

ORIGINAL ARTICLE

ADP-dependent platelet function prior to and in the early course of pediatric Liver transplantation and persisting thrombocytopenia are positively correlated with ischemia/reperfusion injury

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Keywords

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Summary

Little is known about the role of platelets in relation to ischemia/reperfusion injury (IRI) of the liver graft especially in children. Thrombocyte function was prospectively analysed in 21 consecutive pediatric liver transplantation (pLT) patients by platelet aggregometry secondary to adenosine diphosphate (ADP), collagen, and the von Willebrand factor activator ristocetin (VWF:rco). Post-OP serum levels of ALT were used to divide patients into groups with high (highHD, $n = 8$) and low (lowHD, $n = 13$) hepatocellular damage. Clinically, highHD-patients showed impaired plasmatic coagulation and elevated serum bilirubin levels early after pLT when compared with lowHD-patients. Further, platelet counts markedly decreased between pre-OP and postreperfusion (postrep.) in the highHD group ($P = 0.003$) and did not recuperate by POD6. In lowHD individuals thrombocytopenia improved from both pre-OP ($P < 0.05$) and postrep. ($P < 0.001$) respectively towards POD6. Experimental thrombocyte testing revealed that before graft reperfusion only ADP-dependent platelet aggregation correlated with reperfusion injury, thrombocytopenia and early graft function. During the first 48 h after graft reperfusion, all inducers tested demonstrated elevated platelet aggregation levels in the highHD group. Our data suggest a possible role of platelets and their aggregative status in liver IRI subsequent to clinical pLT. Reperfusion-independent ADP-triggered platelet function may be a determinant for IRI in the pediatric hepatic graft recipient.

Introduction

Primary hemostasis in patients with severe liver disease is frequently compromised resulting from a reduced number of thrombocytes that also show altered functional properties. These changes in platelet counts and function may contribute to increased blood loss during and after liver transplant surgery.

In addition to their role in blood coagulation, platelets also have nonhemostatic properties that are important for a number of processes in the context of liver transplantation (LT), such as inflammation, tissue repair, regeneration, and ischemia/reperfusion injury. Clinically, it has been shown that persisting or development of thrombocytopenia subsequent to reperfusion is an unfavorable prognostic marker for early liver graft function and outcome

in adult LT [1–3]. Experimental prevention of platelet adhesion and accumulation in the graft after reperfusion for example attenuates ischemia/reperfusion injury (IRI) [4,5]. IRI occurs when blood flow to an organ or tissue is interrupted for some period of time and subsequently re-established. Modulation of thrombocyte function by prior activation in an *ex vivo* rat-model amplified the extent of liver injury [6] and diminished the effect of IRI modulating therapy in the reperfused liver [7]. The extent of hepatocellular damage (HD) after reperfusion of the graft is correlated with primary nonfunction or poor early graft function negatively influencing the overall outcome of LT [8–10].

Our group has reported previously on preoperative von Willebrand factor (vWF)-dependent platelet aggregation and functional vWF plasma levels in adult LT and correlated these directly with alanine transaminase (ALT) levels early after LT [11]. In the same study, vWF-dependent aggregation levels were elevated by day 6 following LT. Taken together these data suggest a correlation of the functional status of recipient platelets and their impact on IRI. However, the sequence of events that leads to tissue injury in this situation is incompletely understood and even less is known about platelets and their function in the course of pediatric LT (pLT) and subsequent IRI.

The aim of this study was to determine direct platelet aggregation in the course of pLT. We evaluated whether a group of individuals with elevated markers of hepatic injury following reperfusion was paralleled by alterations in aggregative responses to various stimuli if contrasted to an uneventful control group. One focus was whether differences among the two groups investigated were detectable prior to reperfusion as a factor determined in recipients independent of grafting.

Material and methods

Patients

Twenty-one consecutive pediatric patients (<16 years of age) undergoing LT under our program were studied for hepatocellular damage, platelet counts and aggregation. The 21 pLT-patients, included in this study were divided into two groups according to corrected serum ALT levels. Patients with a corrected peak serum-ALT of <200 IU were assigned to the lowHD group ($n = 13$), and patients with serum ALT levels higher than 200 IU ($n = 8$) were assigned to the highHD group. The distribution of indications for pLT is summarized in Table 1.

Material

Adenosine diphosphate (ADP), Collagen and the von Willebrand factor inducer ristocetin as well as the whole-

Table 1. Donor characteristics.

	HighHD	LowHD	P-value
Age (years)	37.0	17.9	0.015
Sodium (mmol/l)	145	149	0.369
Bilirubin (mg/dl)	0.87	0.44	0.123
AST (IU)	12.5	17.0	0.650
ALT (IU)	10.0	10.0	0.618
γ GT (IU)	9.0	10.0	0.18
Resuscitation	2/8 (25%)	4/13 (31%)	0.831
Vasopressors	6/8 (75%)	8/13 (62%)	0.874

blood aggregometer were purchased from Chrono Log[®] NOBIS Labordiagnostik GmbH, Endingen, Germany.

Clinical chemistry

Serum ALT, aspartate transaminase (AST), gamma glut- amyl transpeptidase (γ GT), total serum bilirubin (TSB), sodium as well as Quick (prothrombin time-dependent), partial thrombin time (PTT), hematocrit and platelet count were routinely analysed in clinical chemistry laboratories.

Aggregometry

Standard techniques for measuring platelet aggregation like the Bohr method either depend on platelets already having released their products or having aggregated because of centrifugation, or other preparation steps to obtain platelet-rich plasma, or they involve extensive preparation of plasma before assessment of aggregability. Because of these limitations, in this study *ex vivo* whole blood impedance platelet aggregometry to assess platelet function has been used. This method avoids the complex processing of blood samples and thus is more likely to represent the *in vivo* status of platelets at the time the blood sample was obtained. The blood-volume required for one assay is about 10% of that required for testing in platelet-rich plasma, an advantage crucial for evaluation of pediatric patients' platelet reactivity. Impedance whole blood aggregometry as utilized in this study is reported to be equivalent as platelet function test when compared with standard aggregometry with platelet-rich plasma [12].

In brief, citrated blood was collected from patients 1 h prior to initiation of anesthesia (pre-OP), during the second hour of surgery (intra-OP), 20–5 min prior to clamping of the portal vein (prerep), 20–40 min after reperfusion (postrep), and on postoperative days (POD) 1, 2, 4, and 6 (POD 1 –POD 6). One aliquot each was centrifuged for 20 min (800 g) and supernatant platelet-poor plasma was stored at –80 C for vWF analysis.

The remaining blood was analysed for platelet function in a whole blood impedance aggregometer (Chrono Log[®] NOBIS Labordiagnostikca GmbH, Germany) according to the manufacturers' instruction. In order to further limit the blood-sample volume dilution of the sample was modified (1:3 instead of 1:2 dilution with 0.9% NaCl solution) as described elsewhere [13]. Using this method, platelet adhesion to two alloy wires following various aggregating stimuli such as ADP (5.0 and 10.0 μM), ristocetin (0.63 $\mu\text{g/ml}$) and collagen (5.0 $\mu\text{g/ml}$) were quantified. Increasing layers of platelets subsequent to platelet aggregation lead to an increase of resistance for an electric flow [stated in ohm (Ω)] between the two wires which is proportional to the extent of platelet aggregation. Aggregation analysis was performed within 3 h after sampling.

Statistical analysis

Student's *t*-test was performed for values with normal distribution. Groups of values not passing normality testing were subjected to Mann–Whitney Rank Sum test for statistical analysis. Proportions were compared by *z*-test. Correlations were evaluated utilizing linear regression testing. SIGMA STAT, Version 2.03, SPSS MR, München, Germany served as statistical analysis software. Data are stated as \pm standard deviation of the mean unless otherwise stated.

Results

Study groups

In this study, hepatocellular damage (HD) was assessed by serum ALT levels as transaminases are well described as reference markers for HD subsequent to reperfusion of liver grafts [14–17]. Because of extensive variation of the graft-/body-weight (G/BWr) ratios in pediatric liver transplant recipients (range in this study 0.93–8.41, median 3.41), ALT levels were corrected for G/BWr as suggested before [18]. In our total study collective, we demonstrated a peak of ALT and AST on POD 1, drastically descending towards POD 6 (data not shown). Mean transaminase levels in the highHD collective were 386.6 ± 368.9 IU/l for ALT and 409.9 ± 384 IU/l for AST on POD 1, in contrast to the lowHD group with 67.4 ± 59.9 IU/l for ALT and 67.7 ± 64.1 IU/l for AST on POD 1 (Fig. 1 and data not shown).

Donor demographics

Donor demographics are shown in Table 1. Donor age in highHD patients was higher (mean 37 years; range 9–58 years) than in lowHD individuals (18 years, 2–45 years; $P < 0.05$). Other donor characteristics including liver

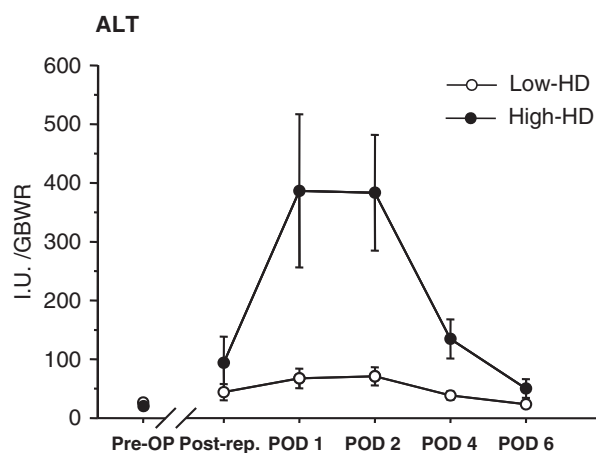


Figure 1 Markers of hepatocellular damage (HD). Course of mean BW/GW ratio-corrected ALT serum levels (IU) in the early course of pLT. Mean \pm SD. Closed circles – highHD group; open circles – lowHD patients. rep – reperfusion; POD – postoperative day.

enzymes, TSB, serum sodium, rate of resuscitation and necessity of vasopressors were statistically not different among high- and lowHD individuals. Two of the grafts in the lowHD group were obtained from living donors, whereas all grafts in the highHD group were derived from brain-dead donors.

Recipient characteristics

The distribution of indications for pLT is summarized in Table 2. Biliary atresia was the main indication for pLT in both study groups (75.0% highHD group and 61.5% lowHD group, $P = 0.874$). Recipient characteristics showed no statistical difference among the two groups concerning UNOS-status, age, body weight or gender (Table 3). Preoperative hematocrit, platelet counts and humoral coagulation testing such as Quick (representing integrity of factors II, V, VII and X as percentage of a normal reference) and PTT were comparable. There was no difference in mean preoperative hemoglobin levels or

Table 2 Indication for pLT

	HighHD	LowHD
Biliary atresia	6	8
Viral hepatitis	1	0
Polycystic liver disease	1	0
cryptogenic	0	1
Mucoviscidosis	0	1
Hemochromatosis	0	1
Byler's disease	0	1
Re-LTX	0	1
Total	8	13

	HighHD	LowHD	P-value
UNOS 1 + 2a	0/8 (0%)	3/13 (23.1%)	0.401
Age (years)	4.37	2.9	0.359
Body weight (BW) (kg)	14.3	11.8	0.478
Gender (M/F)	6/7	4/4	0.781
Hemoglobin level pre-OP (g/dl)	9,94	10.49	0.439
Platelet count pre-OP (counts/ μ l)	202.000	159.000	0.344
Quick (%) pre-OP	82.6	62.3	0.163
Packed red cells peri-OP (ml/kg BW)	162.5	148.0	0.779
Platelet administration peri-OP (%)	46.2	37.5	0.948
Platelet administration post-OP (%)	15.4	25.0	0.978
Partial thrombin time (s) pre-OP	50.0	48.8	0.865
Segmental-LT (%)	8/8 (100.0 %)	10/13 (76.9 %)	0.408
Cold ischemic time (min)	457	420	0.676
Warm ischemic time (min)	45.5	42.3	0.586
Graft/body weight ratio	3.34	3.45	0.903

Table 3. Recipient and LTX characteristics.

perioperative supply of packed erythrocytes in highHD when compared with lowHD individuals. The rate of segmental-pLT (split-, reduced- or living donated grafts) was statistically not different among high- and lowHD as were the cold and warm ischemic times (Table 3).

Liver function

Quick and TSB were compared among the pediatric patients in high- and lowHD groups to evaluate whether post-OP reperfusion injury was paralleled by impaired liver function. Patients with lowHD as compared with highHD demonstrated both a lower TSB after reperfusion during the whole observation period as well as an improvement of TSB from pre-OP values towards POD 6 values (paired *t*-test $P = 0.018$) (Fig. 2a). This improvement of TSB after transplantation was not observed for the collective with pronounced post-OP HD. Quick values were significantly better on POD 1, 2 and 6 in the lowHD group (Fig. 2b).

Platelet aggregation

Platelet function was tested by evaluating platelet aggregation in whole blood upon addition of various and differently acting agonists including ADP, collagen and ristocetin. The latter facilitates vWF-dependent platelet aggregation in a nonshear stress environment. Overall platelet function demonstrated a decrease in aggregation following initiation of surgery for all agonists reaching a nadir at the anhepatic phase (ristocetin) and postreperfusion (postrep.) (collagen and ADP) that was followed by a constant rise in aggregative responses towards POD 6 (data not shown).

In all children included in this study. ADP-induced aggregative responses correlated with ALT serum levels on POD 1 and 2 with a significant linear regression prior to

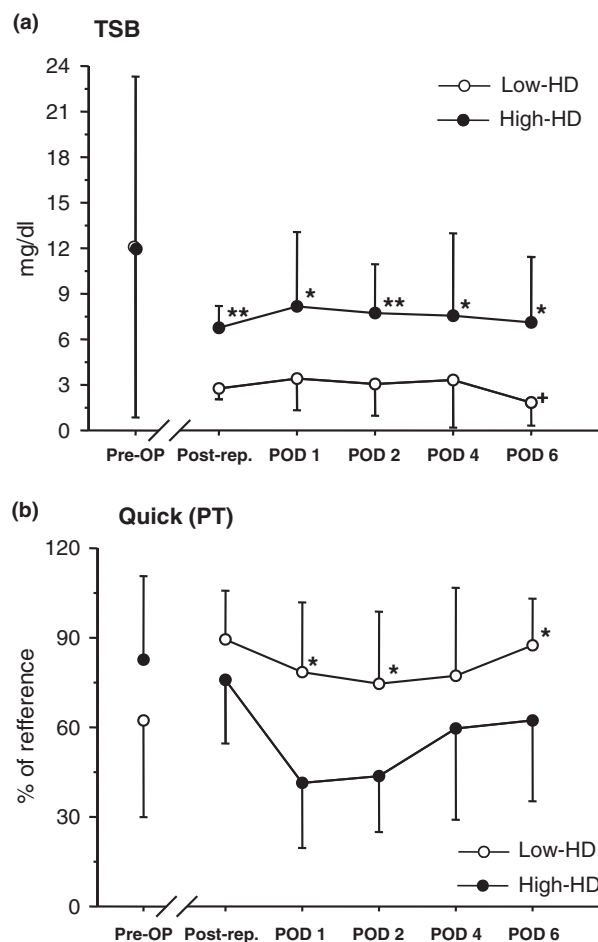


Figure 2 Markers of liver function. Course of mean total serum bilirubin (TSB; a) and prothrombin time (PT as % of normal reference; b). Mean \pm SD HD – hepatocellular damage; rep – reperfusion; POD – postoperative day; closed circles – highHD group; open circles – lowHD patients. * $P < 0.05$; ** $P < 0.001$ low- vs. highHD group, Student's *t*-test. * $P < 0.01$ lowHD group, preop vs. POD6, paired *t*-test.

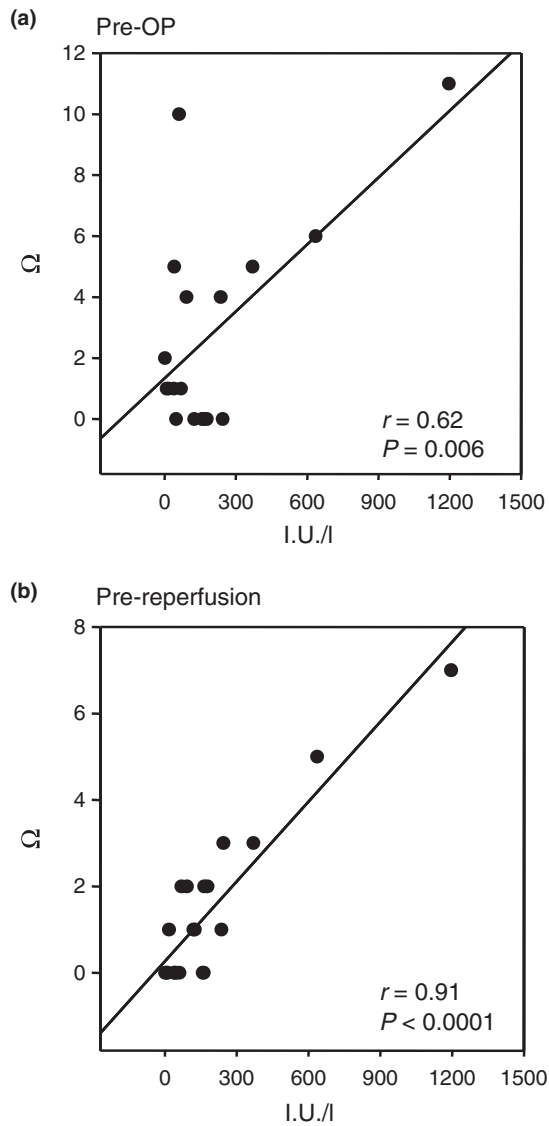


Figure 3 Correlation of ADP depending platelet function with markers of hepatocellular damage. Max. platelet aggregation levels (resistance in Ω) subsequent to ADP co-incubation (final concentration: $5 \mu\text{M}$) of all patients included in this study 1 h prior to initiation of anesthesia (pre-OP) and during 20–5 min prior to clamping of the portal vein (prerep.) are plotted against ALT-levels on POD1 (POD – postoperative day).

surgery (Fig. 3a), peri-operatively ($5 \mu\text{M}$ ADP vs. ALT POD1: $r = 0.59$, $P = 0.007$; $5 \mu\text{M}$ ADP vs. ALT POD 2 $r = 0.55$, $P = 0.014$; data not shown), and 5-min prereperfusion (Fig. 3b). As to be expected, ADP-induced mean aggregative responses were significantly pronounced in highHD group when compared with the lowHD group prior to reperfusion, ($5 \mu\text{M}$ ADP: 2.63Ω vs. 0.77Ω , $P < 0.05$; $10 \mu\text{M}$ ADP: 4.63Ω vs. 1.69Ω , $P < 0.01$) (Fig. 4a).

In contrast to prereperfusion aggregative levels, significant differences in platelet function were observed early

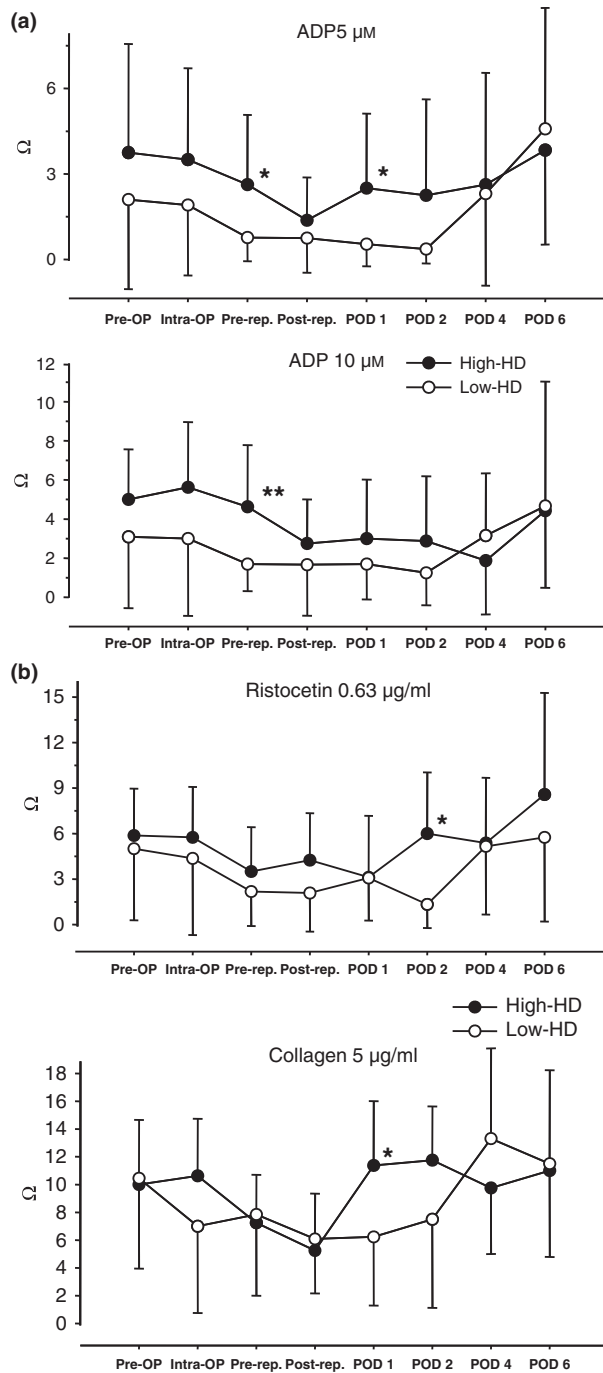


Figure 4 Platelet aggregation levels in the course of pLT. The mean of max. aggregation (resistance in Ω) evaluated with whole blood aggregometry for various time points is plotted. Inducers of aggregation (final concentrations in brackets): ADP, the vWF-activator ristocetin and collagen. Mean \pm SD HD – hepatocellular damage; rep – reperfusion; POD – postoperative day; closed circles – highHD group; open circles – lowHD patients. * $P < 0.05$ /** $P < 0.01$ low- vs. highHD group.

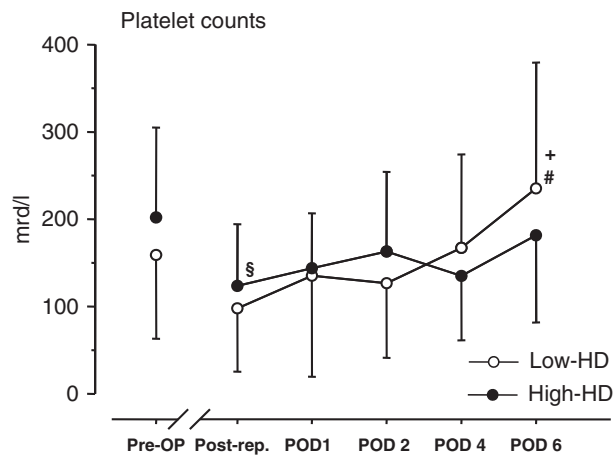


Figure 5 Course of platelet counts. HD – hepatocellular damage; rep – reperfusion; POD – postoperative day; closed circles – highHD group; open circles – lowHD patients. Mean \pm SD [§] $P < 0.01$ highHD pre-OP vs. postrep. [†] $P < 0.01$ lowHD pre-OP vs. POD 6/[#] $P < 0.001$ postreperfusion vs. POD 6, paired t -test.

postrep. among the high- and lowHD groups for all inducers of platelet aggregation tested in this study. In that sense, platelet aggregation after reperfusion (POD2) subsequent to induction with ADP (Fig. 4a), ristocetin and collagen (Fig. 4b) was significantly elevated in the highHD group as compared with lowHD individuals ($P < 0.05$). Evaluating all children included in this study, aggregative responses subsequent to ADP stimulation on POD1 and POD 2 (data not shown) linearly correlated with recipient serum ALT-levels on POD1. This linear regression was also observed for ristocetin on POD2 (ALT POD1 vs. POD 2 Ristocetin 0.63 mg/ml: $r = 0.65$, $P = 0.002$; ALT POD2 vs. POD2 Ristocetin 0.63 mg/ml: $r = 0.59$, $P = 0.0062$; data not shown).

Platelet counts

Platelet counts preoperatively and in the postrep. period up to POD 6 (Fig. 5) as well as rate of necessity for platelet transfusion perisurgery and postsurgery (Table 3) showed no statistical differences among both collectives investigated. However, in the highHD group platelet levels decreased between pre-OP and postrep. (paired t -test; $P = 0.003$) and did not recuperate by POD 6. In contrast, no significant drop was observed for lowHD individuals. In these patients thrombocytopenia improved from both pre-OP (paired t -test; $P < 0.05$) and postrep. levels (paired t -test; $P < 0.001$) towards POD 6.

Discussion

This is the first study to evaluate direct platelet aggregation in the course of pediatric LT. We were able to

demonstrate a correlation of recipient platelet function and I/R-damage markers in the early phase following reperfusion of the liver allograft. Beyond that, ADP triggered platelet aggregation levels were characterized by a strong linear correlation with markers of liver injury already before reperfusion respectively which was in contrast to other inducers tested.

Serum alanine transaminase (ALT) levels of included patients were utilized as marker of liver IRI. After correction for the G/BW_r a peak of ALT levels on POD 1 characteristic for IRI could be demonstrated in the highHD group that was absent in the lowHD collective. As direct evidence for the negative clinical impact of an accentuated IRI on early graft function in our study cohort, we demonstrated lower mean Quick levels and elevated mean TSB levels in the early phase after pLT in the highHD group.

In general, donor characteristics demonstrated no statistical differences between high- and lowHD patients. Only mean donor age was significantly lower in lowHD patients as compared with highHD ones. Increasing donor age is discussed to influence outcome of LT negatively [8,19]. However, analysis of 110 split LT revealed that donor age was not predictive for outcome [20]. The discrepancy of these findings may be resulting from the fact that split-liver donors are by definition mostly optimal donors, including a lower mean age in comparison to whole organ donors. This approach is supported by the report of Marino *et al.* [19] who observed a negative age-impact on outcome only beyond 45 years of age. As all patients in our highHD group were grafted from donors with good quality characteristics and as the mean age was 37 years in this group, it seems highly unlikely that donor age was influencing early outcome in our study. Furthermore, in a previous study investigating von Willebrand factor and IRI in adult recipients of liver allografts lowHD recipients had an increased donor age as compared with highHD recipients (47.1 years lowHD vs. 39.5 years highHD) [11]. Comparison of all other donor, recipient, and transplant procedure related data showed no major differences between the highHD and lowHD groups.

Remarkably superior ADP-dependent aggregative levels of platelets prior to graft reperfusion were demonstrated to be positively correlated with serum markers of hepatocellular reperfusion damage. This was in contrast to other stimuli tested. These data suggest that a certain increase of the ADP-dependent functional status of platelets in the pediatric recipients of liver grafts may result in a critical promotion of platelet adherence and activation on liver graft sinusoidal endothelial cells after reperfusion [21]. Whether differential thrombocytic expression of purinergic receptors, binding ADP on platelets, or ADP degrading ADPDases (e.g. CD39) may be responsible for the

altered ADP-aggregative responses seen with accelerated IRI in this study, needs further evaluation. Published data on experimental xenotransplantation have demonstrated that an ADP-diminishing concept can modulate early post-transplantation complications associated with disordered thromboregulation and thrombocytopenia [22,23]. Apyrase, an ADP-degrading soluble ATPDase, significantly prolonged cardiac xenograft survival if systemically administered. In this experimental setup, functional assays showed inhibition of platelet aggregation suggesting effective systemic anti-aggregative effects of the administered apyrase. Histologic studies showed that apyrase administration abrogated local platelet aggregation [24].

Postoperatively, a general increase of platelet aggregability was observed in patients with pronounced markers of IRI for all inducers tested in this study including ADP, Collagen and vWF dependent aggregation when compared with lowHD-patients. This goes along with previous data on vWF-dependent platelet function, we observed in adult LT patients with and without IRI [11].

With increased IRI platelet levels dropped pre-OP towards postrep. and without significant recuperation by POD 6. This is in keeping with observations in adult LT demonstrating persisting or developing thrombocytopenia subsequent to reperfusion as a prognostic unfavorable marker for early liver graft function and outcome [1–3,25]. In contrast, no significant drop was observed for lowHD individuals and thrombocytopenia improved from both pre-OP (paired *t*-test; $P < 0.05$) and postrep. (paired *t*-test; $P < 0.001$) respectively towards POD 6 in this group. This could point to a lower rate of platelets adhering to the sinusoidal lining with diminished sequestration in the reperfused graft allowing a rise in platelet count subsequent to grafting. A decrease in recipient platelet counts subsequent to reperfusion after LT [26] and in the perfusate in an *ex vivo* perfusion model [27] was observed previously. Further, patients after LT demonstrated a twofold to sixfold uptake of administered radio isotope-tagged platelets when compared with a healthy control indicating the liver as an important site of platelet deposition, leading to thrombocytopenia [21]. A drop in platelets in a canine LT model was only demonstrated with preserved livers in contrast to fresh grafts [28] pointing to hypothermic ischemia-injury as trigger for platelet accumulation in the reperfused organ. Thrombopoietin mRNA-levels were reported to be decreased in pLT patients prior to operation and increased significantly subsequent to LT in acute liver failure children [29]. From our data, it cannot be excluded, that a difference in thrombopoietin levels among the two groups investigated in this study may play a role for the differential course of thrombocytopenia following pLT [25].

In conclusion, we evaluated for the first time direct platelet function in the course of pLT. The demonstrated heightened platelet aggregation levels subsequent to various inducers in patients of pronounced IRI in the early course after pLT – when compared with controls – point to the important role of platelets for the scenario of liver IRI subsequent to pLT. Clinically, this was supported by enhanced IRI being paralleled by pronounced thrombocytopenia and reduced early liver function in this study. Further, the data on ADP-induced aggregative responses prior to reperfusion suggested a role of purinergic signaling-dependent platelet function for hepatocellular damage after liver grafting that is determined in the recipient prior to pLT. Whether platelet hyperaggregability in selected patients could play a role for early [30] or late [31] vascular complications that are observed in up to 50% of all re-pLT cases [30] is not explored yet. However, modulation of platelets in the course of pLT for example by prostaglandin E1 (PGE1) treatment [32] to reduce graft damage subsequent to I/R improving the early outcome needs further investigation.

Authorship

JSaE: designed the study. AA, RYT, JSaE, RG, ST, XR WTK and LF: performed the study. AA, RYT, MS, RG, AK, ST and LF: collected data. JSaE and LF: analysed the data. JSaE, SR, WTK and LF: wrote the paper.

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