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ORIGINAL ARTICLE

Recipient levels and function of von Willebrand factor prior to liver transplantation and its consumption in the course of grafting correlate with hepatocellular damage and outcome*

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Summary

Von Willebrand factor (vWF) is a major platelet adhesion molecule at sites of vascular injury, such as observed in ischemia/reperfusion injury following orthotopic liver transplantation (OLT). Thirty-three OLT patients were divided into groups with elevated or low markers of hepatocellular damage (high and low-HD). Whole-blood aggregometry was performed to evaluate platelet function. Multimeric analysis was utilized to evaluate functional vWF levels in the course of OLT. Donor and recipient demographics were comparable among groups. Low-HD patients demonstrated better preserved coagulation parameters on POD 1–6 if contrasted to high-HD patients. One year graft survival for the high-HD group was lower than low-HD patients ($P = 0.037$). Preoperative vWF-dependent platelet aggregation and functional vWF plasma levels correlated directly with alanine transaminase levels early after OLT as did the decrease of functional vWF to reperfusion. In summary, these data suggest that vWF may serve as a significant mediator of platelet recruitment and hepatocellular injury in the graft following reperfusion.

Introduction

Ischemia/reperfusion (I/R) injury occurs when blood flow to an organ or tissue is interrupted for a period of time and subsequently re-established. The extent of hepatocellular damage (HD) after reperfusion of the graft has been correlated with primary non-function and poor early graft function with a negative influence on the overall outcomes of orthotopic liver transplantation (OLT) [1]. Although the sequence of events that leads to tissue injury in this situation is incompletely understood, platelets seem to play an important role. Persistent thrombocytopenia after reperfusion is an unfavorable indicator for early liver graft function and subsequent outcomes [2]. Adhesion of platelets to the sinusoidal lining and sequestration in the reperfused graft are assumed to be major factors in this phenomenon [3]. Prevention of platelet adhesion and accumulation in rat allografts after reperfusion attenuates I/R injury [4,5]. Prior activation of platelets in an *ex vivo* model amplifies the extent of liver injury [3] and decreases susceptibility of the reperfused liver to I/R-injury modulating therapy [6]. These data suggest a correlation of the functional status of recipient platelets and their impact on I/R injury.

Von Willebrand factor (vWF) is a major mediator of platelet-aggregation at sites of high shear stress and vascular injury [7] as observed with I/R injury. VWF plasma-levels of cirrhotic patients have been reported to be

increased up to fivefold higher than control reference levels [8]. Platelet activation is initiated by interactions of vWF and the specific receptor on platelets, termed glycoprotein Ib (GPIb). The vWF protein comprises of large, multimeric structures of up to 100 identical subunits. Mainly high molecular weight (HMW) multimers are responsible for the physiological interaction with GPIb leading to aggregation and activation of platelets.

Platelet function has been reported to be altered in cirrhotic patients independently of the hematocrit. To date, alterations in platelet function have been described in the scenario of compromised hemostasis in cirrhotic patients [9] [10]. Both vWF-dependent platelet function and vWF antigen alterations during the course of OLT have been examined for intraoperative blood loss [11] and for post-transplant vascular thrombosis [12].

This study was conducted to correlate vWF-dependent platelet aggregation as well as total and functional vWF-plasma levels pre-OLT with markers of hepatocellular injury, early graft function and overall outcome.

Patients and methods

Study groups

This study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. Thirty-three consecutive adult OLT patients were included in this study during a time period of 42 weeks at the University Hospital of Hamburg after giving their informed consent prior to inclusion in this study. Recipients were divided into two groups, one with normal postoperative courses [peak serum alanine transaminase (ALT) <500 IU on postoperative day (POD) 1 or 2], defined as low-HD and a second group with nadir serum ALT level on POD 1 or 2 above 500 IU, designated as the high-HD.

Recipient and donor demographics

Indications for OLT are specified in Table 1. Donor-related data such as age, liver enzymes, serum sodium, rate of resuscitation and necessity of vasopressors were statistically not different among high- and low-HD (Table 2).

Table 1. Indication for OLT.

	High-HD	Low-HD
Posthepatitis B cirrhosis	0	1
Posthepatitis C cirrhosis	3	4
Cryptogenic	2	4
Ethanol	2	4
Primary sclerosing cholangitis	0	4
Biliary others	1	1
Polycystic liver disease	0	3
HCC	1	1
Autoimmune hepatitis	1	0
Primary nonfunction	0	1
Total	10	23

Table 3 compares recipient characteristics. There were no statistical differences among the two groups concerning UNOS state, age, body weight or gender. Preoperative hematocrit and coagulation testing such as thrombin time, quick [prothrombin time (PT), representing integrity of factors II, V, VII and X as percentage of a normal reference] and partial thrombin time (PTT) were comparable. The rate of split-OLT procedures was statistically not different among high- and low-HD, however, there was a trend towards the high-HD group (60.0 vs. 34.8;$P = 0.336$). The mean of cold and warm ischemic time was almost identical (Table 3). There was no statistical difference in ABO distribution between the two groups (data not shown).

Reagents

The ADP, Collagen and ristocetin as well as the whole-blood aggregometer were purchased from Chronolog (Endingen, Germany). Primary anti-vWF and secondary antibody (AB) for multimeric analysis were purchased from Dako (Hamburg, Germany), the vWF-Elisa from American Diagnostica (Pfungstadt, Germany).

Clinical chemistry

Serum ALT, aspartate transaminase (AST), gamma glutamyl transpeptidase, bilirubin, sodium as well as throm-

Table 2. Donor characteristics.

	High-HD		Low-HD		
	mean median *n*/total	SD range % of total	mean median *n*/total	SD range % of total	*P*-value
Age (years)	39.5	16.4	47.1	15.0	0.205
Sodium (mmol/l)	145.7	5.0	143.9	6.5	0.414
Bilirubin (mg/dl)	0.45	0.2–1.82	0.7	0.2–2.5	0.056
GOT (IU)	19.0	5–90	10.0	5–45	0.360
GGT (IU)	13.0	3–352	8.0	5–27	0.175
Resuscitation	1/10	10%	3/23	17%	0.990

Table 3. Recipient and OLT characteristics.

	High-HD		Low-HD		
	Mean *n*/total	SD % of total	Mean *n*/total	SD % of total	*P*-value
UNOS 1 + 2a	2/10	20.0%	2/23	8.7%	0.739
Rate of cirrhosis	8/10	80.0%	17/23	73.9%	0.947
Rate of re-transplantation	0/10	0%	4/23	17.4%	0.409
Age (years)	51.9	8.8	45.2	16.4	0.234
Body weight (kg)	69.6	12.4	69.2	12.1	0.923
Gender (M)	6/10	60.0%	11/23	47.8%	0.705
Hematocrit* (%)	33.3	5.8	34.4	5.1	0.67
PT*† (%) preoperative	73.5	26.3	75.4	26.1	0.853
Thrombin time* (s)	21.5	6.6	21.6	3.8	0.971
Partial thrombin time* (s)	41.1	6.2	40.7	11.2	0.914
Units of platelets during surgery	12.4	12.0	7.4	6.7	0.132
Split-OLT	6/10	60.0%	8/23	34.8%	0.336
CIT (min)	435.7	87.5	439	175.1	0.948
WIT (min)	33.7	6.8	32.1	7.3	0.554

*Preoperative.

†PT was assessed as quick in % of normal reference, higher percentage equals shorter PT.

bin time, PT (stated as Quick in % of a normal reference), PTT, hematocrit and platelet counts where routinely analysed in clinical chemistry laboratories. Tranaminases assays were performed at 24 °C. ALT values have to be taken by the factor 2.0 to be comparable with data, derived from 37 °C assays. However, data in this study are presented as original 24 °C assay values.

Aggregometry

Citrated blood was collected from patients in the course of 1 h prior to initiation of anaesthesia (preoperative), 20–40 min after reperfusion and on POD 1, 2, 4 and 6 (POD 1–POD 6). An aliquot was centrifuged for 20 min with 800 ***g*** (platelet poor plasma) and supernatant plasma was stored at −80 °C for vWF analysis.

The remaining blood was analysed in a whole blood impedance aggregometer (Chronolog, Endingen, Germany) utilized in this study for platelet function according to manufacturers instruction. The only modification was a 1:3 instead of 1:2 dilution with 0.9% NaCl solution as performed and characterised before [13] to limit blood sample volume. In brief, platelet adhesion was quantified to two alloy-wires following various aggregating stimuli, as utilized in this study such as ADP (10 mM), collagen (2 mg/ml) and the vWF activator ristocetin (1.25 μg/ml). Increasing layers of platelets subsequent to platelet aggregation to accelerate resistance for an electric flow [stated in ohm (Ω)] between the two wires that is proportional to the extent of platelet aggregation. Aggregation analysis was performed within the first 3 h after sampling.

Quantitative and qualitative analysis of vWF

Total vWF antigen in patients plasma was determined with a commercial enzyme-linked immunostained absorbent assay (ELISA) according to the manufacturers' instructions. For multimeric analysis of vWF patients plasma was subjected to discontinuous 1.6% aggarose SDS-gel electrophoreses as described previously [14]. After immunoblotting, vWF-multimeric patterns were visualized by horseradish peroxidase labeled secondary AB mediated chemiluminescence and analyzed utilizing digital imaging (Flur-S-Multiimager; BioRad, München, Germany). Definition of high molecular weight multimers (MWM) as those beyond the lower 10 bands detected in immunoblot analysis was utilized as previously suggested for analysis [14] and included with cirrhotic patients before [8]. The ratio of the digitally acquired integral under the curve of high MWM versus low MWM of each sample (SOFTWARE; BioRad) (Fig. 7a) was defined as unit of measurement for the extent of functional plasma vWF.

Statistical analysis

On normal distributed values Student's *t*-test and paired *t*-test were performed with data presented as mean ± SD. Groups of values not meeting normality testing were subjected to Mann–Whitney rank sum test for statistical analysis presented as median plus data range. Proportions were compared by *z*-test. Correlations were evaluated utilizing linear regression testing. Probability of survival was determined utilizing Kaplan–Meier survival analysis. Statistical significance was assumed with a $P < 0.05$. Sigma

Stat, Version 2.03 (SPSS, München, Germany) served as statistical analysis software.

Results

Markers of hepatocellular damage

We considered the serum ALT as representative for hepatocellular damage and demonstrated its peak on POD 2, decreasing POD 6 (data not shown). The high-HD collective had a median on POD 1 of 649 (range 182–2453) and on POD 2 of 928 IU/l (range 528–1475); in contrast the low-HD group on POD 1 measured 209 (range 36–483) and on POD 192 (range 37–476).

Liver function and survival

Follow up time was 54–1095 days with a median of 892 days. The 1-year probability of graft survival for the high-HD group of 60.0% was significantly below that of the low-HD group with 85.6% ($P = 0.037$) (Fig. 1). One year probability of patient survival was statistically not different among high-HD and low-HD patients (80.0 vs. 90.2%; $P = 0.532$, data not shown).

The PT (Quick – factors II, V, VII and X dependent) and PTT where compared among high and low-HD groups to evaluate whether postoperative reperfusion injury is paralled by impaired liver function. Patients in the low-HD had both a shorter PTT when contrasted to the high-HD group as well as an improvement of the PTT from preoperative times towards POD 4 and 6 values (Fig. 2a). The latter was not observed for the group with pronounced postoperative hepatocellular damage. PT values were significantly better on POD 2 and 4 in the low-HD group (Fig. 2b).

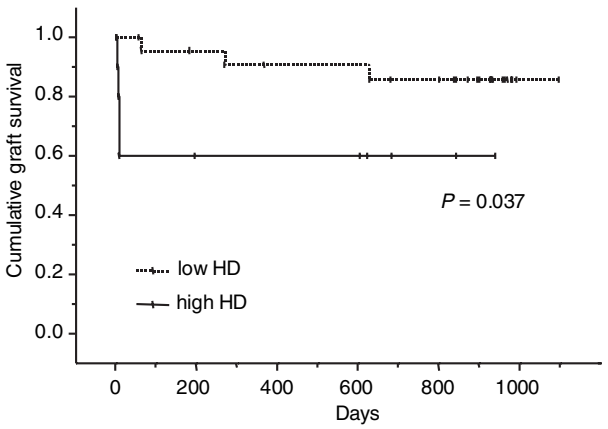

Figure 1 Kaplan–Meier analysis of graft survival. Low hepatocellular damage (HD) – recipients with peak serum-ALT <500 IU on postoperative day (POD) 1 or 2. High-HD – patients with nadir serum ALT level on POD 1 or 2 above 500 IU.

Platelet aggregation and counts

Platelet function was abnormal for all recipients included in this study over the whole observation period for all aggregation agonists tested when contrasted to a healthy reference (data not shown). For all agonists, a decrease in aggregation following initiation of surgery was observed reaching a nadir subsequent to reperfusion, that was followed by a constant return in aggregatory responses (data not shown). Latter finding was more marked for collagen and ristocetin induced, vWF-dependent aggregation, whereas response to the weak agonist ADP tended to recover more slowly after reperfusion.

Significant differences were observed especially in the preoperative phase among the high- and low-HD groups, for platelet function in the recipient (data not shown). Enhanced median aggregatory responses were assessed in low-HD patients when contrasted to high HD individuals for collagen preoperative (7.0 Ω vs. 2.5 Ω; $P = 0.024$)

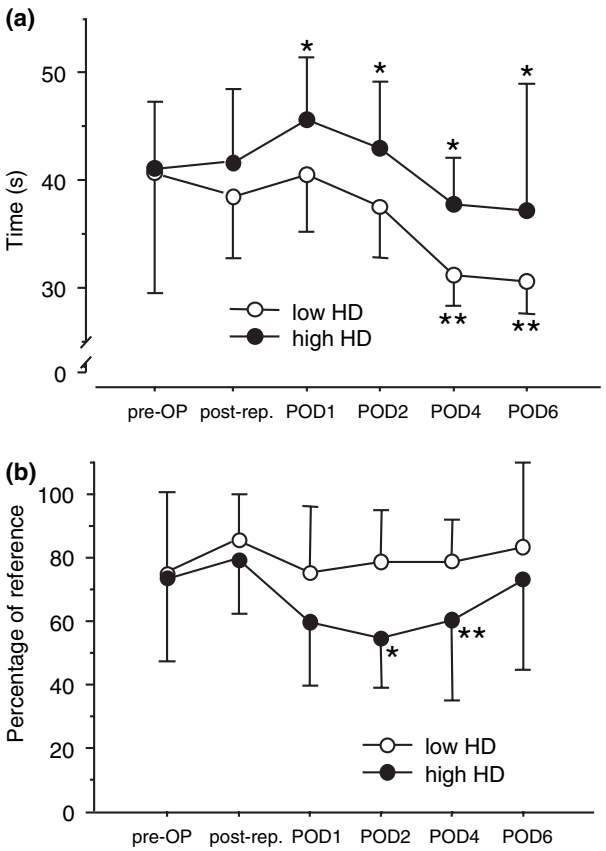

Figure 2 Coagulation parameters as marker of early graft function. HD, hepatocellular damage on day 1 and 2. Mean, ± SD $*P < 0.05$ low vs. high-HD group, Student's *t*-test. $**P < 0.01$ preoperative versus postreperfusion, paired *t*-test. (a) Partial thrombin time (s); (b) PT (stated as Quick in % of normal reference; higher values implicate shorter PT and vice versa).

and intraoperative (10.0 Ω vs. 2.5; $P = 0.005$), for ADP preoperative (3.0 Ω vs. 0,0 Ω; $P = 0.006$).

There were no significant differences observed for the preoperative ristocetin-dependent aggregation curves. On POD 6, mean aggregation levels subsequent to ristocetin stimulation were different (high-HD 7.69 Ω ± 4.28 vs. low-HD 2.75 Ω ± 2.12; $P = 0.004$); otherwise no significant differences where observed for platelet function between the two groups.

Platelet counts and correlation of preoperative aggregation with reperfusion damage

Whole blood aggregometry delivers absolute values of electrical resistance, increasing with the platelet mass, aggregating around the wires in the sample tube [15]. A comparison of platelet counts among groups was comparable in the observation period except preoperative values revealing significant differences (high-HD 66.7×10^3/µl ± 29.0 vs. low-HD 141.0×10^3/µl ± 109.4; $P = 0{,}045$) (Fig. 3a). As platelet counts could influence the maximal possible platelet-aggregate layer and subsequently the maximum of impedance following induction of aggregation, impedance values derived prior to surgery were corrected for individual platelet count in order to make them comparable among differential platelet counts [1 AU = (resistance (Ω)/platelet count (n/µl))*10^3)].

Platelet count corrected vWF-dependent platelet aggregation was significantly increased in the high HD group prior to surgery when contrasted to the low HD group (73.92 AU ± 39.29 vs. 46.37 AU ± 33.48; $P = 0.042$) (Fig. 3b). This was in contrast to other stimuli of platelet aggregation tested. After correction for platelet count, ADP still demonstrated a significantly decreased median aggregation response with platelets in the high-HD group if contrasted to low-HD (0.0 AU/0.0–27.03 vs. 25.0 AU/0.0–171.43; $P = 0{,}017$), collagen did not demonstrate differences among the two groups. Platelet count corrected aggregation subsequent to vWF co-incubation did correlate positively with ALT levels on POD 1 ($r = 0.55$; $P < 0.002$) and POD 2 ($r = 0.54$; $P < 0.002$) after OLT (data not shown). No such correlation was observed for ADP or collagen.

VWF plasma levels and multimeric analysis

In both, high- and low-HD recipients, total vWF antigen blood levels were elevated (when compared with the control reference range) at 341 ± 120% and 263.9 ± 125% decreasing in both groups to mean levels of 136 ± 80% and 147 ± 78% respectively. However, vWF antigen levels were not significantly different among groups ($P = 0.121$ preoperative/0.735 postreperfusion; Fig. 4). The drop in total vWF levels was significant for both groups ($P < 0{,}001$; paired t-test). The changes in total vWF antigen between the two time points positively correlated with ALT levels on POD 1 ($r = 0.5$, $P = 0.019$; data not shown).

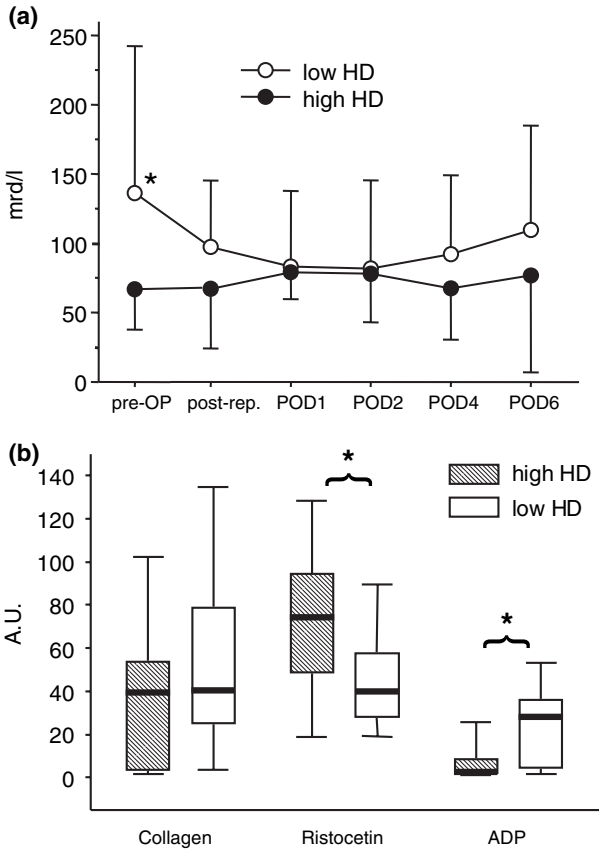

Figure 3 Platelet count and function. (a) Platelet counts in the course of OLT. HD, hepatocellular damage on day 1 and 2. *$P < 0.05$ low versus high HD group, Student's t-test. (b) Median platelet count corrected preoperative aggregation (thick lines in box plots) measured as resistance (Ω), was evaluated with whole blood aggregometry. 1 arbitrary unit (AU) = (resistance (Ω)/platelet count [n/µl)]*10^3). *$P < 0.05$ low versus high HD group, Mann–Whitney rank sum test. Inducers of aggregation (final concentration): collagen (2 µg/ml), the vWF-activator ristocetin (1.25 µg/ml) and ADP (10 µm).

High molecular weight vWF multimers are biologically active. Therefore we evaluated the vWF multimeric pattern of plasma from patients prior to OLT and after reperfusion to determine levels of *functional* vWF antigen as displayed in Fig. 5a. Levels of HMW vWF multimers prior to surgery revealed a positive correlation with serum ALT levels on POD 1 ($r = 0.42$; $P = 0.023$) and POD 2 ($r = 0.5$; $P = 0.006$) (Fig. 5b), as did the reduction of HMW vWF multimer levels between prior OLT and 20 min postreperfusion (with ALT levels POD 1: $r = 0.55$; $P = 0.006$; POD 2: $r = 0.67$; $P = 0.0004$; Fig. 5c).

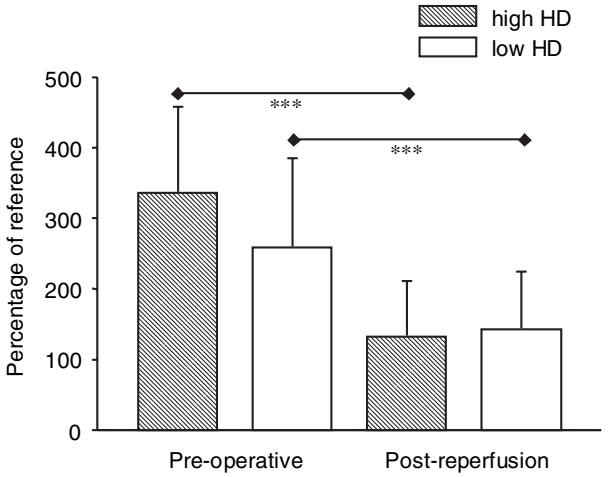

Figure 4 Analysis of recipients total vWF antigen. Total plasma vWF antigen. Determined by a commercial ELISA kit according to manufactures instructions. ***$P < 0.001$; paired *t*-test.

Discussion

The AST and ALT are standard reference markers for hepatocellular damage [16–19] and are considered to be the most reliable currently established noninvasive parameters of preservation and reperfusion injury [20]. The cut off for ALT to determine whether a patient experiences increased or low levels of hepatocellular damage was selected in this study below those levels utilized to identify patients with manifest primary non or poor function [21]. The purpose was to enable the inclusion of individuals with moderate extent of hepatocellular damage following OLT. Significantly worse graft survival as observed for patients in the high-HD group however does indicate a certain clinical relevance of limited extent of hepatocellular damage. The clinical relevance was further supported by diminished levels of liver synthesis in the group of heightened hepatocellular damage in the early postoperative phase, monitored by less preserved liver-depending coagulation markers [22].

Donor characteristics demonstrated no statistical differences between high and low-HD patients concerning the apparent graft quality. Mean platelet levels were demonstrated to be higher in recipients with low-HD prior to surgery, leading to correction of aggregatory levels for these differences to rule out platelet count depending differences at that time point. There was a trend towards high-HD patients for split liver procedures. However preoperative findings would not be influenced. Secondly, comparing our own experience of 202 split-liver transplantations with whole liver procedures in the same time frame, revealed comparable probability of graft and patient survival with a small trend in favor of split procedures (data not shown). Contrasting further recipient and transplant procedure characteristics revealed statistically comparable data including standard testing for coagulation.

Impedance whole blood aggregometry, as utilized in this study to examine platelet function, has been reported to be equivalent to standard platelet function tests like aggregometry with platelet rich plasma [23]. In contrast to vWF independent platelet function, positive correlations of ristocetin-dependent platelet function levels with markers of early hepatocellular damage following reperfusion of the liver graft suggested an increased capability of recipient vWF to interact with platelet receptors. This result was in contrast to other aggregatory stimuli tested and is in keeping with known characteristics of cirrhotic patient studies [9]. It is possible that vWF-triggered overactivation of platelets in recipients with pronounced hepatocellular damage may result in desensitization of platelet ADP receptors and poor ADP-dependent aggregatory potential [24], as observed here *in vitro*. Enhanced pre-activation may account for the pronounced, pre-existing thrombocytopenia in the high-HD patient group. Prior activation of platelets also increased the extent of injury in an experimental liver I/R model [3].

The vWF antigen levels, were elevated up to fivefold of the reference in our total collective prior to surgery, as observed by others in cirrhotic patients [25]. Multimeric analysis of plasma-vWF determined functional vWF, as only HMW multimers are assumed to be active in platelet binding and activation [26]. Taken together, the data derived from total and functional protein levels and *ex vivo* functional vWF analysis in this study suggest, that marked levels of antigen and function in the recipient for this important platelet adhesion and activation factor prior to OLT may facilitate pronounced platelet deposition on liver graft sinusoidal endothelial cells (SEC) after reperfusion. Platelets have been shown to induce SEC apoptosis upon adhesion subsequent to reperfusion of cold ischemic rat livers [5]. The increased levels of apoptosis of SEC are assumed to present a pivotal mechanism in I/R injury of the liver [27]. Among others the significance of platelet adhesion to the graft endothelium during reperfusion as pivotal mechanism for liver injury is supported by the observation that the presence of platelets aggravated reperfusion injury in an *ex vivo* rat liver perfusion model [3].

In summary, liver graft recipients with heightened plasma HMW-vWF multimer levels along with increased vWF-dependent platelet activation may demonstrate an increased risk for hepatocellular damage following liver grafting. This phenomenon is paralleled by worse early graft function and decreased graft survival. Increased consumption of total and functional vWF in the course of graft reperfusion may indicate a significant role of vWF in platelet adhesion to the graft endothelium following reperfusion. Whether preoperative functional vWF levels

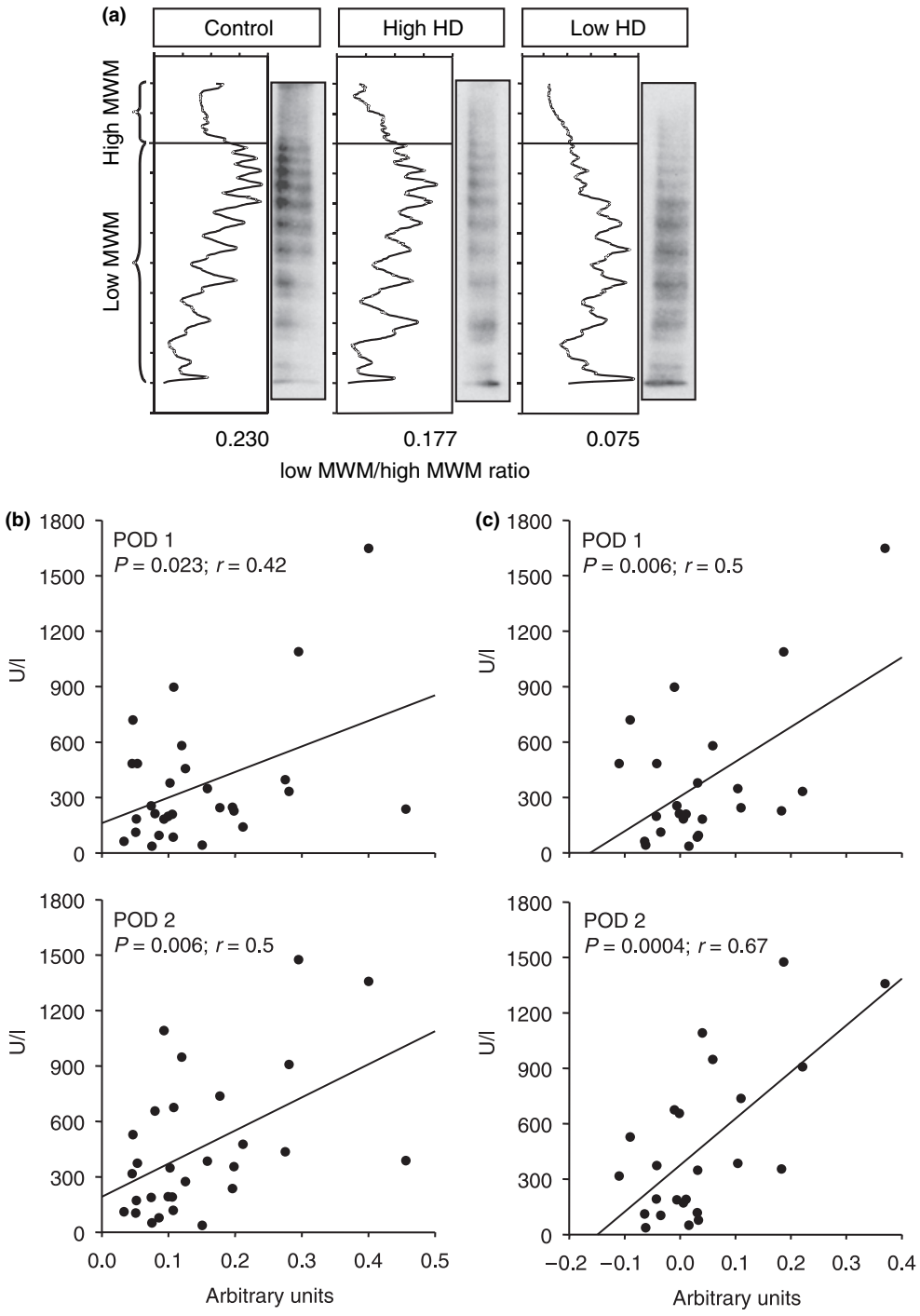

Figure 5 Analysis of recipients functional vWF. (a) Only highest molecular weight multimers (MWM) perform biological activity of vWF (functional vWF). Patients plasma was subjected to discontinuous 1.6% agarose SDS-gel electrophoreses. After immunoblotting, vWF multimeric patterns were visualized by horseradish peroxidase labeled secondary AB-mediated chemiluminescence and analyzed utilizing digital imaging. Levels are stated as ratio of the area under the curve of high MWM (defined as those beyond the lower 10 bands) and that below the curve of low forms (bands 1–10). Typical examples for a normal control, as well as a high- and a low-HD patient vWF multimeric patterns are displayed. Linear correlation of functional vWF levels preoperative (b) and consumption of functional vWF (change of functional vWF preoperative towards postreperfusion) (c), respectively, with markers of I/R injury (ALT levels on POD 1 and 2). Arbitrary units describe the quotient of digitally assessed area under the curve (Flur-S-Multiimager; BioRad) of high and low molecular weight vWF multimers (b) and their differences between preoperative and postreperfusion values (c).

may be of clinical importance needs to be evaluated in a larger study.

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