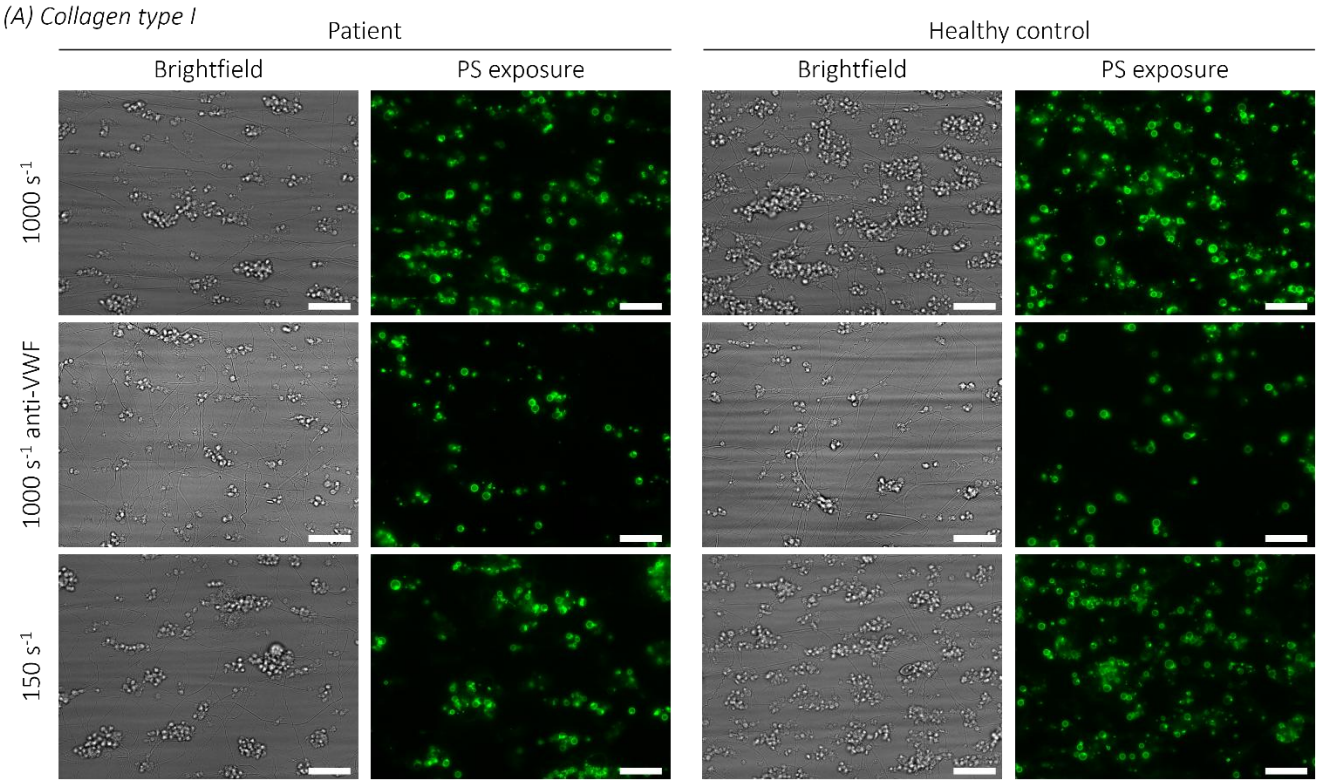
To investigate the mechanism of higher PS exposure and platelet aggregation on collagen III and laminin/VWF and the role of high plasma VWF, we performed additional experiments. In 2 patients, the flow was reduced from 1000 s^{-1} to 150 s^{-1}. This lowered the proportion of PS exposing platelets considerably from 7.64% to 4.87%, 12.35% to 3.28% and 19.06% to 6.59% on collagen I, collagen III and laminin/VWF microspots respectively. In one patient, an anti-VWF antibody was added to the plasma prior to the flow experiment. The antibody blocked PS exposure completely on collagen III (12.04% to 0.07%) and had intermediate impact on collagen I (4.69% to 2.52%) and laminin/VWF (17.43% to 13.40%). Similar results were seen for platelet aggregation parameters under flow. Data are shown in **Figure 11**.

5.2.5.8. Pre-, peri- and post-transplant hemostatic complications and blood product transfusion

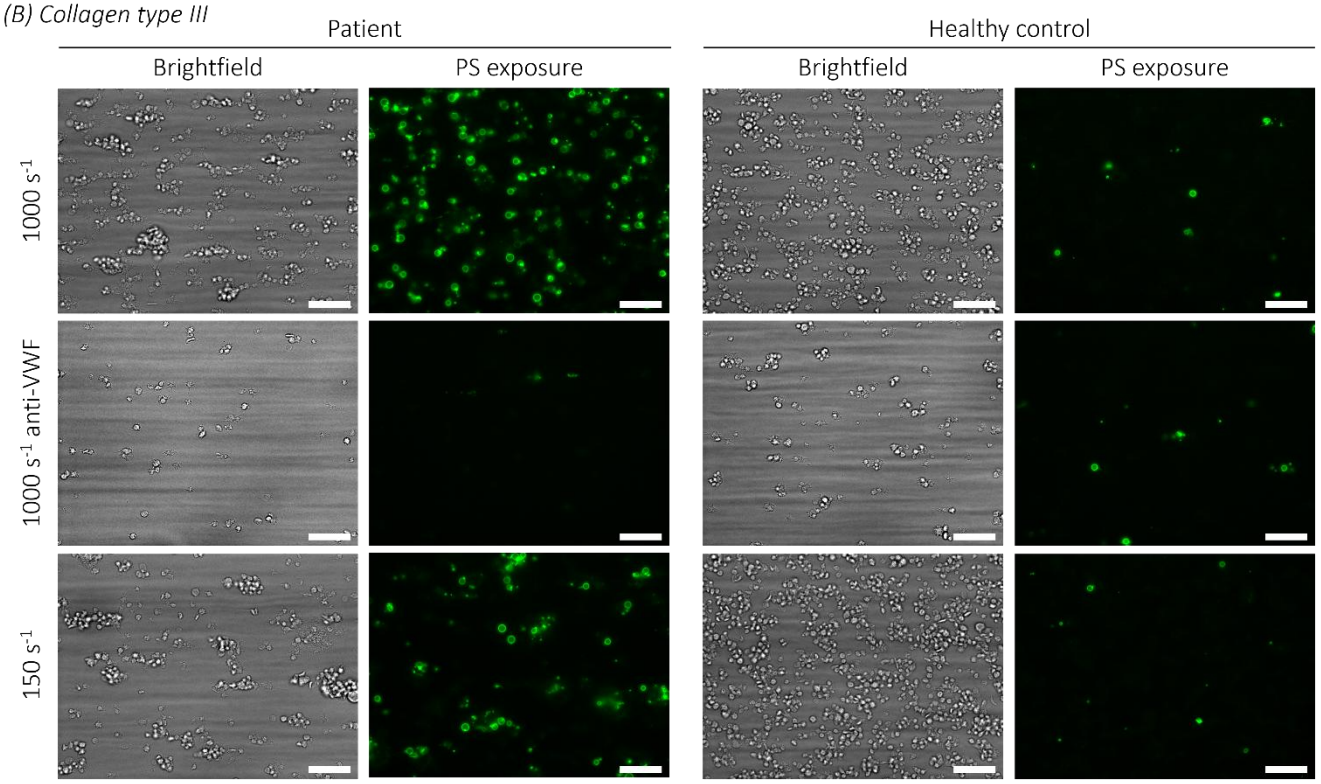
Before liver transplantation, 5 patients had experienced variceal bleeding and 2 patients had episodes of spontaneous, non-portal hypertension-related bleeding. None of the patients showed venous thromboembolism before liver transplantation. No bleeds were seen for patients in subgroup 1 (of the cluster analysis). This group had normal to high values for microfluidic parameters.

Six patients (22.2%) did not require RBC transfusion during transplantation. The remaining 21 patients needed RBC transfusion but the total amount was less than 1 blood volume. The median blood volume transfused was 0.24 of total calculated blood volume (IQR: 0.11-0.45). No statistical difference was seen in RBC use between patients in cluster 1 and 2 (median of 0.37 blood volumes for subgroup 1 and 0.33 blood volumes for subgroup 2; $p = 0.58$). Only four patients (14.8%) were treated with platelet concentrates just before and during surgery. Among these patients, one patient was in cluster subgroup 1 and 2 patients were in subgroup 2 (cluster analysis was not available for the fourth patient). Fresh frozen plasma use, adjusted for circulating blood volume, was not significantly different between subgroups.

Three patients experienced post-transplant bleeding. In one case, bleeding occurred on the first day after surgery due to hemorrhage at the biliary anastomosis, which was surgically controlled. Another patient with severe thrombocytopenia developed a subdural hematoma on day 9 and a third patient experienced both surgical bleeding and hematochezia on days 9 and 16 post-transplant, respectively. Additionally, there was one thrombotic complication in the cohort, with a patient presenting with hepatic artery thrombosis on day 6. In total, two patients died: one who experienced the thrombotic complication, and another who had multi-organ failure on the first day after surgery. Post-transplant hemorrhagic complications occurred in both cluster analysis subgroups. The post-transplant thrombotic complication occurred in subgroup 2, the group with moderately impaired hemostasis before transplantation.


(A) Collagen type I
Patient
Healthy control
Brightfield
PS exposure
Brightfield
PS exposure
1000 s-1
1000 s-1 anti-VWF
150 s-1

(B) Collagen type III



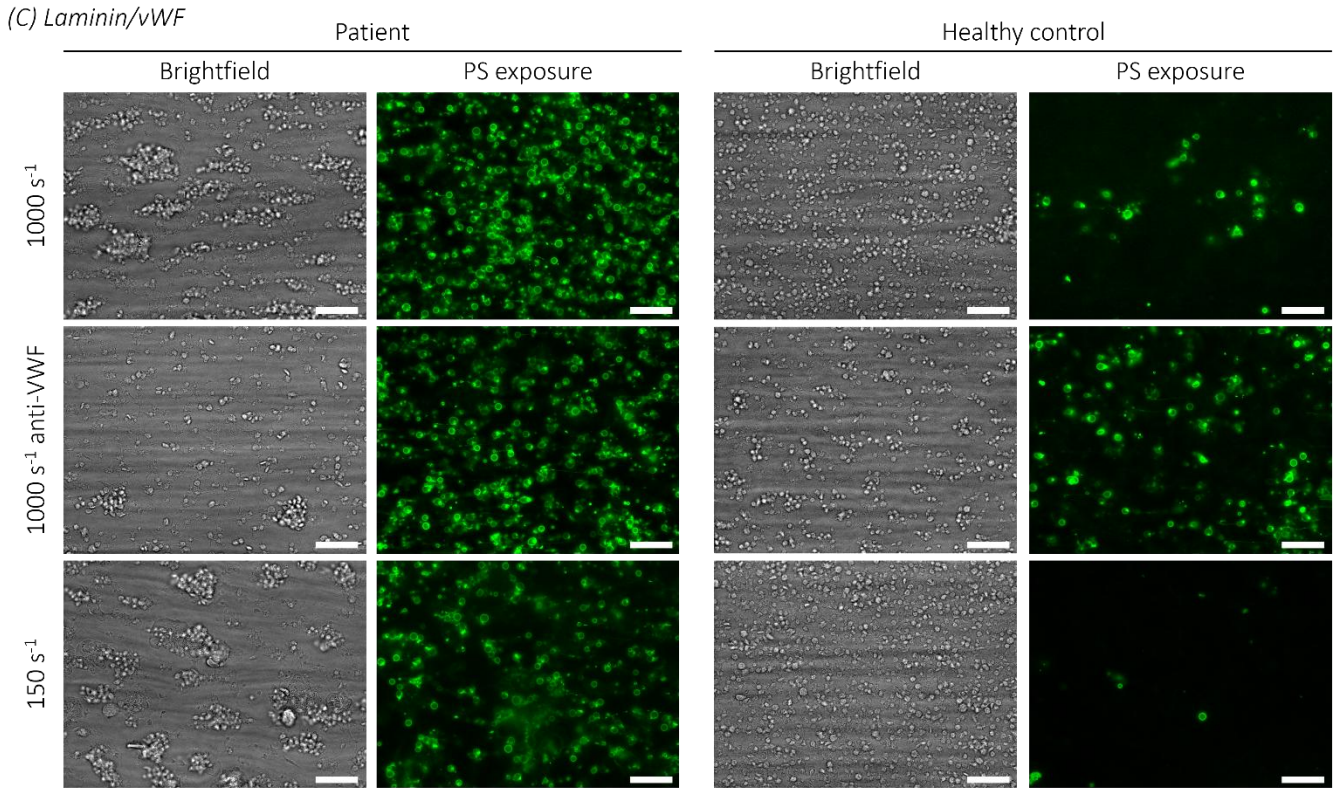


Fig. 11. Microfluidic thrombus formation from a representative patient with high PELD score and a healthy control at various wall shear rates. Further conditions as in Fig.3. Results from collagen I (A), collagen III (B) and laminin/VWF (C) at high shear rate (1000 s^{-1}), at high shear rate (1000 s^{-1}) with an anti-VWF mAb and at low shear rate (150 s^{-1}). Scale bar = 20 µm. PELD, pediatric end-stage liver disease score; PS, phosphatidylserine; VWF, von Willebrand factor.

5.2.6. DISCUSSION

In this proof-of-principle study, we examined detailed platelet responses in 27 pediatric patients with severe liver disease just before liver transplantation and compared these results with 15 age-matched controls. This is one of the largest prospective studies with pediatric patients and age-matched controls, reporting a comprehensive study on platelet properties in pediatric patients with cirrhosis. Few studies have examined platelet activation and function in this population [54]. Furthermore, our research combines multicolor platelet flow cytometry and advanced microfluidics, an approach not previously applied to evaluate hemostasis in patients with cirrhosis.

The patient platelets had lower activatability in response to high agonist concentrations in flow cytometry, and showed high variability in adhesion, aggregation, and activation in microfluidic thrombus parameters. High platelet aggregation and procoagulant activity were demonstrated in a subgroup of patients on the less reactive surfaces collagen III and laminin/VWF. Platelet responses on collagen III and laminin/VWF surfaces were particularly dependent on the level of platelet adhesive VWF [188] that was substantially elevated in patients. Although most patients had biliary atresia, heterogeneity was observed in severity of liver disease with PELD scores ranging from -10 to 44 and variable hepatic arterial resistive index [189]. Consistent with demographic data on liver transplantation, the majority of patients were very young, with a median age of 21 months [190].

The reduced platelet activation tendency in patients in comparison to healthy controls was most pronounced for the formation of procoagulant, PS-exposing platelets after stimulation with CRP-XL and α-thrombin. This platelet defect was specifically observed in patients with severe liver disease and PELD scores >10. Interestingly, in the absence of agonists, patients had a small but significant increase in PS-positive platelets. In previous publications [52, 56, 61] decreased platelet activatability assessed with P-selectin (alpha granule release), was seen in various etiologies of cirrhosis in adults.

In accordance with the literature [19, 119, 191], we observed relatively higher levels of VWF antigen and activity in the patient group, whereas the level of ADAMTS13 was lower, although still in hemostatically effective range (>40%) for most patients. We did not see an elevation of the most active ultra-large VWF multimers. Rather, we observed a lower proportion of high-molecular weight multimers in the patient group, which is also concordant with earlier work in adults [19], and is in line with the somewhat reduced VWF activity. Spontaneous attachment of VWF to platelets was not demonstrated, which is in line with the lower proportion of high-molecular weight multimers in patients.

Based on the clustering analysis, we defined a subgroup of patients with higher platelet counts and thrombus parameters. Interestingly, these were patients with severe liver disease, reflected by a high PELD score, but a lower arterial resistive index on Doppler ultrasound. Since platelet count was well correlated with the arterial resistive index ($r = -0.72$; $p < 0.0001$), patients with a lower resistive index and a higher platelet count likely had less severe portal hypertension [192]. In adults with cirrhosis, studies have demonstrated that increased hepatic arterial resistance is associated with the severity of portal hypertension and portal resistance [193, 194].

PELD correlated well with static assays where platelet count did not interfere, however, microfluidic experiments are more sensitive to platelet count, and in this way, better reflect in vivo primary hemostasis. Interestingly, although the number of patients was too small, the group with lower resistive index and higher thrombus parameters did not have a history of bleeding before liver transplantation.

There is evidence that VWF plays a major role in thrombus parameters especially on surfaces such as collagen III [188], which explains the comparable thrombus parameters between patients and controls on this adhesive surface, although variability was observed between patients. Moreover, in the static flow cytometry assay, which is completely independent of VWF, we observed clearly reduced platelet responses. These combined results suggest that VWF compensates for decreased platelet functionality, as previously proposed [19]. Indeed, a moderate correlation was seen between VWF antigen and thrombus parameters under flow. To further explore the participation of VWF, we performed an experiment with a low shear rate, to minimize the contribution of VWF, and an experiment with a VWF A1 domain blocker. Both experiments resulted in a reduction of thrombus parameters. This reduction was complete on collagen III but not on an adhesive surface consisting of a combination of laminin and VWF and on collagen I, which is particularly GPVI dependent [195].

Since this was an observational, proof-of-principle study, drawing definitive conclusions on the clinical aspects was challenging. True hemostasis-related bleeding or thrombosis is relatively uncommon in this patient population. Even though platelet function appeared somewhat impaired in the static platelet assay, patients with higher platelet counts (cluster subgroup 1) and severe liver disease showed normal to enhanced platelet thrombus formation in the microfluidic assay. Most patients, including those with moderately impaired thrombus formation in the microfluidic assay, did not receive platelet concentrates and did not experience excessive bleeding during surgery. In patients with increased platelet thrombus formation under flow conditions, the use of platelet

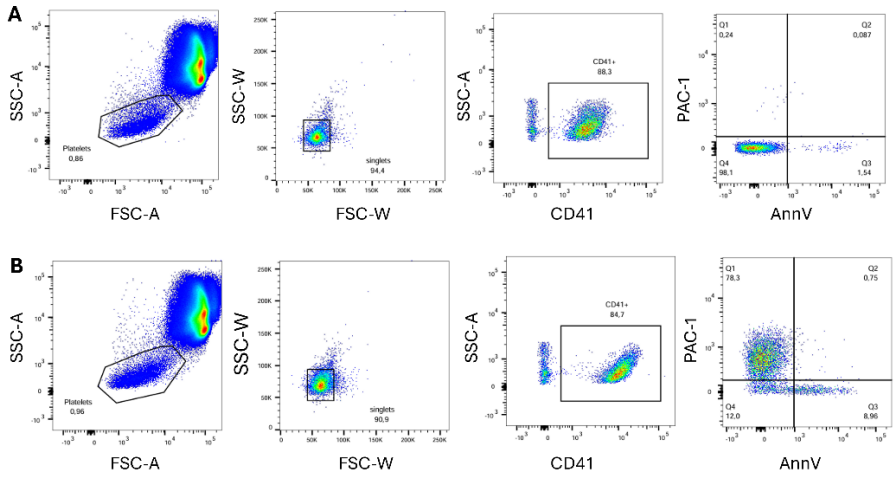
concentrates may be contraindicated before or during liver transplantation, although this concept requires clinical validation.

Three months post-liver transplantation platelet alterations were improved but not yet fully reversed. Platelet count increased but did not yet reach the levels of controls. Static agonist-induced platelet activation did surprisingly not improve and was even lower for some parameters. Platelet thrombus formation did improve but high patient variability was maintained. The reduction in VWF levels after liver transplantation was remarkable. Post-transplant patients received immunosuppressive medication which may also affect platelet function [196]. This could complicate comparisons between patients before- and after transplantation.

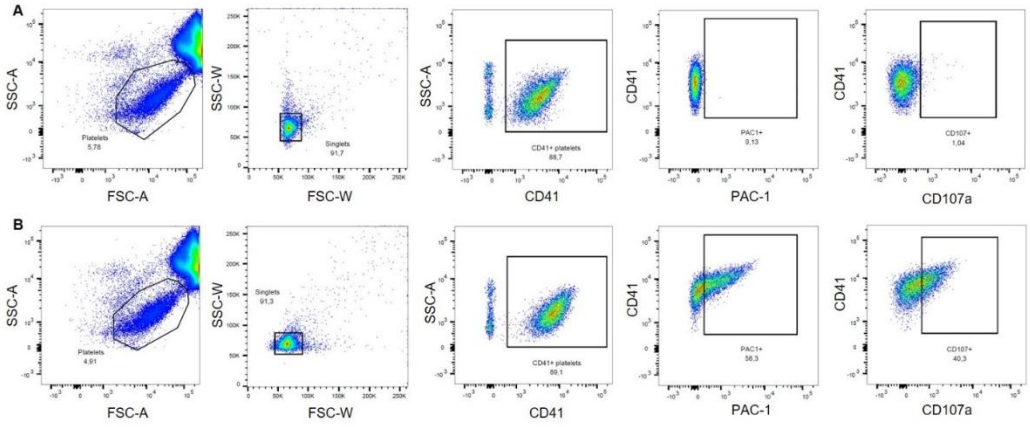
This study had some limitations. There was significant overlap in platelet activation and platelet thrombus formation between patients and the control group. However, this also underlines the relatively preserved primary hemostasis. We managed to include 9 patients 3 months post liver transplantation with a complete data set. Although this is a small sample size, it could already give an indication on reversibility of alterations. As the majority of patients had biliary atresia, the sample sizes in patients with other etiologies were too small for a meaningful comparison of the results.

In conclusion, in this proof-of-principle study, we demonstrated that children with cirrhosis have an increased proportion of partially activated platelets in the circulation, which are less effectively activated ex vivo. In a subgroup of pediatric patients with severe liver disease and higher platelet count, the low platelet activatability is likely overcome by high plasma VWF under high-shear flow conditions. Despite moderate hemostatic impairment, with lower platelet count and reduced thrombus formation, in the remainder of patients, no excessive bleeding was seen before, during or after liver transplantation. These data support a relatively preserved primary hemostasis in pediatric patients undergoing liver transplantation. Whether interventions supporting platelet function may benefit the subgroup of patients with apparently impaired platelet function and/or may harm the patients with higher platelet thrombus formation under flow requires additional clinical study.

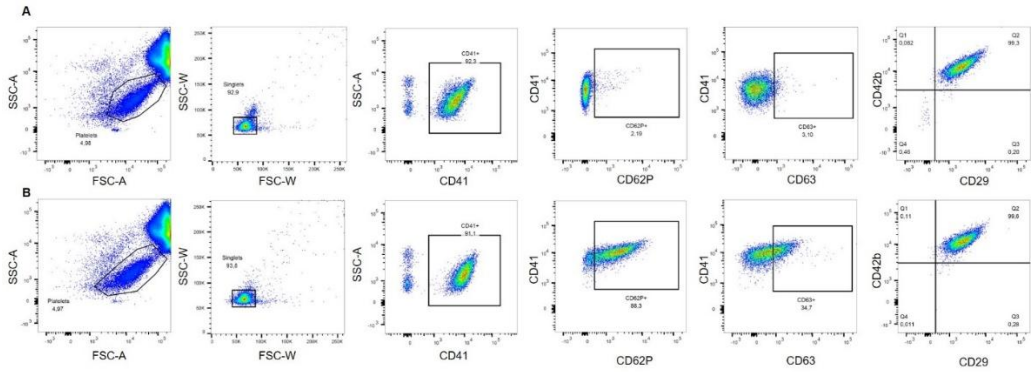
5.2.7. SUPPLEMENTARY MATERIALS



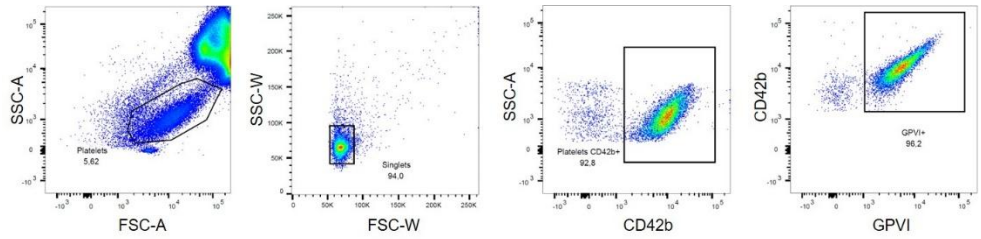
Phosphatidylserine exposure. (A) Without stimulation. (B) Maximal stimulation with α-thrombin and CRP-XL.



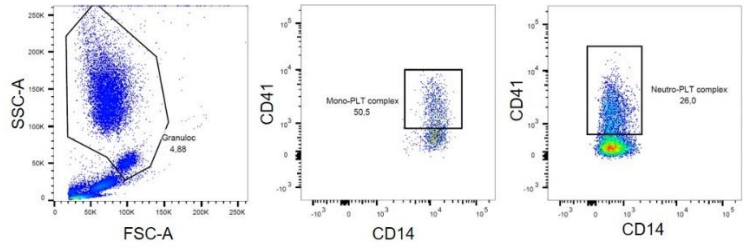
Platelet receptors and activation markers, panel 1. (A) Without stimulation. (B) Stimulation with TRAP-6 (3.3 µM).



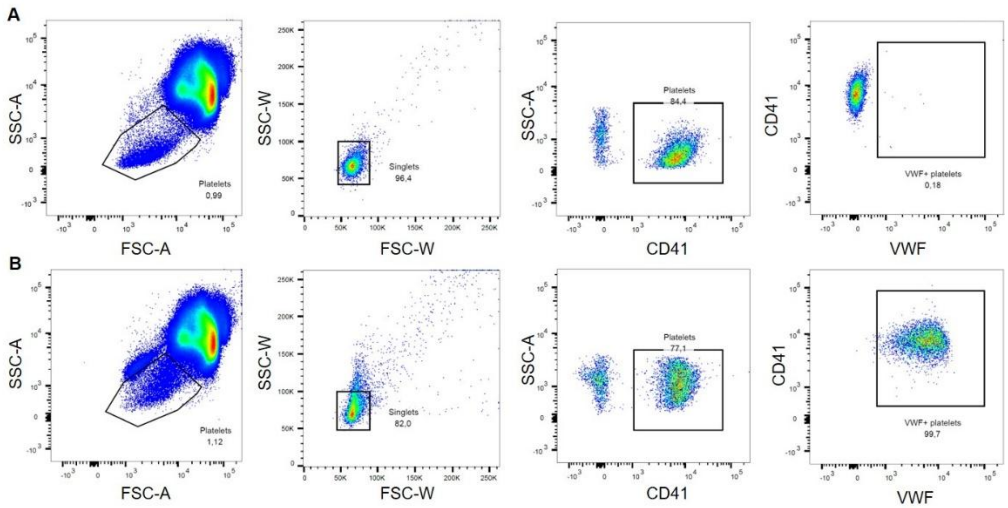
Platelet receptors and activation markers, panel 2. (A) Without stimulation. (B) Stimulation with TRAP-6 (3.3 µM).



Platelet GPVI expression.



Platelet-leucocyte complexes.



Platelet-bound von Willebrand factor. (A) Without stimulation. (B) Stimulation with ristocetin (2 mg/mL).

Fig. S6. Flow cytometry gating strategy and dot plots for different platelet panels.

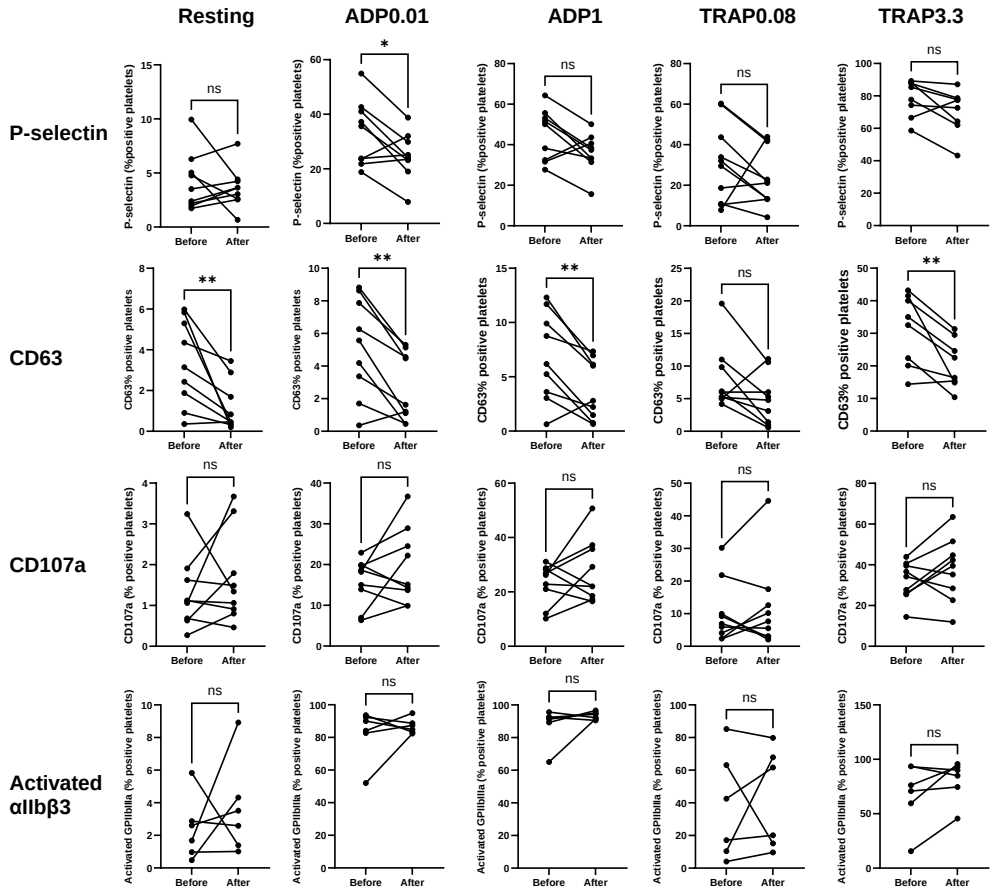
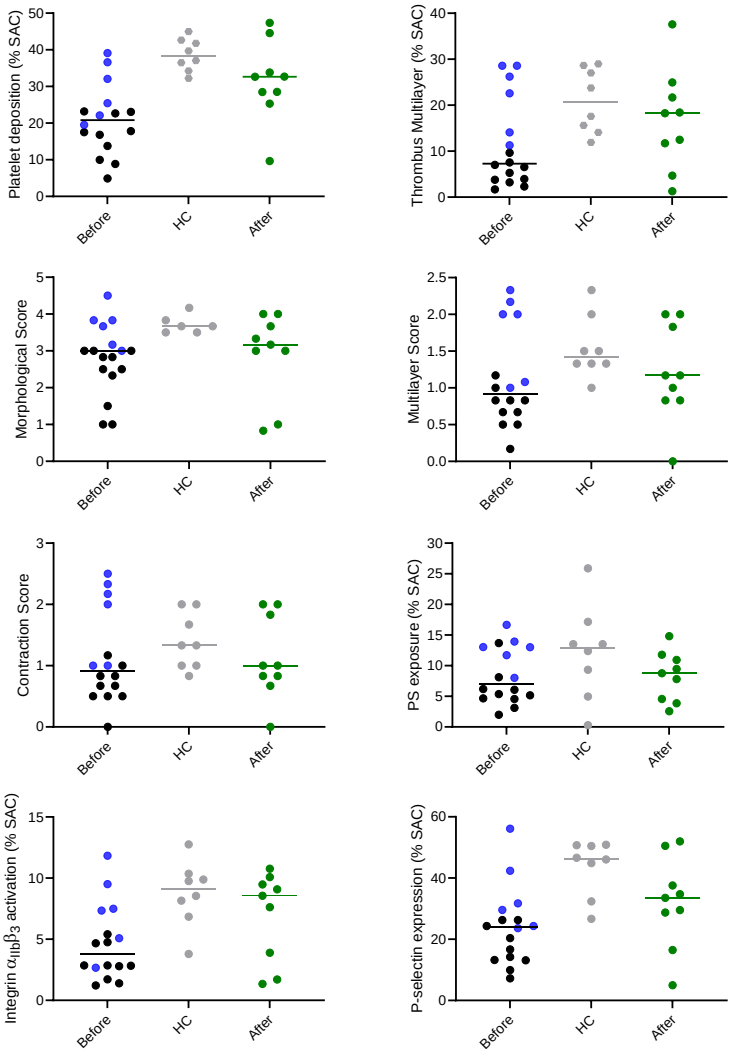
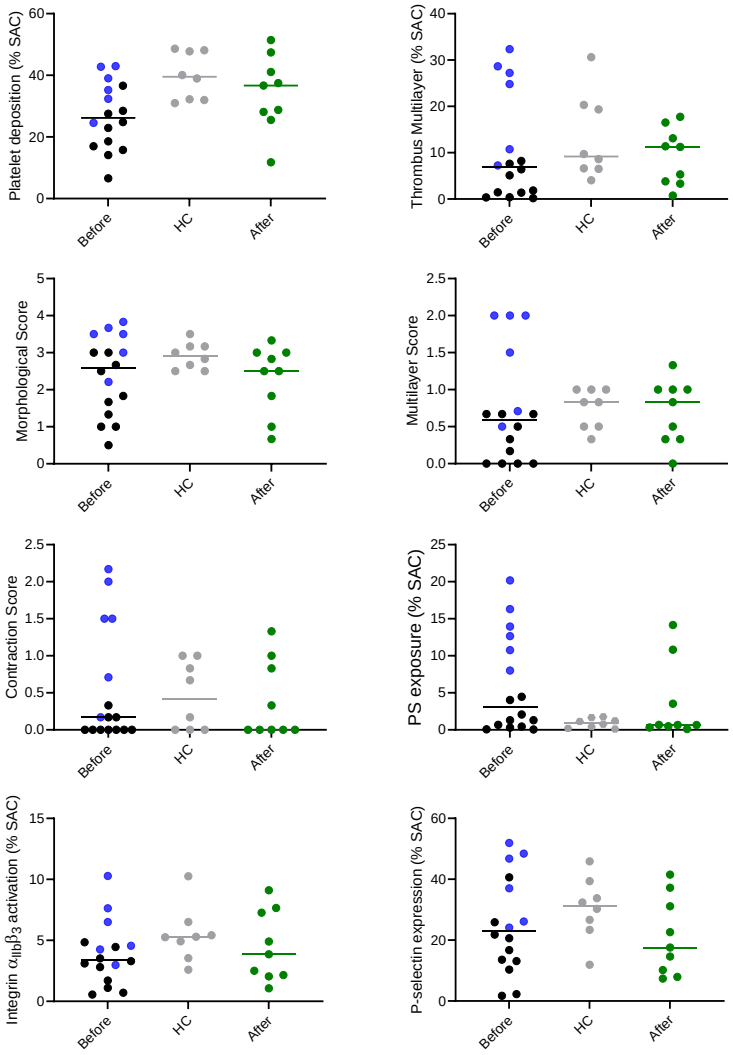


Fig.S7, Platelet flow cytometry results in patients before and after liver transplantation (n = 9). Platelet activation markers are expressed as % positive platelets.

(A) Collagen I


Platelet deposition (% SAC)
Thrombus Multilayer (% SAC)
Morphological Score
Multilayer Score
Contraction Score
PS exposure (% SAC)
Integrin $\alpha_{IIb}\beta_3$ activation (% SAC)
P-selectin expression (% SAC)
Before
HC
After

(B) Collagen III



(C) Laminin/VWF

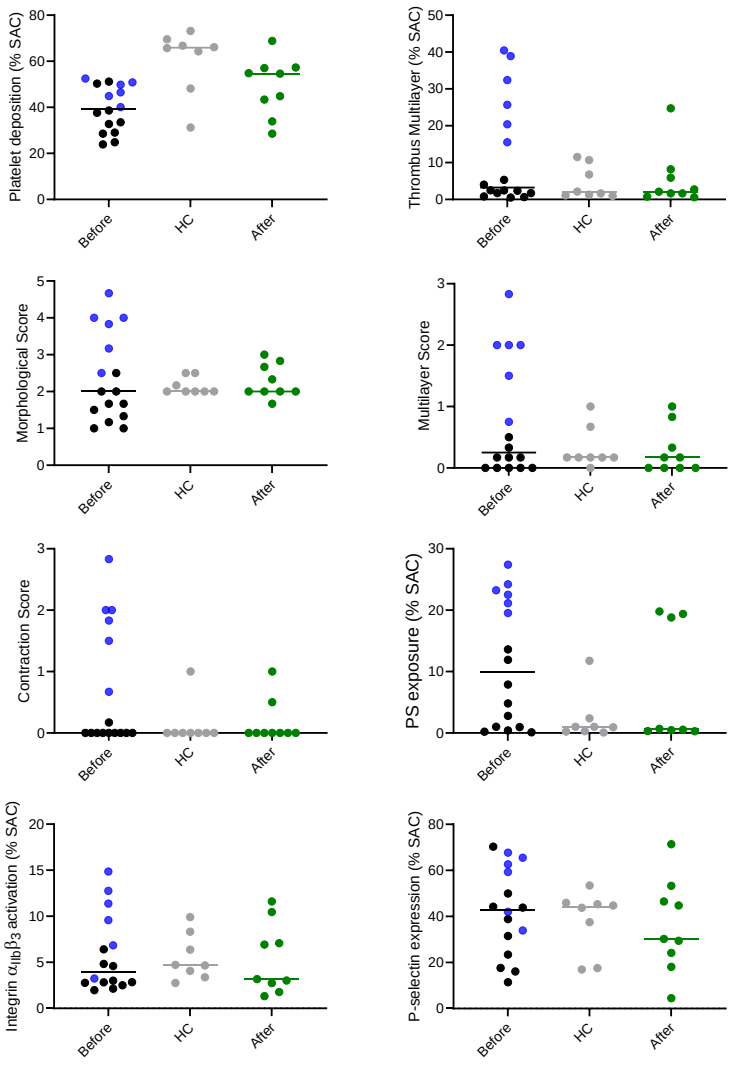


Fig. S8. Graphical representation of whole-blood thrombus formation on indicated surfaces in pediatric patients, before, and 3 months after liver transplantation, and in healthy controls. (A) collagen I (B) collagen III (C) laminin/VWF. Black bullets: pediatric patients before liver transplantation, blue bullets : patients in principal component subgroup 1, green bullets: patients after liver transplantation, grey bullets: healthy controls. SAC: surface area coverage

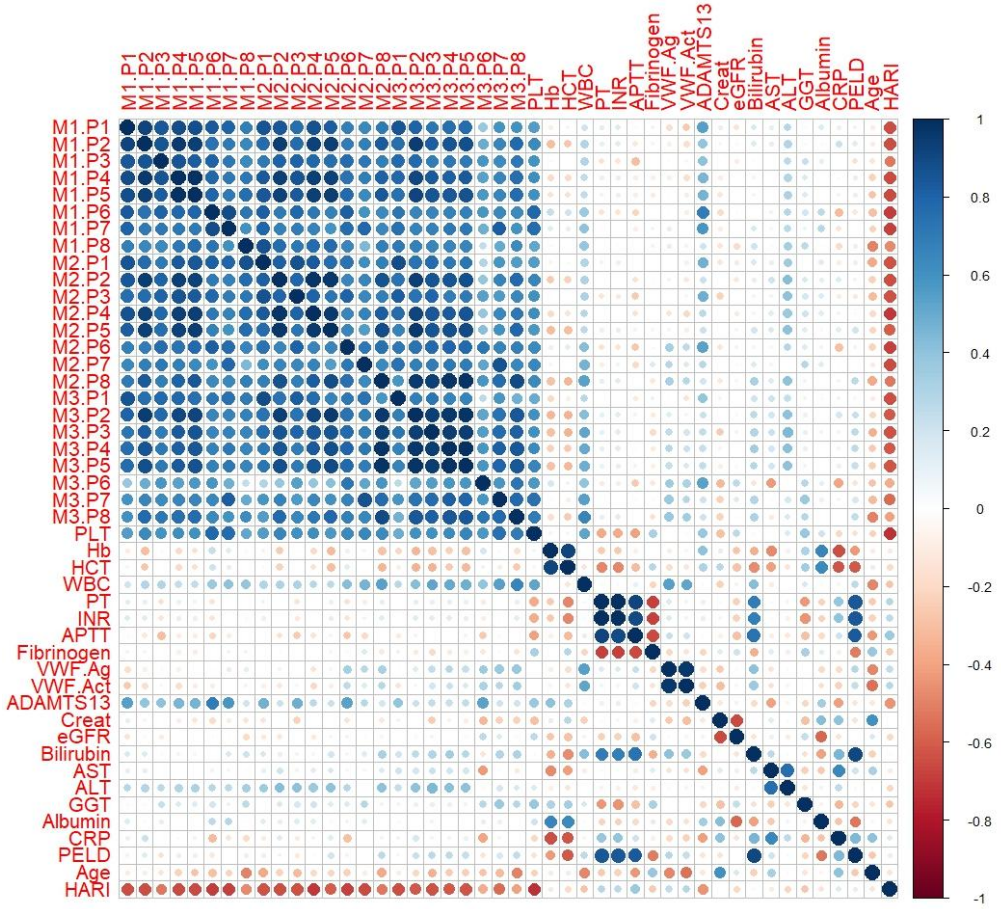


Fig. S9. Correlation matrix. Pearson correlation matrix showing excellent correlation between thrombus parameters and platelets and inverse correlation with patient age and hepatic artery resistive index.

Table S1. Details on fluorochrome-coupled antibodies and probes used in flow cytometry experiments.

Marker	Clone (if Ab marker)	Fluorochrome
CD41a	HIP8	PE
PAC-1	PAC-1	FITC
Annexin V	/	APC
CD107a	H4A3	PE-Cy7
CD42b	REA185	PerCP-Vio700
CD29	TS2/16	APC-Vio700
CD62P	REA389	APC
CD63	ME491	BV510
CD14	REA599	FITC
GPVI	HY101	PE
VWF	EPSISR15	Alexa fluor 488

5.3. RESULTS PAPER: PERSISTING THROMBOMODULIN RESISTANCE AT 3 MONTHS AFTER LIVER TRANSPLANTATION IN CHILDREN WITH CIRRHOSIS.

5.3.1. ABSTRACT

Background

The coagulation cascade in pediatric cirrhotic patients appears rebalanced, similar to adults, with few true hemostasis-related bleeds or thromboembolic events before liver transplantation. Vascular thrombosis is an important post- liver transplantation complication. Few papers have addressed the recovery of the coagulation cascade after liver transplantation.

Objectives

We aimed to assess the coagulation cascade, with both measurement of individual factors and a global hemostasis assay, before living donor liver transplantation, and to investigate its recovery three months after transplantation, when liver function has normalized.

Methods

From January 2022 to July 2023, pediatric cirrhotic patients were prospectively enrolled 1 day before liver transplantation. An age-matched control group was included for comparison. Routine hemostasis tests, levels of coagulation factors and natural anticoagulants, and thrombomodulin-modified thrombin generation were determined on automated coagulation analyzers at inclusion and 3 months after liver transplantation.

Results

Twenty-seven pediatric patients with cirrhosis, primarily of cholestatic origin, and 10 controls were enrolled. Sixteen patients were sampled 3 months after liver transplantation. Pediatric end-stage liver disease scores ranged from -10 to 44. A rebalanced coagulation cascade was confirmed in cirrhotic children, indicated by a thrombomodulin-modified thrombin generation assay similar to controls, although with higher interpatient variability. Interestingly, 3 months post-transplant, coagulation was not completely normalized. In the majority of patients resistance to thrombomodulin persisted.

Conclusion

This study confirmed a rebalanced coagulation system in pediatric cirrhotic patients before liver transplantation. Three months post-transplant thrombomodulin resistance persisted.

Whereas this contributes to thrombotic complications observed after liver transplantation, remains to be elucidated.

5.3.2. INTRODUCTION

The concept of rebalanced hemostasis is well-established in the adult cirrhotic population. Although the literature is more limited, it appears that the hemostatic system in pediatric patients with cirrhosis is also rebalanced in a similar manner [119, 120]. This is in line with the paucity of true hemostasis-related bleeds in these patients. Most bleeding events pre-transplant are variceal in nature and are mainly caused by high portal pressure. Similarly, thrombotic events are rarely seen in this patient group before liver transplantation.

Vascular thrombosis and bleeding are, however, important post-transplantation complications. The incidence of hepatic artery thrombosis after liver transplantation varies between 3.6 % and 7.4 % and presents a major cause of graft loss [197-199]. The actual cause of hepatic artery thrombosis is mostly unknown but surgical risk factors, like technical issues with the arterial anastomosis, are mainly identified. In our center, a 1-year incidence of 5.7% was observed for portal vein thrombosis [197]. Portal vein thrombosis is associated with several risk factors: patients age, weight, biliary atresia, warm ischemia time and technical variant grafts [200]. Peripheral thromboembolism is well-documented in adult patients but is less commonly reported in pediatric patients [201]. Deep venous thrombosis was seen in 6.5% of pediatric patients (n = 92) after liver or multivisceral transplantation in a study by Borst et al [202]. Although surgical factors are recognized as risk factors for vascular complications, nonsurgical factors, like hypercoagulability, may also play a role [203, 204]. Furthermore, a decrease in the rate of venous thrombosis was seen after the introduction of early post-operative heparin in a “before and after” study design [205]. The majority of bleeding events post liver transplantation are surgical and often require a repeat intervention.

Few papers have addressed the recovery of the coagulation cascade after liver transplantation. In the paper of Werner et al [119], several coagulation parameters were assessed at 1 month after liver transplantation. In our study, we aimed to assess the coagulation cascade, with both measurement of individual factors and a global hemostasis assay, before liver transplantation, and to investigate its recovery three months afterward.

5.3.3. METHODS

5.3.3.1. Patient population

From January 2022 to July 2023, pediatric patients with cirrhosis were prospectively enrolled 1 day before living donor liver transplantation at Cliniques Universitaires Saint-Luc (Brussels, Belgium). Informed consent was obtained from the parents or legal guardians. Blood samples were collected from the patients both immediately before and three months after liver transplantation. An age-matched control group of healthy individuals undergoing minor surgery was also included. The study received approval from the local ethics committee (2020/12MAR/157) in accordance with the Declaration of Helsinki.

5.3.3.2. Blood draw

Blood was collected in a citrate tube (Monovette® plastic 3.0 mL, 3.2% citrate, 106 mmol/L, Sarstedt) after drawing a discard tube. Samples were homogenized immediately after sampling. Platelet-poor plasma (PPP) was prepared within 30 minutes of venipuncture through two centrifugation steps (2×15 minutes at 2,500g). After the first centrifugation, the plasma was decanted and subjected to a second centrifugation. The aliquots were then immediately stored at −80 °C until analysis.

5.3.3.3. Analysis

Studied parameters were measured using an automated coagulation analyzer, the ACL-TOP 750 (Werfen, Barcelona, Spain). Routine parameters were measured with the following reagents: RecombiplastIN 2G (Werfen) for prothrombin time, SynthASil (Werfen) for activated partial thromboplastin time, HemosIL Thrombin time for thrombin time and QFA Thrombin (Werfen) for fibrinogen. For determination of the natural anticoagulants, following assays were executed: chromogenic protein C (HemosIL Protein C), free protein S (HemosIL Free Protein S) and antithrombin (HemosIL Liquid Antithrombin). Intrinsic coagulation factors were measured using factor deficient plasma (VIII, IX, and XI) and SynthASil (Werfen). Extrinsic coagulation factors were measured with factor deficient plasma (II, V, VII, X) and RecombiplastIN 2G (Werfen). For all parameters calibration was performed with HemosIL calibration plasma, which is traceable to international standards.

The ST Genesia is a benchtop analyzer for thrombin generation measurements. The system is based on the same principle as the calibrated automated thrombogram (CAT) but is fully automated and allows continuous loading of reagents and samples. Calibration was performed with STG®-ThrombiCal, STG®-FluoSet and STG®-FluoStart. Patient samples were run two times: once for the plasma absorption properties adjustment (STG®-FluoSet) and

once for the actual measurement of thrombin generation using STG®-ThromboScreen and STG®-FluoStart. The STG®-ThromboScreen kit enables to run thrombin generation without and with thrombomodulin (TM) with a fixed concentration of tissue factor and phospholipids. Three levels of controls (STG®-QualiTest) were also run.

5.3.3.4. Statistical analysis

Statistical analysis was performed with GraphPad Prism, version 9.5.1. Normal distribution was checked with a Kolmogorov-Smirnov test. For comparison between patients and control subjects, an Unpaired Student's t test (normal distribution) or Mann-Whitney test (non-parametric distribution) was used.

5.3.4. RESULTS AND DISCUSSION

5.3.4.1. Patient description

Twenty-seven pediatric patients were enrolled prospectively and a blood sample was taken 1 day (IQR: 1-7 days) before living donor liver transplantation. Sixteen patients were sampled 3 months afterward (IQR: 85-92 days). A control group, consisting of 10 age-matched, healthy pediatric controls, was also included. The majority of patients had cholestatic cirrhosis: biliary atresia (67%), progressive familial intrahepatic cholestasis (7%), and cholestatic cirrhosis of unknown etiology (11%). Other causes included α-1 antitrypsin deficiency (4%), drug-related cirrhosis (4%) and chronic liver disease of unknown etiology (7%). Median age was 21 months (IQR: 12-48) for patients and 20 months (IQR: 11-43) for controls. The pediatric end-stage liver disease (PELD) score varied between -10 and 44.

5.3.4.2. A rebalanced coagulation cascade is confirmed in children with liver cirrhosis

Prothrombin time and thrombin time were significantly prolonged in patients compared to controls. In contrast, APTT and fibrinogen levels showed no significant differences, though there was greater interindividual variability among patients, particularly in fibrinogen. Perturbation of routine hemostasis assays was more pronounced in patients with higher PELD score (**Figure 12**). Despite these prolonged hemostasis assays patients presented a rebalanced coagulation cascade with a simultaneous decline in pro- and anticoagulant proteins. This can be appreciated when looking at procoagulant – and anticoagulant drivers of hemostasis (**Figure 13**). Except for factor VIII, coagulation factors were generally lower in patients compared to controls. Natural anticoagulants, protein C and antithrombin, were significantly reduced, while protein S remained normal in most patients. Coagulation parameters showed considerable variability among patients compared to controls, consistent with findings from routine hemostasis assays.

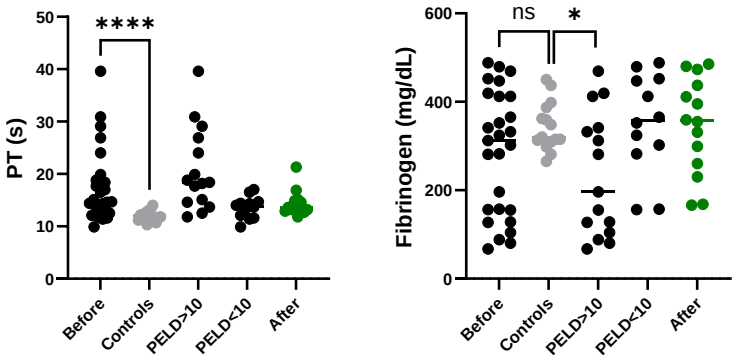


Fig.12. Routine hemostasis assays in patients before and after liver transplantation, compared to controls. APTT: activated partial thromboplastin time, PELD: pediatric end-stage liver disease score; PT: prothrombin time, TT: thrombin time. Note the difference in prothrombin time and fibrinogen between patients with a PELD <10 and >10. Bullets in black: patients before transplantation; bullets in green: patients after transplantation; bullets in grey: age-matched control group.

Without TM, thrombin generation was lower in patients compared to controls. In the presence of TM, no significant difference in thrombin generation was observed between the two groups, although variability between patients was high with some patients showing hypocoagulable or hypercoagulable features. When evaluating % inhibition TM, which measures the percentage inhibition of thrombin generation in the presence of TM, most patients with severe liver disease (indicated by a high PELD score) showed TM resistance. Protein C (r = 0.61) and protein S (r = 0.61) showed a good correlation with the percentage inhibition by TM, whereas FVIII did not demonstrate an inverse correlation with % inhibition (r = 0.27). Werner et al [119] examined various hemostatic parameters before, during, and up to one month after liver transplantation in children with cirrhosis. As seen in our study, the endogenous thrombin potential (ETP) was comparable to age-matched controls before liver transplantation. Magnusson et al [121] found lower ETP in children with prolonged routine coagulation assays, though they did not incorporate TM in their assay. Detailed results of thrombin generation are shown in **Figure 14**.

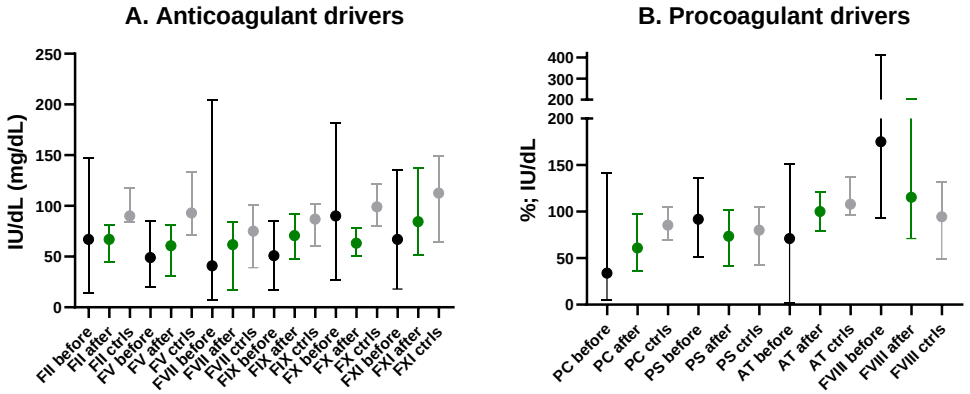


Fig.13. Anticoagulant drivers (A) and procoagulant drivers (B) in patients before and after liver transplantation, compared to controls. AT: antithrombin, ctrls: controls, F: factor, PS: protein S, PC: protein C. Bullets in black: patients before transplantation; bullets in green: patients after transplantation; bullets in grey: age-matched control group.

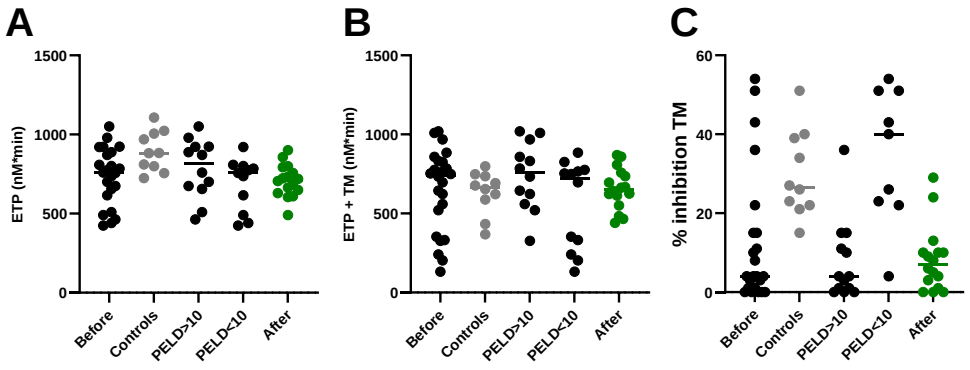


Fig. 14. Thrombin generation (endogenous thrombin potential) in patients compared to controls. **A**. Thrombin generation without TM; **B**. Thrombin generation with TM; **C**. % inhibition TM. Note: high resistance to TM in patients with high PELD score and patients 3 months after liver transplantation. APC: activated protein C, ETP: endogenous thrombin potential; PELD: pediatric end-stage liver disease score; TM: thrombomodulin. Bullets in black: patients before transplantation; bullets in green: patients after transplantation; bullets in grey: age-matched control group.

5.3.4.3. Three months after liver transplantation, coagulation assays were not completely normalized

In 16 patients coagulation cascade analysis was also performed 3 months after liver transplantation. Routine hemostasis parameters mostly normalized (**Figure 12**). Protein C and antithrombin were significantly higher 3 months after liver transplantation, but they remained lower than in the control group, especially protein C. The same trend was seen for the coagulation factors. FVIII lowered after transplantation but remained higher compared to controls. The remaining coagulation factors, with the exception of FII, was significantly higher in patients after liver transplantation but did not return to levels seen in the control group (**Figure 13**). Endogenous thrombin potential with TM improved post-transplantation with lower variability between patients. Interestingly, even 3 months after liver transplantation, patients still showed TM resistance (**Figure 14**). This may be partially attributed to persistently low levels of protein C ($r = 0.31$) and elevated FVIII ($r = -0.13$), although the correlation for protein C was not as strong as that observed before liver transplantation. This is in contrast to a paper by Mimuro [206] et al where protein C activity already normalized at 1 month post liver transplantation. Similar to our study, their study cohort comprised a majority of biliary atresia patients undergoing living donor liver transplantation.

In an adult cohort with cirrhosis TM resistance was identified as an independent risk factor of portal venous thrombosis [105]. In this study it is however not clear if endogenous thrombin potential with TM was higher in patients compared to controls. In our study, virtual all patients who underwent sampling 3 months post liver transplantation had resistance to TM in comparison to controls. It would be interesting in future, larger studies to correlate the incidence of TM resistance to the incidence of vascular complications. In our study only one patient had hepatic artery thrombosis and died 1 month after liver transplantation. No portal vein thrombosis or peripheral thromboembolism were observed.

5.3.5. CONCLUSION

Rebalanced hemostasis was confirmed in a cohort of pediatric patients with cirrhosis, just before liver transplantation. Interestingly, 3 months after liver transplantation coagulation was not completely normalized. In the majority of patients resistance to TM persisted. Whereas this contributes to thrombotic complications observed after liver transplantation, remains to be elucidated.

5.4. RESULTS PAPER: FIBRINOLYTIC PROFILE DEPENDS ON DISEASE SEVERITY IN PEDIATRIC PATIENTS WITH CIRRHOSIS: ILLUSTRATION BY 2 DIFFERENT PLASMA-BASED FIBRINOLYSIS ASSAYS

5.4.1. INTRODUCTION

The fibrinolytic state in cirrhotic patients is still a debated subject. Historically, cirrhotic patients were thought to be in a hyperfibrinolytic state which was thought to contribute to bleeding risk [207]. More recently, this hyperfibrinolytic state and its association to bleeding risk has been put into question. Patients with cirrhosis show simultaneous changes in pro- and antifibrinolytic mechanisms. Typically, a decrease in plasminogen, α2-antiplasmin and thrombin activatable fibrinolysis inhibitor (TAFI) and an increase in tissue plasminogen activator (tPA) and plasminogen activator inhibitor 1 (PAI-1) are seen [5]. Depending on the type and severity of liver disease, hypo, normo- and hyperfibrinolysis have been described [124]. Few reports on fibrinolysis are available in pediatric patients with cirrhosis. Similar to adult patients with cirrhosis hypo-, normo- and hyperfibrinolysis have been described [119]. Which assays can best be used to evaluate fibrinolysis remains uncertain and cut-offs for various available assays are not always firmly established. Since the process of fibrinolysis in clotting blood or plasma is slow, activators are added in most assays to estimate fibrinolytic potential more reliably and rapidly. Whereas fibrinolytic assays in whole blood may better represent physiology, plasma-based assays have been much more extensively used to estimate fibrinolytic potential in cirrhosis. One of the plasma-based assays used has been extensively clinically validated in the context of thrombosis in the general population, and may therefore have clinical relevance in patients with cirrhosis as well [208].

5.4.2. MATERIAL AND METHODS

5.4.2.1. Study population

Pediatric patients with cirrhosis, listed for liver transplantation at Cliniques Universitaires Saint-Luc, Brussels, Belgium, were enrolled prospectively. Informed consent was obtained from the parents or legal representative. A blood draw was performed just before and 3 months after liver transplantation in patients. In addition, an age-matched control group was included comprising healthy controls requiring minor surgery. The study was approved by the local ethical committee (2020/12MAR/157) based on the Declaration of Helsinki.

5.4.2.2. Blood collection

Peripheral blood was collected in a citrate tubes (Monovette plastic 3.0 mL, 3.2% citrate, 106 mM (Sarstedt, Numbrecht, Germany); 9:1 blood:citrate ratio) after drawing a discard tube. Citrate plasma was prepared by double centrifugation at 2500g during 10 minutes. Samples were stored at −80 ◦C until analysis.

5.4.2.3. Fibrinolysis assays

Clot lysis time was performed as previously described [209]. In short, a mixture of tissue factor (Dade Innovin, Siemens Healthineers, Forchheim, Germany, 1/1000 final dilution), $CaCl_2$, phospholipids and tPA (56 ng/mL) were added to plasma. During clot formation and clot lysis, turbidimetry at 405 nm was performed at 37°C. The time in minutes between the midpoint of coagulation and midpoint of fibrinolysis (CLT) was registered. Lysis timer was also executed as described before [210]. Patient plasma was mixed with silica and tPA (160 ng/mL). Coagulation was then triggered with human α-thrombin (4 NIH/mL) and $CaCl_{2.}$ Results were expressed as the time at which maximal lysis speed occurred (peak of the first derivative). Where indicated, PAI-1 antigen levels were measured with the Asserachrom PAI-1 (Stago, Asnières-sur-Seine, France) kit. Details on the plasma-based fibrinolysis assays are provided in **Supplementary Table 2**.

5.4.3. RESULTS

Twenty-five pediatric patients with cirrhosis were prospectively included just before liver transplantation. From this group, we also collected samples 3 months after liver transplantation for 16 patients. For comparison, a group of 12 age-matched pediatric healthy controls was sampled. Median pediatric end-stage liver disease (PELD)-score was 11 (6-20 IQR). Median age was 20 months (IQR: 11-48 months) for patients and 28 months (IQR: 11-52) for healthy controls. Indications for liver transplantation were biliary atresia in 72%, progressive familial intrahepatic cholestasis in 12%, other causes (α-1 antitrypsin; drug related) in 8% and unknown etiology in 12% of patients.

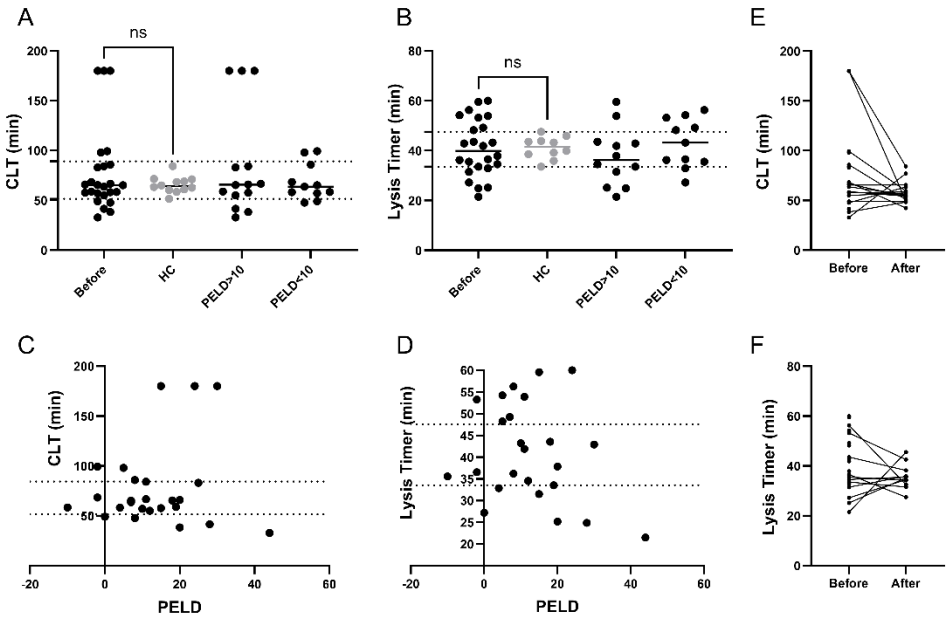


Fig.15. Clot lysis time (CLT) and Lysis Timer results in patients and controls. (A) CLT results in patients before liver transplantation compared with age-matched controls. Note the presence of severe hypofibrinolysis for Pediatric End-Stage Liver Disease (PELD) score of >10. (B) Lysis Timer results in patients before compared with age-matched controls. (C) Correlation between CLT and PELD score. (D) Correlation between Lysis Timer and PELD score. (E, F) Spaghetti plots comparing patients before and 3 months after liver transplantation for (E) CLT and (F) Lysis Timer (F). HC, healthy controls; ns, not significant.

The results for the CLT and Lysis Timer in our study population are shown in **Figure 15**. The CLT (median + IQR) was 64.3 min (60.6-70.6 min) in the controls and 65.2 min (56.1-85.1 min) in patients (p = 0.82). Although results were not significantly different, a higher variation was seen for CLT in patients. After exclusion of severe hypofibrinolytic results (i.e., samples that were not fully lysed or not lysed at all after 3 hours), changes in CLT correlated with severity of liver disease (PELD; r = -0.42; p = 0.05). Five patients (20%) were identified as having hypofibrinolysis, defined as a value above the highest value of the healthy controls. Three of these patients (12%) had severe hypofibrinolysis with CLT higher than 180 min (the limit of the assay). All the patients with severe hypofibrinolysis had PELD scores >10. Hyperfibrinolysis was also demonstrated in 5 patients (20%), identified as a result below the lowest value of the controls, of which 4 patients had PELD >10. Similar results were seen with Lysis Timer. Lysis Timer results (median + IQR) were 41.6 min (37.9-47.6 min) in controls and 37.9 min (32.9-49.3 min) in patients (p = 0.79). However, one patient with

severe hypofibrinolysis with CLT had a normal Lysis Timer result (42 min). PAI-1 level in this patient was 101 AU/mL (reference <16 AU/mL) which is concordant with hypofibrinolysis. After exclusion of patients with extreme hypofibrinolysis with CLT (at the limit of the assay), a strong correlation (r = 0.91; p< 0.001) was seen between the 2 assays (**Figure 16**) for patients. A more moderate correlation between the two assays (r = 0.73; p< 0.05) was seen for healthy controls. Most patients with aberrant fibrinolysis profiles pre-transplantation demonstrated CLT and Lysis timer results similar to healthy controls 3 months after liver transplantation.

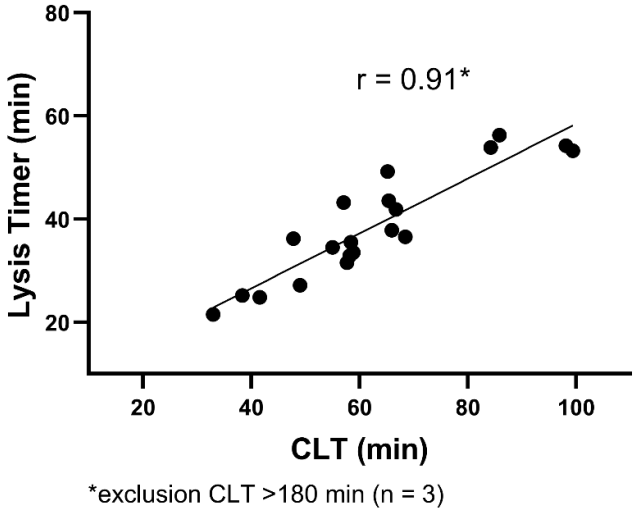


Fig.16. Correlation between clot lysis time (CLT) and Lysis Timer after exclusion of CLT of >180 minutes.

5.4.4. DISCUSSION

In pediatric patients with cirrhosis both hypo- normal-, and hyperfibrinolysis, compared to healthy controls, were demonstrated with 2 plasma-based fibrinolysis assays. The largest variation in fibrinolytic capacity was observed in patients with more severe liver disease. Werner et al [119] also demonstrated higher variation in results for CLT in patients compared to controls with CLT results ranging from hypo- to hyperfibrinolysis. In a cohort of adult patients with acute decompensation and ACLF, patients presented a mixed fibrinolytic phenotype with both clearly prolonged and shortened fibrinolysis [211]. In this adult population, hypofibrinolysis was associated with sepsis, organ failure and mortality. Although our control group was rather small, our pediatric population had similar results compared to the pediatric healthy controls in the paper by Werner et al [119] for CLT and

compared to the adult healthy population tested by Bareille et al [212] for Lysis Timer. Roullet et al [213] defined a Lysis Timer threshold of 31 min for the detection of hyperfibrinolysis in adult patients undergoing liver transplantation by comparing results with euglobulin clot lysis time. A limitation of this study is that we could not sample all patients 3 months after liver transplantation. Specifically, 2 patients had deceased, 1 patient experienced a clinical complication, and 6 patients returned to their country of origin shortly before sampling.

Overall concordance between the two fibrinolysis assays was excellent, with one exception: a patient who exhibited normal fibrinolysis with Lysis Timer but hypofibrinolysis with CLT. This discrepancy might be due to the lower tPA concentration used in CLT making it more sensitive to hypofibrinolysis. The higher tPA concentrations used in the Lysis Timer, however, result in shorter assay time making it more suitable for clinical applications. The CLT assay initiates coagulation with tissue factor whereas the Lysis Timer uses human α-thrombin. Since tissue factor acts upstream in the coagulation pathway, it allows for the detection of coagulation-mediated fibrinolysis defects, such as impaired activation of the thrombin-activatable fibrinolysis inhibitor due to hypocoagulability. In contrast, the Lysis Timer's use of excess exogenous thrombin ensures the conversion of a maximum amount of fibrinogen to fibrin, enhancing fibrin clot formation and structure [214]. This makes the assay more focused on the fibrinolytic system without being dependent on thrombin generation. As a commercially available test, the Lysis Timer could also offer better standardization of reagents and reference values. Conversely, the CLT assay has undergone extensive validation for clinical purposes [208].

In conclusion, our results are in line with previous publications in the pediatric cirrhotic population, confirming both hypo-and hyperfibrinolysis in this patient population, with two plasma-based fibrinolysis assays, of which Lysis Timer has never been tested before in a pediatric population. Aberrant fibrinolytic profiles (hypo- and hyperfibrinolysis) were seen in severe liver disease patients. After liver transplantation, these severe liver disease patients demonstrated fibrinolytic profiles similar to healthy controls.

5.4.5. ACKNOWLEDGEMENTS

We would like to thank Jelle Adelmeijer of the University Medical Centre Groningen and Sandrine Desmet of Cliniques universitaires Saint-Luc for expert technical assistance. We also would like to thank Nodia (Boom, Belgium) for providing the Lysis timer and the accompanying reagents.

5.4.6. SUPPLEMENTARY MATERIAL

Table S2. Details on the two plasma-based fibrinolysis assays.

	Clot lysis time	**Lysis Timer**
Matrix	Citrate plasma (50 µL)	Citrate plasma (100 µL)
Temperature	37°C	37°C
Fibrinolysis activator	tPA (56 ng/mL)	tPA (160 ng/mL)
Coagulation activator	TF (dade innovin, 1/1000 final dilution)	Human α-thrombin (4 NIH/mL) and silica (4 g/L)
Calcium chloride	17 mM	30 mM
Phospholipids	10 µM (Avanti polar lipids)	No phospholipids
Buffer	HEPES buffer 25 mM, 137 mM NaCl, 3.5 mM KCl, 0.1% BSA, pH 7.4	Glycine, HEPES, BSA buffer (+NaCl in 2nd reagent with thrombin)
Assay time	180 min	60 min
Measurement	Turbidity change (405 nm)	Turbidity change (940 nm)
Instrument	Spectramax 340 kinetic microplate reader	Lysis Timer
Software	SoftMax Pro (Molecular Devices Corporation), LysisAnalyzer version 4.0.0.0	LysisControl version 2.0.2
Result	Time between 1/2 coagulation and 1/2 fibrinolysis (min)	Peak of the first derivative or maximal lysis speed (min)

Abbreviations: BSA: bovine serum albumin; CLT: clot lysis time; TF: tissue factor; tPA: tissue plasminogen activator

5.5. RESULTS: INFLAMMATION

5.5.1. NETOSIS

5.5.1.1. Netosis markers are elevated in pediatric patients with cirrhosis, especially in severe liver disease

The discovery of NETs is rather recent and literature has mainly focused on the adult population. Unlike our adult cohort (cfr below), cfDNA was clearly elevated in children with cirrhosis, before liver transplantation, in comparison to age-matched controls. Three months post-transplantation, cfDNA levels significantly decreased, aligning with those of the control group. Since cfDNA is not a specific marker of NET formation, this could also indicate more pronounced cell damage in these patients. MPO-DNA was only significantly increased in patients with PELD score >10 and returned to normal 3 months after liver transplantation. Results are depicted in **Figure 17**.

5.5.1.2. MPO-DNA complexes, but not cfDNA, were increased in adult cirrhotic patients

CfDNA levels showed no significant difference between adult patients and controls ($p = 0.89$). In contrast, MPO-DNA complexes were significantly elevated in patients (median [IQR]: 0.49 [0.17–1.40] AU) compared to controls (median [IQR]: 0.07 [0.06–0.10]; $p < 0.0001$).This is in line with a paper by Zenlander et al [144] who also demonstrated high MPO-DNA and high citrullinated histone H3-DNA in patients with liver cirrhosis and hepatocellular carcinoma. Von Meijenfeldt et al showed increased MPO-DNA in patients with liver disease before and during different phases of liver transplantation [175]. Similar to our results they did not observe high levels of cfDNA in adult cirrhotic patients compared to controls before liver transplantation. Interestingly, Agraz-Cibrian et al demonstrated that peripheral blood neutrophils of patients with cirrhosis had reduced NET formation capacity upon stimulation [215]. Results for adult patients are shown in **Figure 18**.

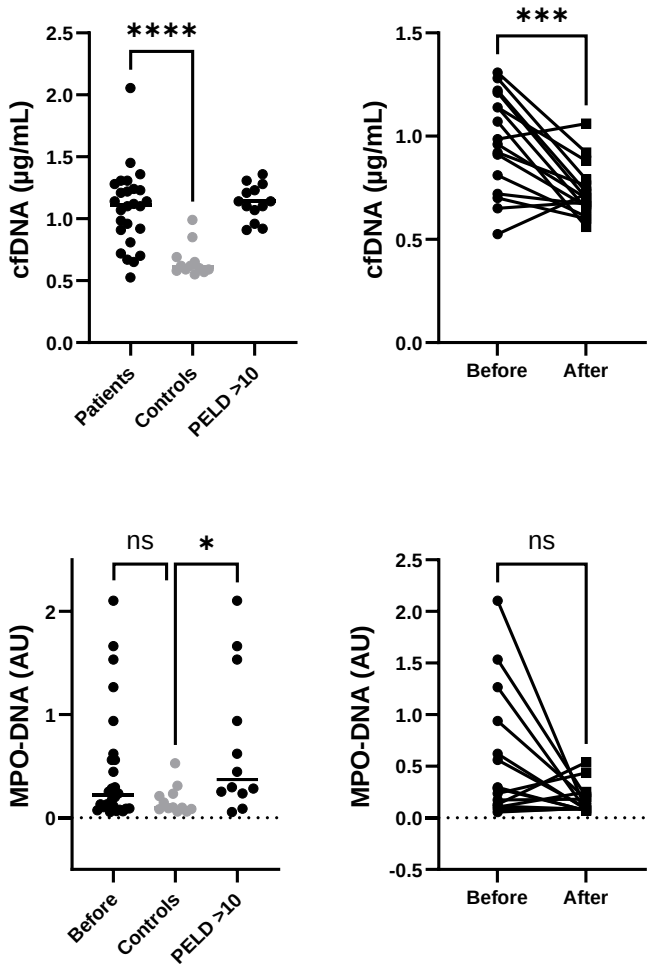


Fig.17. NETosis markers, cfDNA and MPO-DNA complexes, in pediatric cirrhotic patients before and after liver transplantation compared to age-matched controls. Note: higher cfDNA and MPO-DNA complexes in patients with higher PELD-score**.** Abbreviations: cfDNA, cell-free DNA; MPO, myeloperoxidase; PELD, pediatric end-stage liver disease score. Levels of significance: **** p<0.0001 (Unpaired Student's t-test); ***p<0.001 (Paired Student's t-test); *p<0.05 (Mann-Whitney test); ns, not significant (Wilcoxon test).

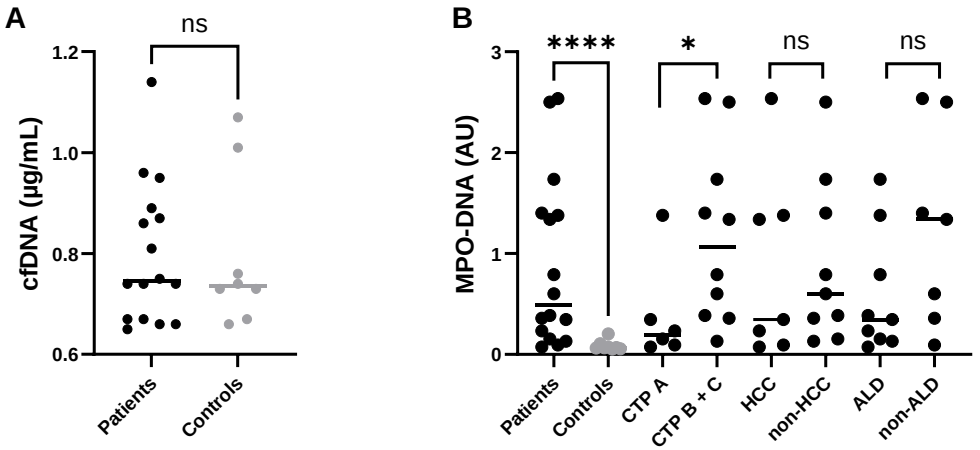


Fig.18. Markers of NETosis in adult patients before liver transplantation compared to controls. (A) Cell-free DNA. (B) MPO-DNA. Note the significant difference between CTP A patients and CTP B+C patients in MPO-DNA. Levels of significance: ***p < 0.001 (Mann-Whitney test); *p <0.01 (Mann-Whitney test); ns, not significant (Mann-Whitney test). Abbreviations: ALD: alcoholic liver disease, CTP: Child-Turcotte-Pugh, HCC: hepatocarcinoma; MPO: myeloperoxidase.

5.5.1.3. Netosis markers correlate with severity of liver disease

A moderate correlation was seen between PELD score and cfDNA (r = 0.47; p <0.05) and MPO-DNA (r = 0.40; p <0.05) in pediatric patients. For both NETosis markers different correlations were seen for the PELD components (**Figure 19**). A very good correlation was seen between total bilirubin levels and cfDNA (r = 0.79; p <0.0001).

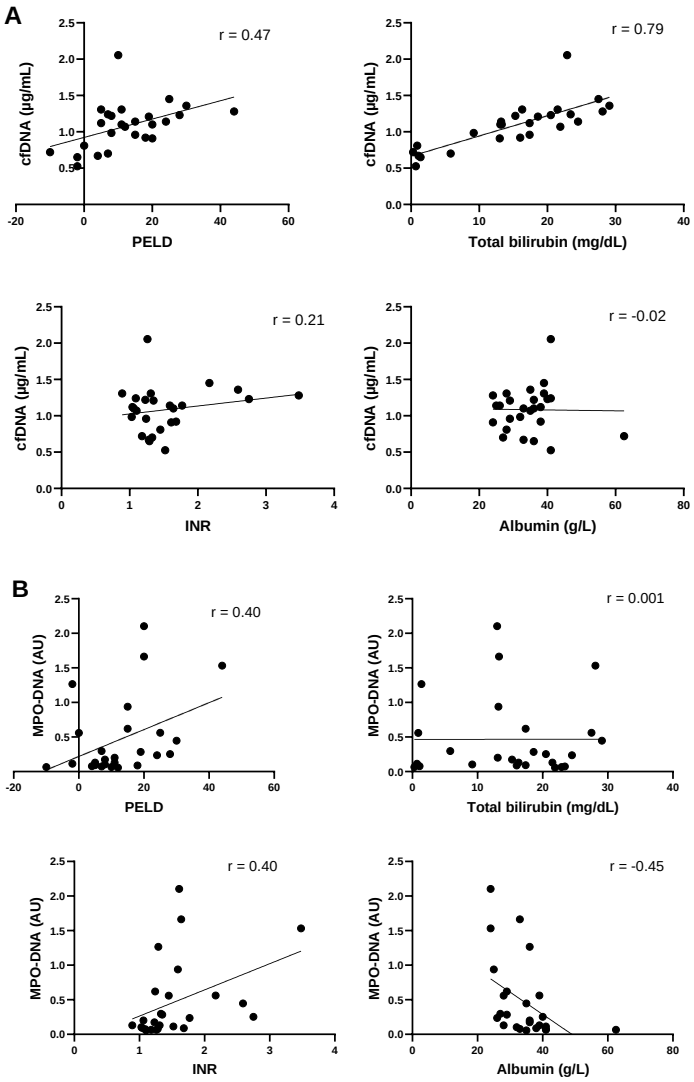


Fig.19. Correlation between NET markers, PELD score and its determinants in pediatric patients with cirrhosis. (A) Correlation between cfDNA, PELD, total bilirubin, albumin and INR. (B) Correlation between MPO-DNA complexes, PELD, total bilirubin, albumin and INR. Abbreviations: cfDNA, cell-free DNA; INR, international normalized ratio; MPO, myeloperoxidase; PELD, pediatric end-stage liver disease score.

Although correlation with MELD score was poor (r = 0.29; p = 0.28), adult patients with high severity of liver disease (CTP B and C) demonstrated higher MPO-DNA in comparison to patients with CTP A (**Figure 18**). No correlation was observed between MPO-DNA complexes and bilirubin (r = 0.16; p = 0.56) and creatinine (r = -0.004; p = 0.99). Correlation with INR was weak and not significant (r = 0.35; p = 0.18). A moderate inverse, though not significant, correlation was found between albumin and MPO-DNA complexes (r = -0.43; p = 0.10). We did not demonstrate a difference in MPO-DNA between patients with or without hepatocellular carcinoma, nor did we see a difference between alcoholic liver disease and other etiologies. This was based on a limited sample size of adults with cirrhosis. Moreover, patients with HCC had lower severity of liver disease (5 patients with CTP A, 2 patients with CTP B and 0 patients with CTP C).

5.5.1.4. Moderate correlation between MPO-DNA complexes and coagulation parameters

No significant correlation was seen between thrombomodulin-modified thrombin generation and MPO-DNA for pediatric patients (r = 0.29; p = 0.17; **Figure 20**) and for adult patients (r = 0.40; p = 0.12; **Figure 21**). A moderate inverse correlation between MPO-DNA complexes and fibrinogen was seen in pediatric (r = -0.39; p = 0.05) but not in adult patients (r = -0.04; p = 0.90).

Von Willebrand antigen and activity demonstrated a moderate correlation with MPO-DNA in pediatric (r = 0.16; p = 0.44 and r = 0.44; p <0.05, respectively; **Figure 20**) and adult (r = 0.50; p <0.05 and r = 0.49; p = 0.06, respectively; **Figure 21**) patients. No correlation was seen between cfDNA and VWF. The correlation between MPO-DNA complexes and VWF may suggest a link with endothelial dysfunction and inflammation [216]. In S. aureus sepsis NETs were attached to liver sinusoids by the intermediary of VWF [217]. In an in vitro experiment, Grassle et al. [218] demonstrated that shear-activated VWF can bind to DNA released from activated neutrophils.

Two different papers did not demonstrate a link between thrombin-antithrombin complexes and MPO-DNA [144, 175]. Similarly, Blasi et al [219] did not show a link between cfDNA, MPO-DNA and thrombin-antithrombin complexes in patients with acute decompensation and acute-on-chronic liver disease. In the paper by von Meijenfeldt et al, an association between cfDNA and coagulation activation was demonstrated in patients during and after liver transplantation, suggesting that this marker could contribute to the hemostatic rebalance during liver transplantation [175]. CfDNA during liver transplantation is likely the result of cell damage and not NETosis. These papers conclude that NETs do not seem to contribute to systemic activation of the coagulation system.

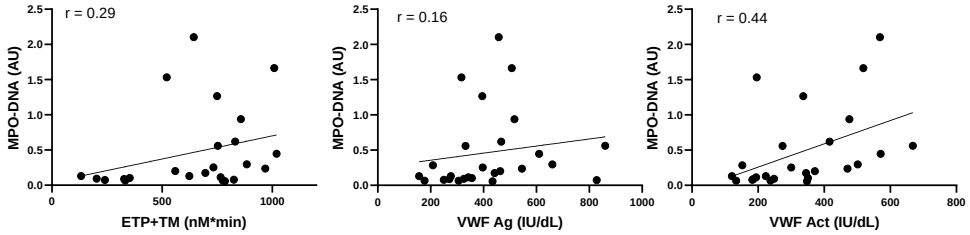


Fig.20. Correlation between MPO-DNA complexes and coagulation parameters in pediatric patients with cirrhosis. Abbreviations: ETP, endogenous thrombin potential; MPO, myeloperoxidase; VWF, von Willebrand factor.

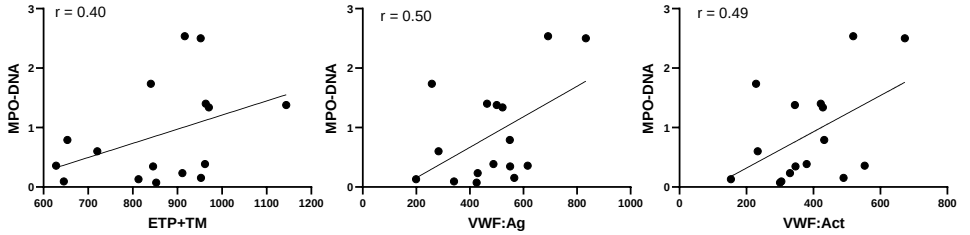


Fig.21. Correlation between MPO-DNA and coagulation parameters in adult patients with cirrhosis. Abbreviations: ETP, endogenous thrombin potential; MPO, myeloperoxidase; VWF, von Willebrand factor.

5.5.2. CONCLUSION

NET formation appears to be associated with advanced liver disease, as indicated by elevated levels of cfDNA and MPO-DNA complexes correlating with higher PELD scores in pediatric patients and increased MPO-DNA complexes correlating with CTP B and C cirrhosis in adult patients. Patients with CTP B and C cirrhosis were often in a decompensated state in our study. In contrast to adult patients, pediatric patients showed higher cfDNA compared to age-matched controls. Since cfDNA is not a specific marker of NET formation, this could also indicate more pronounced cell damage in patients versus controls. The exact mechanism driving increased NET formation in cirrhosis is unclear, but chronic systemic inflammation is likely a contributing factor [144, 220]. While NETs can provide a scaffold for thrombus components, the connection between NET formation and coagulation activation is still ambiguous. As our study was observational, we could only demonstrate moderate correlations between NET formation and certain coagulation parameters (fibrinogen and von Willebrand factor), without establishing a causal link

between NET formation and hemostasis. These observed correlations may simply reflect liver disease severity rather than direct mechanistic relationships.

5.6. RESULTS ADULT COHORT

5.6.1. STUDY PARTICIPANT CHARACTERIZATION AND CONVENTIONAL ASSAYS

We enrolled 16 adult cirrhotic patients with cirrhosis and 8 apparently healthy controls. In one patient liver transplantation did not take place due to a graft problem. This patient died 3 months later. In the remaining patients, blood samples were taken either the day before or on the day of liver transplantation. Additionally, a portal blood sample was collected from 9 patients. Based on the limited post-transplant sample size (n = 7), we chose to use these samples exclusively for comparison with pediatric post-transplant samples (see 5.6.3). For seven patients we also obtained a sample 3 months after transplantation. Median age was 59 years for patients and 60 years for controls. Twenty-five percent of patients were female, compared to 50% of the controls. The most common indication for liver transplantation was alcoholic steatohepatitis. The median MELD-score was 15 and varied between 9 and 32. Six patients were in CTP A, 4 in CTP B and 6 in CTP C. Forty-four percent of patients presented with decompensated cirrhosis and 44% had hepatocellular carcinoma. Details on patient and controls are shown in **Table 6**.

The patient blood samples showed a significantly lower platelet count and hemoglobin level. Mean platelet volume was not significantly different between patients and controls. Patients had a higher international normalized ratio (INR; $p < 0.001$) and longer activated partial thromboplastin times ($p < 0.01$) when compared to healthy controls. Values for fibrinogen were not significantly different ($p = 0.07$) between patients and controls, except for patients with CTP B and C ($p < 0.05$). Plasma levels of AST, ALT, bilirubin, GGT and C-reactive protein were higher in patients compared to controls (**Table 6**). For the majority of patients, normal values were seen for creatinine and glomerular filtration rate. None of the patients were on dialysis.

Table 6. Characteristics of patients before liver transplantation and healthy controls.

	Cirrhotic adult patients (n = 16)	Healthy volunteers (n = 8)
Age in years	59 (52-62)	60 (54-62)
Gender F/M	4/12	4/4
Etiology		
ASH	7	NA
viral	2	NA
Alpha-1 AT	2	NA
NASH	1	NA
PBC	1	NA
mix ASH/NASH	1	NA
mix ASH/viral	1	NA
mix NRH/viral	1	NA
HCC	7/16	NA
MELD	15 (11-16)	NA
CTP	9 (6-11)	NA
A	6	NA
B	4	NA
C	6	NA
Hemoglobin, g/dL	10,9 (9,1-13,3)	14,1 (13,0-15,0)
Platelets ,10^9/L	86 (60-124)	241 (232-264)
MPV, fL	9,9 (9,5-11,0)	9,4 (9,4-9,7)
WBC, *10^9/L	4,7 (3,1-6,4)	5,8 (4,6-6,7)
INR	1,52 (1,40-1,84)	1,00 (0,96-1,02)
APTT, s	34.4 (29.9-35.7)	27.9 (27.4-28.9)
Fibrinogen, mg/dL	312 (189-359)	372 (341-405)
D-dimers, ng/mL	976 (587-5164)	344 (295-512)
Total bilirubin, mg/dL	2,5 (1,2-3,4)	0,5 (0,4-0,6)
AST, U/L	46 (35-78)	18 (18-25)
ALT, U/L	33 (25-42)	15 (13-22)
GGT, U/L	54 (39-175)	18 (14-23)

LDH, U/L	235 (181-256)	195 (162-206)
Albumin, g/L	33 (28-36)	47 (43-48)
CRP, mg/L	8,7 (2,2-15,3)	1,0 (1,0-1,2)
Creatinine, mg/dL	0.91 (0.64-1.0)	0.94 (0.87-0.97)
HVPG (n = 6), mmHg	10,5 (7-12)	NA
Decompensated, #	7	NA
Esophageal varices		
absent	5	NA
grade 1	4	NA
grade 2	4	NA
grade 3	2	NA
unknown	1	NA
Ascites		
absent	9	NA
small to moderate	4	NA
significant	3	NA
Previous hemorrhage	9/16	NA
variceal	7	NA
spontaneous (epistaxis, hematoma)	2	NA
Previous thrombosis	5/16	NA
PVT	4	NA
DVT	1	NA

Values are represented as median (IQR). ALT: alanine aminotransferase, ASH: alcoholic steatohepatitis, AST: aspartate aminotransferase, CRP: C-reactive protein, CTP: Child-Turcotte-Pugh, DVT: deep vein thrombosis, GGT: gamma glutamyl transferase, HCC: hepatocellular carcinoma, NRH: nodular regenerative hyperplasia, INR: international normalized ratio, LDH: lactate dehydrogenase, MPV: mean platelet volume, NA: not applicable, NASH: non-alcoholic steatohepatitis, MELD: Model for end-stage liver disease score, PBC: primary biliary cholangitis, PVT: portal vein thrombosis, WBC: white blood cell count

5.6.2. PRIMARY HEMOSTASIS

5.6.2.1. Impaired agonist-induced platelet activation in patients under stasis

As observed in pediatric patients in the previous paragraphs, the proportion of PS-exposing platelets was significantly lower in adult cirrhotic patients compared to healthy controls following maximal stimulation with α-thrombin and CRP-XL (**Figure 22**; p <0,0001). In unstimulated platelets, the proportion of procoagulant, PS-exposing platelets was higher in patients compared to controls (p <0.05). Low PS exposure was correlated with higher severity of liver disease (MELD; r = -0.50; p <0.05; **Figure 23**).

Additional flow cytometry measurements with diluted blood indicated that the patient's platelets had decreased α-granule release (CD62P expression); αIIbβ3 activation (PAC-1 mAb) and lysosomal release (CD107a expression) in comparison to control platelets, when stimulated with a high concentration of the thrombin receptor agonist TRAP-6 (3.3 µM; **Table 7**). A similar trend was observed with the GPVI agonist CRP-XL but this did not reach significance due to the small sample size of patients and controls. In the paper by Nassar et al [109], decompensated cirrhotic patients tested before transjugular intrahepatic portosystemic shunt (TIPS) procedure also showed significantly lower αIIbβ3 activation in response to TRAP-6 (20 µM), consistent with our findings. However, they reported significantly higher P-selectin expression in response to ADP (20 µM), which may be attributed to the 20-fold higher agonist concentration used in their study.

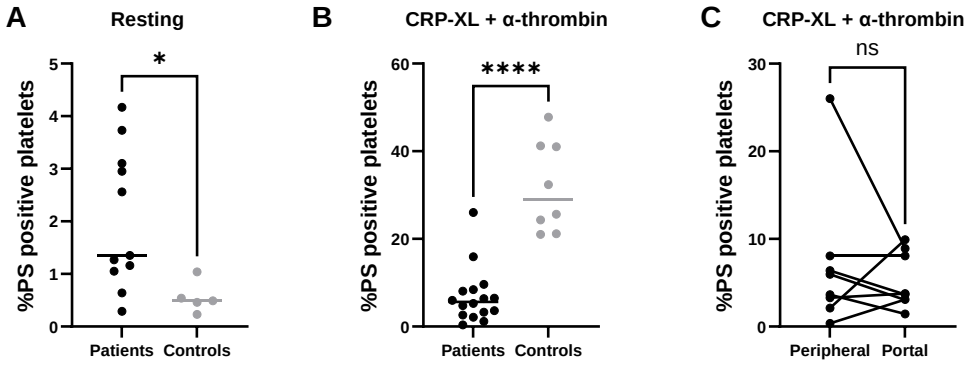


Fig. 22. Flow cytometric assessment of platelet phosphatidylserine (PS) exposure in patient blood before liver transplantation compared to healthy controls. (A) PS exposure of unstimulated platelets from patients and healthy controls. (B) PS exposure of platelets stimulated with CRP-XL and α-thrombin (maximally effective concentrations) of platelets from cirrhotic patients and healthy controls. (C) PS exposure of platelets stimulated with CRP-XL and α-thrombin in a peripheral blood sample compared with a portal blood sample. *Abbreviations*: CRP-XL: collagen-related-peptide

(cross-linked), PS: phosphatidylserine. Levels of significance *p < 0.05 (Unpaired Student's t-test); ****p < 0.0001 (Mann-Whitney test); ns : non-significant (Wilcoxon test).

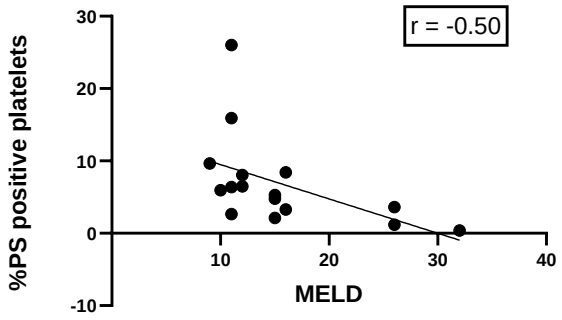


Fig.23. Correlation between PS-exposing platelets and MELD. Abbreviations: MELD, model for end-stage liver disease score; PS, phosphatidylserine.

Table 7. Platelet flow cytometry results in adult patients and healthy controls.

Agonist	Activation marker	Patients		Healthy volunteers		p-value
		n	%positive platelets	n	%positive platelets	
Resting	CD62P	16	4,8 (2,9-7,8)	8	4,4 (2,6-7,8)	0,70
	activated $\alpha_{IIb}\beta_3$	10	1,8 (0,2-4,2)	5	6,1 (2,0-10,7)	0,14
	CD63	16	1,8 (0,6-3,5)	8	1,7 (0,5-3,1)	0,85
	CD107a	16	1,2 (0,9-1,7)	7	0,7 (0,6-0,9)	0,14
ADP 0.01	CD62P	16	39,5 (31,6-49,4)	8	44,6 (32,0-51,5)	0,70
	activated $\alpha_{IIb}\beta_3$	10	90,3 (80,8-92,8)	5	95,8 (93,0-96,9)	0,05
	CD63	16	3,9 (1,9-6,6)	8	4,8 (2,0-6,5)	0,90
	CD107a	16	11,7 (7,5-22,2)	8	20,5 (17,4-40,0)	0,14
ADP 1	CD62P	16	50,5 (43,7-61,2)	9	58,9 (47,9-62,7)	0,47
	activated $\alpha_{IIb}\beta_3$	10	94,3 (76,7-95,2)	5	97,3 (95,4-98,0)	0,13
	CD63	16	5,6 (2,3-7,0)	8	5,0 (2,1-7,1)	0,83
	CD107a	16	17,6 (10,6-25,6)	8	28,3 (23,3-44,7)	0,07
TRAP 0.8	CD62P	16	22,2 (17,0-40,3)	8	37,8 (21,0-56,8)	0,24
	activated $\alpha_{IIb}\beta_3$	10	18,6 (7,9-53,4)	5	60,7 (32,4-91,0)	0,14
	CD63	16	4,5 (2,0-6,5)	8	4,8 (3,7-8,5)	0,70
	CD107a	16	4,8 (3,2-8,6)	8	7,2 (3,4-22,0)	0,39
TRAP 3.3	CD62P	16	85,6 (78,3-89,0)	8	93,6 (90,1-95,4)	**0,02**
	activated $\alpha_{IIb}\beta_3$	10	76,3 (40,1-92,2)	5	97,4 (96,1-98,2)	**0,02**
	CD63	16	32,0(24,0-42,5)	8	34,9 (27,3-49,7)	0,62
	CD107a	16	32,8 (26,2-37,3)	8	49,7 (44,7-52,5)	**0,02**
CRP 150	CD62P	6	23,4 (12,5-71,6)	3	95,3 (92,7-97,0)	0,07
	activated $\alpha_{IIb}\beta_3$	6	23,6 (5,2-61,2)	3	97,9 (87,1-98,5)	0,07
	CD63	6	4,0 (0,6-16,1)	3	37,7 (31,1-54,2)	0,07
	CD107a	6	7,1 (1,3-37,4)	3	64,7 (38,6-66,6)	0,07
Agonist	**Platelet receptor**	**Patients**		**Healthy volunteers**		**p-value**
		n	**MFI**	**n**	**MFI**	
Resting	CD42b	15	12678 (11495-15076)	8	13114 (12042-13400)	0,86
	CD29	15	p0,58	8	3095 (2432-3526)	0,24
	GPVI	12	5851 (4655-7304)	4	8095 (7645-8597)	0,14

Platelet activation markers are expressed as % positive platelets and platelet receptors as Mean Fluorescence Intensity (MFI). Results are expressed as median with IQR. The p-value comparing

patients and healthy controls is also shown. Bold values are statistically significant. A Student's t-test was performed for normally distributed parameters, while the Mann-Whitney test was used for non-normally distributed parameters. Multiple comparisons were corrected using the Benjamini-Hochberg method. Abbreviations: Me-S-ADP, 0.01 µM (ADP0.01); Me-S-ADP, 1 µM (ADP1); TRAP-6, 0.8 µM (TRAP0.8); TRAP-6, 3.3 µM (TRAP3.3); collagen related peptide, cross-linked, 150 ng/mL (CRP150).

5.6.2.2. Platelet-leukocyte formation is elevated in adult cirrhotic patients

In contrast to pediatric patients, circulating levels of platelet-monocyte and platelet-neutrophil complexes were higher in adult cirrhotic patients compared to controls (**Figure 24**). This finding aligns with the study by Stoy et al [64], which reported elevated levels of platelet-complexed leukocytes in cirrhotic patients. Notably, in that study, complex levels decreased with increasing MELD scores, a trend that was not observed in our cohort. The authors proposed that platelet-leukocyte complexes may serve as sensitive markers of platelet activation in patients with cirrhosis. These complexes are likely involved in inflammatory responses as well [221, 222]. Sturgeon et al demonstrated that neutrophils interacting with cirrhotic platelets underwent higher oxidative burst in comparison to interaction with healthy platelets [223].

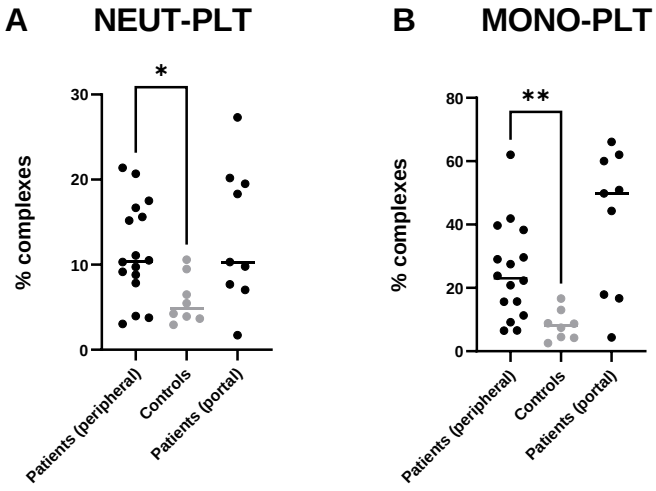


Fig.24. Leukocyte-platelet complexes in peripheral and portal patient blood compared to controls. (A) Neutrophil-platelet complexes. (B) Monocyte-platelet complexes. Levels of significance *p < 0.05 (Unpaired Student's t-test); **p < 0.01 (Unpaired Student's t-test)

5.6.2.3. Von Willebrand factor levels are increased in adult patients with cirrhosis

Significantly higher VWF antigen and activity levels were measured in patient plasma samples when compared to the control group (**Figure 25**, $p < 0.0001$). The levels of ADAMTS13 (processing VWF multimers) did not differ significantly between patients and controls. ADAMTS13 levels did not correlate with MELD-score ($r = 0.06$).

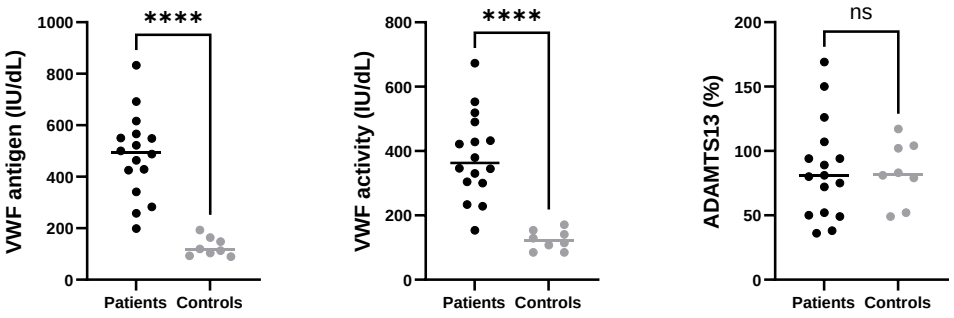


Fig.25. Levels of von Willebrand factor and ADAMTS13 in patients before liver transplantation and healthy controls. *Abbreviations:* ADAMTS13: a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13, VWF: von Willebrand factor. Levels of significance ****$p < 0.0001$ (Unpaired Student's t-test); ns, not significant (Unpaired Student's t-test).

5.6.2.4. Platelet activation does not differ between peripheral and portal blood

In 9 patients a portal blood sample was taken during liver transplantation and analyzed with platelet flow cytometry. Queck et al [224] demonstrated increased platelet activation in the portal circulation compared to the hepatic vein. Blood samples were collected during TIPS procedure, and platelet activation was assessed through levels of soluble GPVI and soluble P-selectin. Additionally, bacterial translocation and oxidative stress were measured using LPS and soluble NADPH oxidase 2-derived protein, respectively. They hypothesized that increased LPS-driven inflammation in the portal vascular bed could enhance platelet activation.

In our study, no differences were observed between peripheral and portal blood in α-granule release (CD62P expression), αIIbβ3 activation (PAC-1 mAb), or dense granule/lysosomal release (CD63/CD107a expression) following stimulation with various agonists at both low and high concentrations (**Figure 26**). Similarly, spontaneous formation

of platelet-neutrophil and platelet-monocyte complexes was identical between peripheral and portal blood (**Figure 24**). This aligns with findings from Nassar et al [109], who evaluated platelet aggregation, activation, and leukocyte-neutrophil complexes before and after TIPS procedure, noting no differences in platelet aggregation and activation between peripheral and portal blood. Likewise, in the study by Shalaby et al [110], no difference was found in platelet aggregation with whole blood aggregometry between peripheral and portal blood. Driever et al did not demonstrate elevated platelet activation, measuring levels of platelet factor 4, in portal blood in comparison to the systemic and hepatic circulation [108].

5.6.2.5. Conclusion on primary hemostasis in adult cirrhotic patients

In the static flow cytometry assay, which is independent of VWF, we mostly observed reduced platelet responses in response to high agonist concentrations. This was most pronounced for PS-exposing platelets. However, unlike pediatric cirrhotic patients, adult patients exhibited high levels of leukocyte-platelet complexes in unstimulated blood, indicating some degree of spontaneous platelet activation. Elevated levels of von Willebrand factor were observed, while ADAMTS13 levels remained normal in most patients. Interestingly, platelet activation was not found to be elevated in portal blood

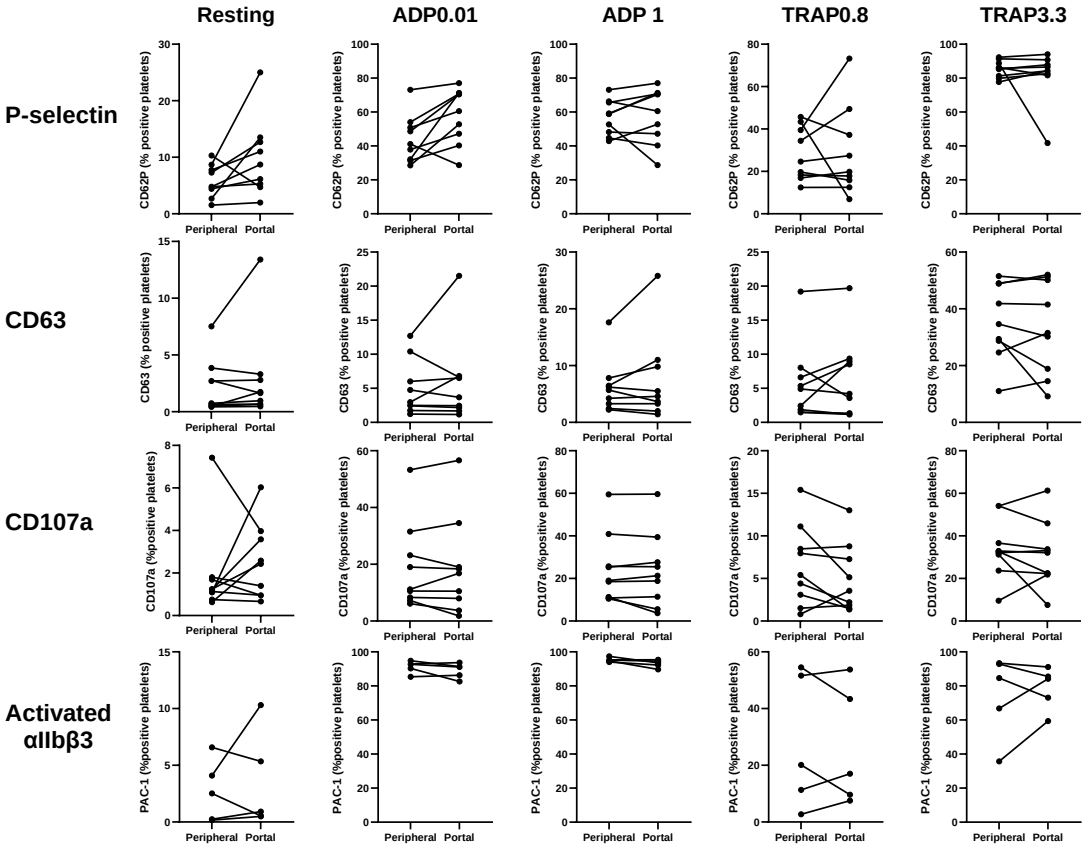


Fig. 26. Platelet flow cytometry: Comparison between peripheral and portal blood in adult patients. Abbreviations: Me-S-ADP, 0.01 μM (ADP0.01); Me-S-ADP, 1 μM (ADP1); TRAP-6, 0.8 μM (TRAP0.8); TRAP-6, 3.3 μM (TRAP3.3).

5.6.3. COAGULATION CASCADE

5.6.3.1. Routine coagulation assays are prolonged in cirrhotic patients

Prothrombin time, activated partial thromboplastin time and thrombin time were significantly prolonged in adult patients compared to controls. Fibrinogen levels were significantly lower in patients with CTP B and C than in controls, with greater interindividual variability observed among the patients. As observed in pediatric patients, perturbation of routine hemostasis assays was more pronounced in patients with higher liver disease severity (higher CTP score; **Figure 27**).

5.6.3.2. Patients with cirrhosis present a rebalanced coagulation cascade

Despite these prolonged hemostasis assays, patients presented a rebalanced coagulation cascade with a simultaneous decline in pro- and anticoagulant proteins. This can be appreciated when looking at procoagulant – and anticoagulant drivers of hemostasis (**Figure 28**). Except for factor VIII, coagulation factors were generally lower in patients compared to controls. Natural anticoagulants, protein C and antithrombin, were significantly reduced, while protein S was more variable between patients.

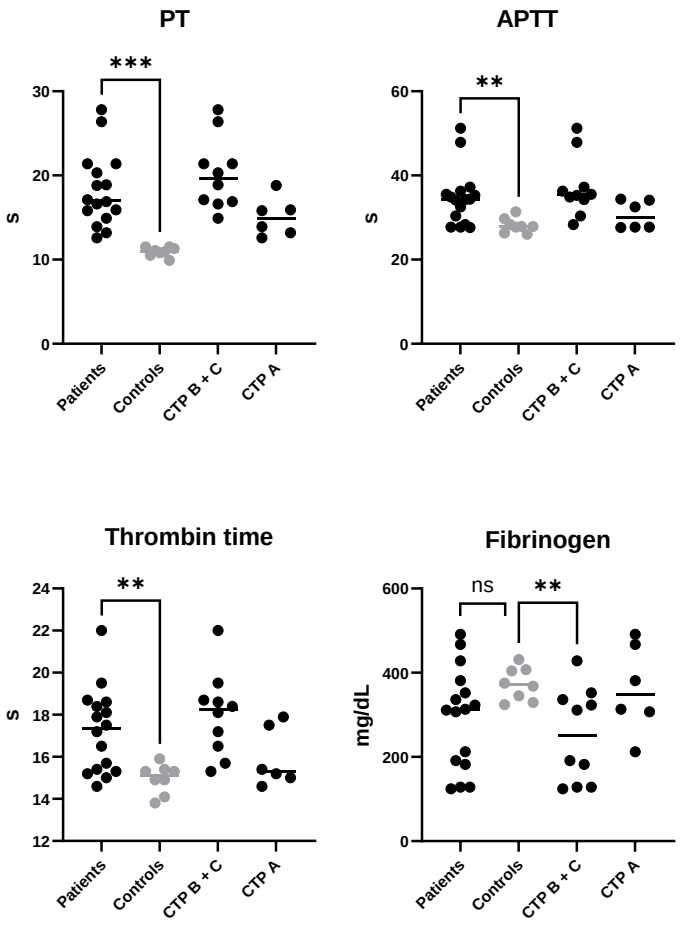


Fig. 27: Routine hemostasis assays in patients before liver transplantation, compared to controls. APTT: activated partial thromboplastin time; CTP: Child-Turcotte-Pugh; PT: prothrombin time. Note the difference in routine hemostasis assays between patients with CTP A and CTP B + C. Bullets in black: patients before transplantation; bullets in grey: age-matched control group. Levels of significance ***p < 0.001 (Unpaired Student's t-test/Mann-Whitney test); **p <0.01; ns, not significant (Unpaired Student's).

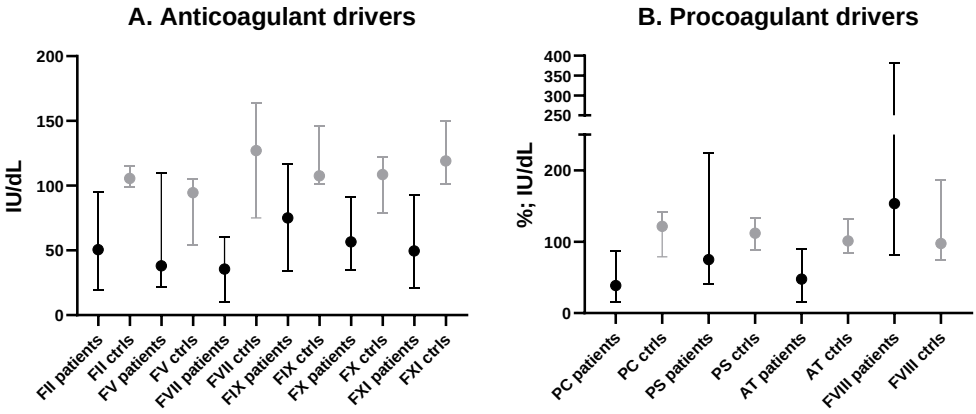


Fig.28. Anticoagulant drivers (A) and procoagulant drivers (B) in patients before liver transplantation, compared to controls. AT: antithrombin, ctrls: controls, F: factor, PS: protein S, PC: protein C. Bullets in black: patients before transplantation; bullets in grey: age-matched control group.

Without thrombomodulin, thrombin generation in patients was not significantly different from controls, except for patients with more severe liver disease (CTP B+C; **Figure 29, A**). In the presence of thrombomodulin, endogenous thrombin potential was significantly higher in patients compared to controls ($p < 0.5$; **Figure 29, B**). This was already demonstrated in previous studies [225, 226]. Both low protein C and increased FVIII were shown to play an important role in this hypercoagulability [227]. Virtually all patients showed TM resistance (low % TM inhibition; **Figure 29, D**). Addition of activated protein C reduced thrombin generation considerably in both healthy controls and patients (**Figure 29, C**) but patients demonstrated higher thrombin generation in the presence of activated protein C in comparison to controls. In contrast to the abstract published by Sinegre et al [228], where no resistance to activated protein C was seen in cirrhotic patients, we demonstrated that, in particular in patients with CTP B and C, % inhibition by activated protein C was lower, indicating that thrombin generation is less effectively inhibited by activated protein C (**Figure 29, E**). Thrombin generation in the presence of activated protein C can detect acquired protein C resistance and likely informs on the levels of antithrombin protein S and FVIII. In our patients, inhibition by activated protein C correlated well with antithrombin levels ($r = 0.67$, $p < 0.01$) and moderately with protein S levels ($r = 0.45$, $p = 0.08$). The inverse correlation with FVIII was poor ($r = -0.12$, $p = 0.65$; **Figure 30**).

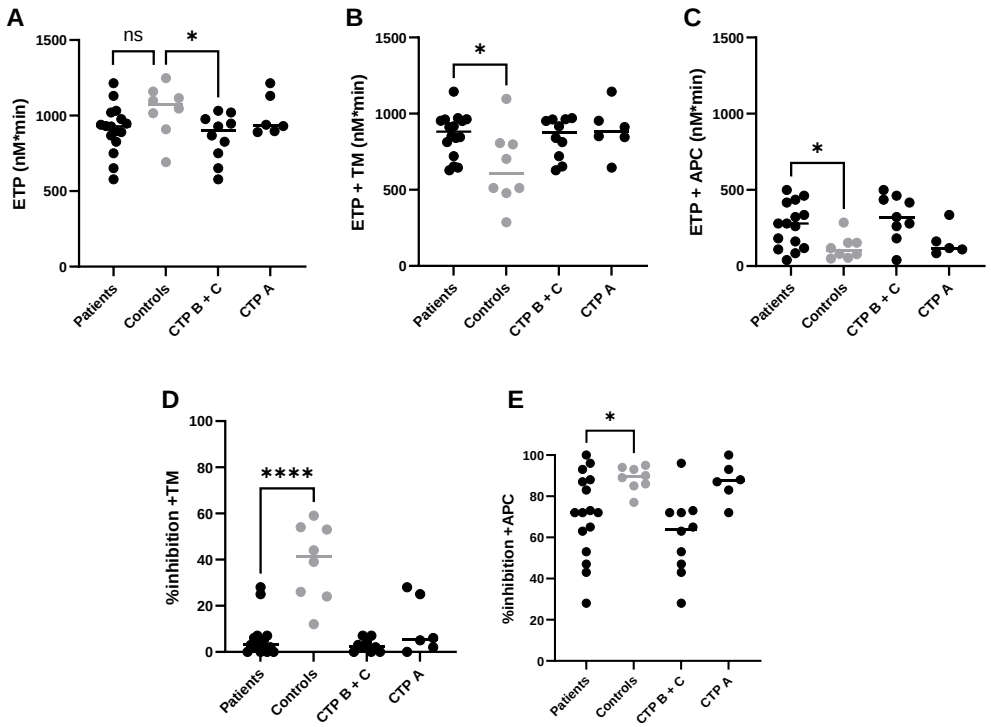


Fig.29. Thrombin generation (endogenous thrombin potential) in patients compared to controls. **A**. Thrombin generation without TM; **B**. Thrombin generation with TM; **C**. Thrombin generation with APC; **D**. % inhibition TM; **E.** % inhibition APC. Note: high resistance to TM in +- all patients independent of liver disease severity and higher resistance to APC in patients with CTP B + C. Abbreviations: APC: activated protein C, ETP: endogenous thrombin potential; CTP: Child-Turcotte-Pugh; TM: thrombomodulin. Bullets in black: patients before transplantation; bullets in grey: age-matched control group. Levels of significance ****p < 0.001 (Mann-Whitney test); *p <0.05; ns, not significant (Unpaired Student's).

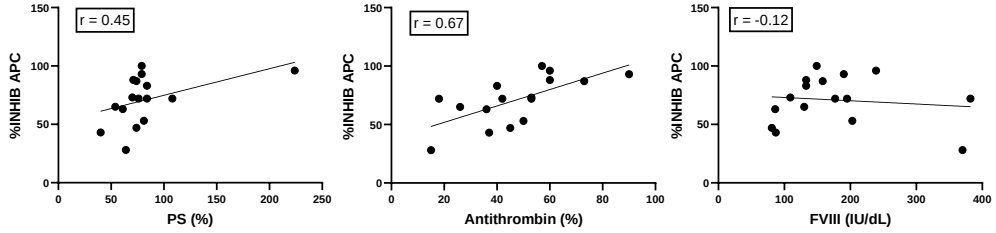


Fig.30. Correlation between % inhibition by activated protein C and coagulation parameters in adult patients with cirrhosis. Abbreviations: APC, activated protein C; %INHIB, % inhibition; PS, protein S.

5.6.3.3. Comparison between pediatric and adult patients

In comparison to pediatric cirrhotic patients, adult patients with cirrhosis had a more hypercoagulable profile, although an overlap between the two groups was shown (**Figure 31**). Pediatric patients are transplanted with a portion of an adult liver. This could result in higher levels of hemostatic proteins, similar to those in adults, after liver transplantation. Three months after transplantation, pediatric patients demonstrated lower levels of coagulation proteins except for factor VIII (**Figure 32**), suggesting they re-establish childhood coagulation factor levels.

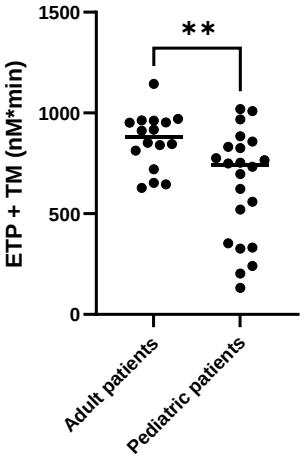


Fig.31. Comparison of thrombomodulin-modified thrombin generation between adult and pediatric cirrhotic patients. Abbreviations: ETP, endogenous thrombin potential; TM, thrombomodulin. Levels of significance **p < 0.01 (Unpaired Student's t-test)

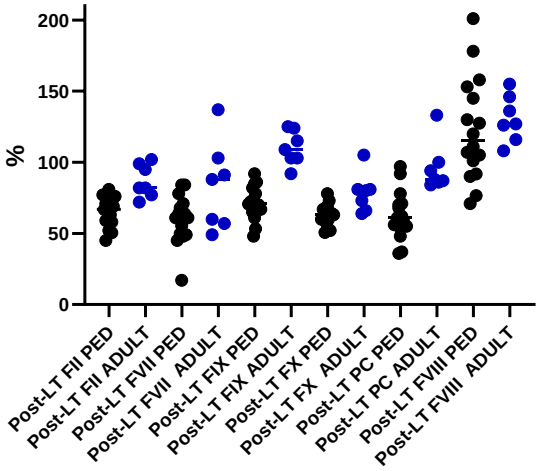


Fig.32. Coagulation factors 3 months after liver transplantation in pediatric compared to adult patients. Black bullets: pediatric patients. Blue bullets: adult patients. Abbreviations: LT, liver transplantation, PC: protein C.

5.6.3.4. Conclusion on the coagulation cascade in adult cirrhotic patients

Despite prolonged routine hemostasis assays, adult cirrhotic patients demonstrated a hypercoagulable coagulation profile with both resistance to thrombomodulin and activated protein C. Resistance to thrombomodulin and activated protein C was more pronounced in patients with advanced liver cirrhosis.

5.6.4. FIBRINOLYSIS

5.6.4.1. Both hypo-and hyperfibrinolysis are seen in adult cirrhotic patients

The CLT and Lysis Timer results for the adult study population are presented in **Figure 33**. One patient was excluded due to tranexamic acid infusion before blood sampling. In patients, the CLT (median + IQR) was 56.9 minutes (46.0–83.1 minutes), compared to 62.3 minutes (55.8–157.3 minutes) in controls (p = 0.21). The Lysis Timer (median + IQR) measured 35.6 minutes (29.9–54.8 minutes) in patients and 43.4 minutes (37.2–68.2 minutes) in controls (p = 0.14). In contrast to pediatric healthy controls, hypofibrinolysis was seen in 2 adult controls, both of whom had elevated PAI-1 antigen levels. Two cirrhotic patients presented with clear hypofibrinolysis with both techniques, though these patients did not show elevated PAI-1 levels. Low plasminogen could be responsible for the longer CLT seen in these patients.

Three patients exhibited hyperfibrinolysis according to the Lysis Timer (<30 min), with CLT values also lower than those of controls (reference values for CLT are not available). Changes in CLT and Lysis Timer did not correlate with severity of liver disease (MELD; r = -0.05 and r = 0.28 respectively). In a cohort of patients with acute decompensation and acute-on-chronic liver failure, patients also presented both hypo-and hyperfibrinolysis [211].

As expected, D-dimer levels were elevated in both pediatric and adults patients and were associated with liver disease severity (**Figure 34**). Interpretation of D-dimer levels both in the context of fibrinolysis and thrombosis is challenging, as explained in the introduction. Interestingly, in a cohort of patients with advanced chronic liver disease, severity of ascites was independently associated to plasma D-dimer levels, suggesting a role of ascites in systemic hyperfibrinolysis [229].

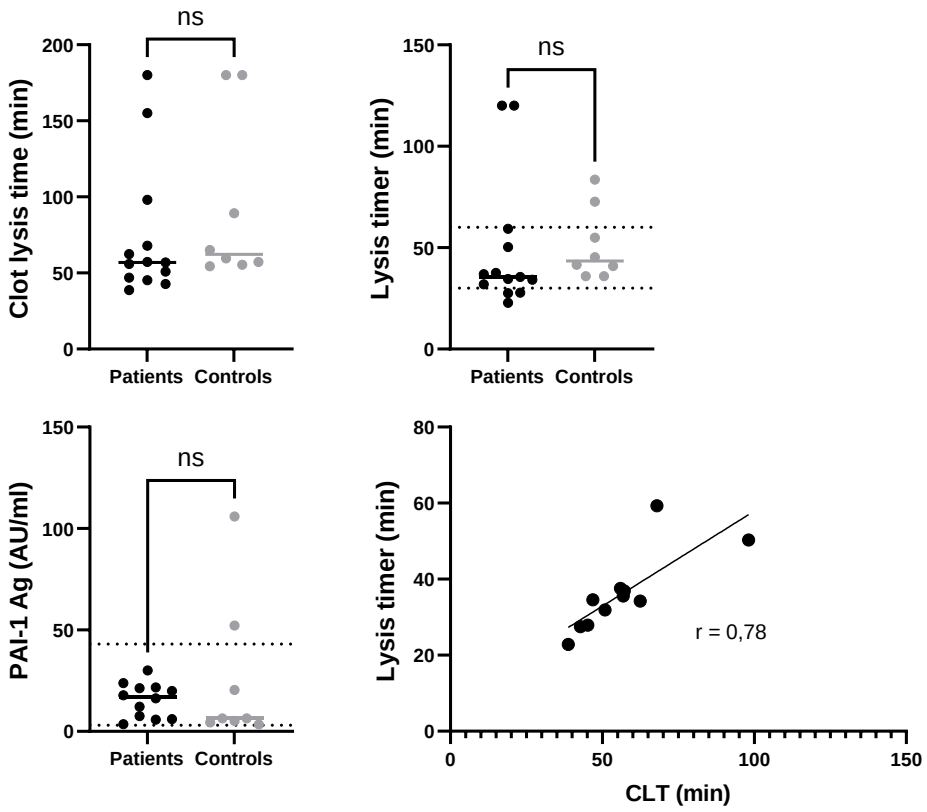


Fig. 33. Clot lysis time and Lysis timer results in adult cirrhotic patients before liver transplantation compared to controls. Results for PAI-1 are also shown, as well as the correlation between Lysis Timer and CLT (after exclusion of severe hypofibrinolytic results). Dashed lines are the reference values according to the manufacturer. Abbreviations: PAI-1 Ag, plasminogen activator inhibitor-1 antigen; CLT, clot lysis time. Levels of significance: ns, not significant (Unpaired Student's t-test/Mann-Whitney test).

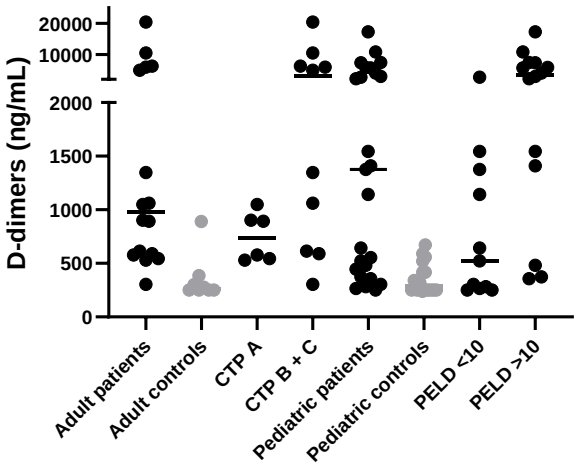


Fig.34. D-dimer levels in adult and pediatric patients compared to controls and based on liver disease severity. Abbreviations: CTP, Child-Turcotte-Pugh; PELD, pediatric end-stage liver disease.

5.6.4.2. FXIII levels are significantly lower in adult patients, but not in pediatric patients

Factor XIII activity was significantly different in adult patients with cirrhosis compared to controls. Nine out of 16 patients had a factor XIII activity <50%. This is high compared to a cohort of 93 adult patients with cirrhosis where only 6.5% had levels <50% [230]. However, patients in our cohort had more severe liver disease and different techniques for FXIII activity measurements were used. Intagliata et al reported that patients with decompensated cirrhosis and acute kidney injury had lower FXIII levels compared to patients without acute kidney injury [231].

Although not significant, an inverse correlation was seen with clot lysis time ($r = -0.49$, $p = 0.05$), which is surprising since FXIII increases resistance to fibrinolysis by strengthening the clot [232]. No correlation was seen with severity of liver disease (MELD score; CTP score).

In the pediatric cohort, FXIII levels were not significantly lower in patients compared to age-matched controls, even among those with a PELD score greater than 10. However, very low FXIII values (<30%) were observed only in patients with PELD scores of 20 or higher. Variability between patients was higher than in controls with observation of both high and low FXIII levels. FXIII levels correlated well with fibrinogen levels ($r = 0.62$; $p<0.01$). Three

months after liver transplantation FXIII levels were not significantly different, although levels <30% had improved to levels >30%. Results are shown in **Figure 35.**

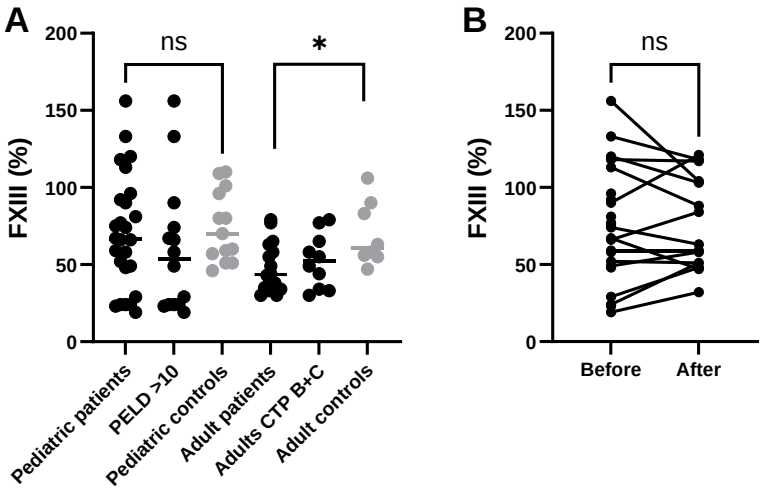


Fig.35. FXIII activity in adult patients and pediatric patients with cirrhosis. (A) Comparison to age-matched controls with whole cohort and with patients with more severe cirrhosis: PELD >10 for pediatric patients and CTP B+C for adult patients. (B) Pediatric patients before and 3 months after liver transplantation.

5.6.4.3. Conclusion on fibrinolysis in adult cirrhotic patients

Consistent with the pediatric cohort, both hypo-normal fibrinolysis and hyperfibrinolysis were observed in adult cirrhotic patients using both plasma-based global fibrinolytic techniques. Factor XIII levels were lower in adult patients compared to both normal controls and pediatric patients The latter exhibited high interpatient variability in FXIII levels.

6. GENERAL DISCUSSION, PERSPECTIVES AND CONCLUSION

This doctoral thesis outlines the hemostatic system in pediatric patients with cirrhosis, primarily due to cholestatic liver disease, both before and 3 months after liver transplantation. Additionally, a small cohort of adult cirrhotic patients with varying etiologies, predominantly alcoholic liver disease, was included for comparison.

We have gained insight into the hemostatic system, and particularly primary hemostasis, in children with cirrhosis prior to liver transplantation. Based on our observations we identified areas for further clinically focused studies. The following paragraphs present the main observations of this thesis, followed by a discussion of potential future perspectives.

Considering primary hemostasis, the coagulation cascade, and fibrinolysis together, we conclude that the hemostatic system is generally well-preserved in most pediatric patients with cirrhosis before liver transplantation. These observations indicate that pro-hemostatic interventions are likely not indicated in the majority of these patients before liver transplantation.

Next to pediatric patients with preserved hemostasis, some patients, often with high severity of liver disease, showed laboratory evidence of either a prothrombotic or pro-hemorrhagic state. For primary hemostasis, we observed 2 subgroups of patients. The first subgroup of patients had higher platelet aggregation and increased phosphatidylserine exposure on von Willebrand factor–dependent surfaces, while the second subgroup demonstrated moderate impairment in platelet thrombus formation.

Several mechanisms underlying alterations in platelet function can be proposed, though they remain speculative. Low-grade platelet activation may lead to platelet exhaustion, potentially resulting in an acquired storage pool defect characterized by impaired release of stimulators of platelet activation from alpha and dense granules. Defective transmembrane signaling might arise from protease-mediated damage to platelet receptors or alterations occurring at the megakaryocyte level. Additionally, extrinsic plasma factors might contribute, such as bile salts exerting an inhibitory effect on platelets, while unconjugated bilirubin demonstrates a pro-aggregatory effect. The complex role of von Willebrand factor has already been extensively discussed.

Thrombomodulin-modified thrombin generation in pediatric patients showed considerable variability, with both hypo- and hypercoagulable characteristics present. Plasma-based global fibrinolytic assays demonstrated normal fibrinolysis for most patients, though some with severe liver disease displayed either hypo- or hyperfibrinolysis. During liver

transplantation, tranexamic acid has been shown to be safe in adult patients and can be used either systematically or in patients with bleeding due to hyperfibrinolysis.

Three months post-liver transplantation platelet alterations were improved but not yet fully reversed in pediatric patients. Platelet count increased but static agonist-induced platelet activation did not improve and was even lower for some parameters. Overall, platelet thrombus formation did improve but variability between patients was maintained. Several factors could explain the persistence of changes in platelet number and function after transplantation. While spleen size typically decreases following liver transplantation, splenomegaly may still persist up to three months post-transplant. Additionally, some level of postoperative inflammation and endothelial dysfunction may remain. Immunosuppressive medications can also affect platelet function.

Persistent thrombomodulin resistance was observed 3 months after liver transplantation and could be a possible contributor to thrombotic complications (hepatic-and portal vein thrombosis), more often seen after pediatric than adult liver transplantation [233]. Pediatric patients are transplanted with a portion of an adult liver. This could result in higher levels of hemostatic proteins, similar to those in adults, potentially increasing the risk of thrombosis. However, 3 months after liver transplantation, pediatric patients had lower levels of hemostatic proteins than adult patients. This pattern suggests they re-establish childhood coagulation factor levels.

Based on a small sample of adult cirrhotic patients, some similarities between children and adults were observed. In adults, platelet activatability in response to high agonist concentrations—particularly for procoagulant platelets—was also reduced. Thrombin generation ranged from normal to hypercoagulable, but unlike pediatric patients, no clear hypocoagulable profiles were observed. Fibrinolytic profiles in adults, similar to those in pediatric patients, varied from hypo-normal to hyperfibrinolysis but the association with liver disease severity was less evident in adults. In adult cirrhotic patients we demonstrated similar platelet activation in the portal circulation in comparison to the systemic circulation, using our validated flow cytometry activation panel. This observation supports recent studies concluding that the portal vein circulation is not inherently more prothrombotic than the systemic circulation.

We also touched on the coagulation-inflammation axis with neutrophil extracellular traps in pediatric and adult patients. In pediatric patients, literature is virtually inexistant on this topic. A moderate correlation was observed with von Willebrand factor activity in both groups. This possibly links VWF to inflammation-NETosis and NETs to coagulation activation. Recent literature has described platelets with a more pro-inflammatory and

procoagulant phenotype (164). In our study, we observed an increase in procoagulant platelets under flow in patients, suggesting that inflammation may play a role as an underlying mechanism next to von Willebrand factor.

Although this proof-of-principle study was not designed to assess clinical endpoints, we can formulate 4 perspectives for future clinical studies or transfusion management guidance, based on the previous observations.

First, the results obtained for primary hemostasis can impact transfusion protocols by supporting a restrictive approach to platelet concentrate transfusion in pediatric patients with cirrhosis, similarly to what is advocated in adults. Whether the subgroup of patients with apparently impaired platelet function may benefit from interventions supporting platelet function requires additional clinical study. Furthermore, we can question the utility of static platelet function assays in guiding platelet transfusion, since key players, such as von Willebrand factor, are not taken into account.

Second, in some patients with advanced cirrhosis we demonstrated hypercoagulable profiles with thrombomodulin-modified thrombin generation. In these patients prophylactic transfusion of fresh frozen plasma is not indicated as is in line with adult cirrhosis guidelines [234]. Patients with advanced cirrhosis are at elevated risk for the adverse effects of plasma infusion since it exacerbates portal hypertension and thereby increases bleeding risk.

Third, additional studies are needed to reassess whether our local guidelines for antithrombotic management can be improved. Specifically, we can evaluate if an (additional) anti-Xa based anticoagulation method would be more appropriate, since thrombomodulin resistance persisted even 3 months after liver transplantation. Antithrombotic management following liver transplantation varies between centers, with 20 different approaches identified [235]. In our center, acetylsalicylic acid is currently used during 6 weeks to prevent thrombotic complications.

Fourth, a very practical perspective that came from this thesis is the plan to set up a multidisciplinary working group for blood product management peri-liver transplantation in pediatric patients, locally at Cliniques universitaires Saint-Luc.

From a more scientific perspective, we can formulate 3 areas of interest. Additional insight in primary hemostasis in adult patients can be achieved by performing a microfluidic assay, similar as in the pediatric cohort (1). In this way, we can observe if similar subsets of patients with enhanced platelet aggregation and phosphatidylserine exposure are identified. Further analysis on the frozen portal vein plasma by performing thrombin

generation and inflammation panels could provide further insights of the hemostatic and inflammatory state in the portal vein circulation (2). Additionally, a deeper investigation into the role of inflammation in the systemic circulation could be conducted through cytokine panel analyses in both pediatric and adult patients and by examining platelet receptors involved in inflammatory processes, such as CLEC-2 and CD40L. Microfluidic flow assays with coated inflammatory proteins (e.g., S100 A8/A9) could provide further insights into the mechanisms underlying procoagulant platelet formation and the contribution of inflammation (3).

In conclusion, our results enhance the understanding of the hemostatic system in pediatric cirrhotic patients. A relatively rebalanced hemostatic system before liver transplantation, with primary hemostasis varying according to platelet count, von Willebrand factor levels, and disease severity, is our key finding. Our results provide the basis for further clinically focused studies and scientific work.

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