Perioperative Von Willebrand Factor Dynamics are Associated with Liver Regeneration and Predict Outcome after Liver Resection

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Abstract:

Von Willebrand Factor (vWF) was found to mediate platelet influx during the early phase of liver regeneration in mice. Further, increased vWF-antigen (vWF-Ag) levels were shown to be predictive for outcome of patients with chronic liver disease. Accordingly, we aimed to assess the relevance of perioperative vWF-Ag dynamics in terms of liver regeneration and clinical outcome in patients undergoing liver resection (LR). Accordingly, we observed that vWF-Ag and its activity – estimated via ristocetin cofactor measurement – increased immediately after induction of liver regeneration and was associated with platelet accumulation within the liver. However, a significant vWF-Ag burst was only observed in patients with unaffected postoperative liver regeneration. E-selectin, as an established marker for endothelial cell activation, was found to correlate with vWF-Ag in the liver vein after induction of liver regeneration (P=0.022). Preoperative vWF-Ag levels significantly predicted postoperative liver dysfunction (LD) (N=95, AUC:0.725, P=0.009). Furthermore, a cut-off of vWF-Ag≥182% was defined to identify patients with a higher risk for postoperative LD or morbidity. This was confirmed within an independent mulitcenter validation cohort (N=133). Ultimately, multivariable analysis revealed that vWF-Ag was an independent predictor of postoperative LD and morbidity.

Conclusion: Within this study we were able to provide evidence that an initial vWF burst is required to allow for adequate platelet accumulation and concomitant liver regeneration after LR and might be abolished as a consequence of intrahepatic endothelial cell dysfunction. We were further able to reveal and validate the potential of preoperative vWF-antigen levels to predict poor postoperative outcome in patients undergoing LR. Despite the pathophysiological relevance of our findings, vWF-Ag seems to be a valuable tool for preoperative risk assessment in patients undergoing LR.

Introduction

The most significant factor determining outcome after liver damage or resection represents the ability of the remnant liver to regenerate[1, 2]. Growing evidence suggests, that an impairment of hepatic regeneration after liver surgery might culminate in postoperative liver dysfunction (LD), which is associated with a markedly increased risk of postoperative morbidity and mortality[2-4]. During initiation of liver regeneration, platelets and their specific activation seem to be of crucial relevance[5]. In this context, von Willebrand Factor (vWF) has recently been demonstrated to be of central relevance during the early period of liver regeneration by mediating platelet adhesion within the liver after partial hepatecomy in mice[6]. In particular, Kirschbaum et al. demonstrated that platelet influx after liver resection (LR) was virtually absent in the presence of a neutralizing antibody against vWF. Further, vWF deficient mice showed markedly reduced liver regeneration[6]. However, clinical evidence for this association is still missing.

VWF is a 2050 amino acids long glycoprotein that is mostly organized in multimers. Activated endothelial cells release vWF-Ag as a response to certain stimuli, including high shear stress and portal hypertension[7, 8] Its primary functions are stabilization of factor VIII and cross linkage of platelets with the subendothelial matrix. Thus, vWF-Ag is widely known as a key player in coagulation and primary hemostasis[9]. However, vWF-Ag also plays a role in platelet mediated liver regeneration. In context of the liver, vWF-antigen (vWF-Ag) has further been implicated as a non-invasive marker for outcome of a variety of chronic liver disease[8]. It has been shown, that increased circulating vWF-Ag levels are associated with increased mortality rates in patients with cirrhosis and in patients with liver damage due to hepatitis C infection[10, 11]. Moreover, patients with acute on chronic liver failure were found to have elevated levels of vWF-Ag compared to

healthy controls but also compared to patients with cirrhosis, and vWF-Ag levels were a valid tool to predict in-hospital mortality in these patients[12]. In addition, multiple studies have reported that reduced liver function and portal hypertension are associated with increased levels of circulating vWF-Ag, that might be released from activated endothelial cells as a response to increased shear stress[5, 8, 13]. However, the clinical relevance of vWF-Ag to assess preoperative liver function and concomitantly predict postoperative clinical outcome after LR has not been evaluated so far. Hence, the aim of this study was 1) to explore the dynamics, functional capacity and the pathophysiologic relevance of vWF-Ag in the process of early human liver regeneration, 2) to investigate the role of vWF-Ag as a predictive marker in patients undergoing LR and 3) to validate our data in an international multicenter fashion to present a clinically applicable tool for prediction of postoperative outcome prior to liver surgery.

Material and Methods

Study Population and Sampling Time Points

Initially an exploration cohort was prospectively recruited and patients were followed for a postoperative period of 90 days at the Medical University of Vienna. Patients with hepatocellular carcinoma (HCC), cholangiocellular carcinoma (CCC) or colorectal cancer liver metastasis (mCRC), were considered eligible for inclusion. Subsequently, as we observed a significant predictive potential of vWF-Ag for postoperative LD and clinical outcome, we validated our explorative results in a prospective set of patients representing a clinical routine cohort. Importantly, patients undergoing LR at 4 different institutions (General Hospital of Vienna [Austria], Rudolfstiftung Hospital [Austria], Paracelsus Medical University [Austria], University Hospital Inselspital Bern [Switzerland]) were included within this validation cohort. A sample size calculation on the data gathered within the exploratory cohort was performed using an online sample size calculator (sample-size.net): Concerning an observed mean difference of 42.429% and a standard deviation of 71.253% vWF-Ag, and an approximate incidence of LD of 20%, the calculation suggested a sample size of 138 patients for a sufficiently powered validation cohort. Specific characteristics of all patients were prospectively recorded (Table 1). Baseline characteristics and surgical procedure of patients and preoperative variables of liver function, routine laboratory parameters and baseline liver pathology were recorded. Of note, no patient received platelet transfusions or was treated with terlipressin or comparable vasopressin analogs. Intraoperative transfusion of erythrocytes and perioperative medication with anti-platelet drugs were documented. VWF-Ag was evaluated one day prior to as well as one (POD1) and five (POD5) days after surgery. The extent of resection was classified as minor or major resections (<3segments=minor, ≥3segments=major), according to the IHPBA-Brisbane-2000

nomenclature[14]. Further, postoperative outcome was prospectively documented and classified in LD, postoperative morbidity and postoperative mortality as described previously[5]. For more detail refer to the supplemental methods section.

We further assessed perioperative vWF-Ag dynamics during "early liver regeneration" in a subset of 30 patients undergoing hemihepatectomy. During hemihepatectomy we routinely start with the preparation of the hepato-duodenal ligament and the transection of the respective branches of the portal vein. As ligation of the portal branches is believed to trigger regenerative processes in the contralateral lobe[15], we collected blood from the remaining liver vein – i.e. from the regenerating liver lobe - after parenchymal transection. Furthermore, liver tissue samples were retrieved at the beginning of surgery and after parenchymal transection for electron microscopy analysis (description in supplemental methods section) in three patients undergoing major hepatectomy.

The Institutional Ethics Committee approved the study and all patients gave written informed consent. Furthermore, the trial was registered at a clinical trials registry (ClinicalTrials.gov-Identifier: exploration set: NCT01700231, validation-set: NCT02118545).

Measurement of vWF-Ag, Ristocetin Cofactor, ADAMTS13-Activity and Routine Laboratory Parameters

Perioperative blood-parameters of liver function were measured in serum samples via local routine laboratory. To assure clinical relevance, also vWF-Ag was analyzed using routine laboratory analysis of each respective institution. Further, within our intraoperative sub-group, we assessed ristocetin cofactor (vWF:RiCo), a measure for vWF-Ag activity, in the routine laboratory, whereas the activity of A disintegrin and

metalloprotease with thrombospondin type 1 motif member 13 (ADAMTS13), the main regulating protease of vWF-multimer length, was assessed using a commercially available ELISA (TECHNOZYM® ADAMTS-13 Activity ELISA, Technoclone, Vienna, Austria).

Statistical Analyses

Statistical analyses were performed using SPSS 23 software (SPSS, Inc., Chicago, IL, USA) and were based on non-parametric tests for either paired or independent samples (Mann-Whitney U test, Wilcoxon test, chi-squared test, Spearman-Rho correlation). Further, receiver operating characteristic (ROC)-analysis was applied to assess the discriminatory potential of vWF-Ag between patients with or without LD and multivariable analysis was used to investigate independency of predictive markers for postoperative LD and morbidity.

For more detailed elaboration of statistical analysis refer to the supplemental material. Boxplot illustrations are given without outliers and extreme values to improve the resolution of interquartile ranges. P-values <0.05 were considered statistically significant.

To validate the ability of preoperative vWF-Ag to detect poor postoperative outcome, a ROC-curve analysis was performed. In addition, this statistical approach–was used to identify the optimal cut-off level with the greatest accuracy of distinguishing high and low risk groups.

Results

Patient Demographics

A total of 95 patients suffering from either mCRC, HCC or CCC who underwent LR between 1/2012 and 12/2013 were included in our prospective exploratory study cohort (in 30 patients detailed intraoperative blood sampling was applied). Subsequently, additional 133 consecutive patients from 4 different institutions (General Hospital of Vienna: N=35, Rudolfstiftung Hospital: N=38, Paracelsus Private Medical University of Salzburg: N=35, University Hospital Inselspital Bern: N=25) and 2 countries (Austria and Switzerland) were enrolled between 2/2014 and 7/2015 and served as a validation cohort. Of note, this international multicenter approach allows validation with high clinical relevance, as it reflects clinical routine among different institutions. Patient characteristics, outcome and laboratory parameters of patients from both groups are listed in Table 1.

VWF-Ag Levels Increase after Partial Hepatectomy

We initially assessed the perioperative time course of vWF-Ag blood levels in patients undergoing LR as illustrated in Fig.1A. In the exploration cohort, perioperative vWF-Ag concentrations revealed a distinct and significant increase on POD1 (median PRE OP=160.0%, median POD1=276.9%, P=0.001) and vWF-Ag levels further increased until POD5 (median PRE OP=160%, median POD5=336.0%, P=0.001; median POD1=276.9%, median POD5=336.0%, P=0.007).

To evaluate whether the extent of LR would affect postoperative vWF-levels, vWF-Ag was analyzed in patients undergoing minor or major resection (Fig.1B). While preoperative vWF-Ag concentrations were found to be comparable in patients

undergoing major or minor LR, we found significantly deteriorated postoperative (POD1) vWF-Ag levels, in patients after major LR (median minor=252.5%, median major=312.0%, P=0.001). VWF-Ag levels further increased until POD5 and the trend of elevated vWF-Ag levels in patients undergoing major resection compared to those with minor resections could also be observed at this later time point. However, this observation did not reach statistical significance (median minor=279.0%, median major resection=377.0%, P=0.075; Fig.1B).

As both erythrocyte transfusion and anti-platelet drugs might interfere with our readout, we subsequently evaluated perioperative dynamics of vWF-Ag in patients that did or did not receive intraoperative blood transfusion (Supplemental Fig.1A) and patients that were perioperatively treated with anti-platelet drugs (Supplemental Fig.1B). Importantly, we found similar dynamics as for our exploration cohort and could not assess any differences between the cohorts.

Liver Cirrhosis and Diminished Liver Function are Associated with higher Levels of vWF-Ag prior to Surgery

When looking at dynamics of vWF-Ag between the perioperative time points, similar patterns were found in patients with fibrosis or cirrhosis (Supplemental Fig.2A), sinusoidal obstruction syndrome (Supplemental Fig.2B), steatosis (Supplemental Fig.2C) and steatohepatitis (Supplemental Fig.2D). In line with the current literature, we observed higher levels of vWF-Ag at baseline in patients suffering from cirrhosis compared to non-fibrotic patients (median no fibrosis=146.0 %, median cirrhosis=183.0 %, P=0.036). Further, we observed significantly higher levels of vWF-Ag in patients with cirrhosis compared to patients with fibrosis on POD5 (median

fibrosis=335.5 %, median cirrhosis=420.0 %, P=0.039). Yet, patients suffering from fibrosis, steatosis, sinusoidal obstruction syndrome or steatohepatitis, did not show significant deviations in terms of vWF-Ag prior to surgery. In addition, we evaluated the relation of preoperative vWF-Ag to markers of liver damage (aspartate-aminotransferase, alanine-aminotransferase, albumin), liver function (bilirubin, INR, MELD score), cholestasis (gamma-glutamyl transferase, alcalic phosphatase), and to preoperative platelet counts. Indeed, we were able to document significant, yet weak correlations with most of the mentioned markers, as visualized in Supplemental Fig. 3. Of note, there was no association between levels of vWF-Ag and platelet count prior to surgery (R=-0.103, P=0.321).

Perioperative vWF-Ag Levels are Associated with Poor Postoperative Outcome

Further, we aimed to evaluate the association of perioperative vWF-Ag levels and postoperative clinical outcome after LR. Patients suffering from postoperative LD were found to have significantly higher preoperative vWF-Ag values than patients that were not affected by postoperative LD (vWF-Ag median: no LD=158.7%, LD=194.3%, P=0.009). This elevation in vWF-Ag levels could also be observed on POD1 (median no LD=268.0%, median LD=325.0%; P=0.035; Fig.1C) and on POD5 (median no LD=304.3%, median LD=420.0%; P=0.002; Fig.1C).

As postoperative LD is the main determinant for postoperative morbidity, we further compared perioperative vWF-Ag dynamics in patients with and without postoperative morbidity. Similar to LD, vWF-Ag levels were significantly increased in patients suffering from postoperative morbidity (PRE OP: median no morbidity=146.0%, median morbidity=171.6%, P=0.002; POD1: median no morbidity=240.5%, median

morbidity=317.0%, P=0.001; POD5: median no morbidity=274.2%, median morbidity=395.0%, P=0.001; Fig.1D).

VWF-Ag Increases Immediately after Induction of Liver Regeneration

As vWF-Ag has been suggested to be involved in the process of liver regeneration and as we had observed a striking association with clinical outcome in our exploration cohort, we subsequently aimed to generate a more detailed picture of perioperative vWF-Ag dynamics and its correlation to platelets. Using scanning electron microscopy, we were able to document a striking accumulation of blood cells, predominantly platelets, immediately after induction of liver regeneration (representative example is given in Fig.2A). Furthermore, in our subset of 30 patients, vWF-Ag increased immediately after induction of liver regeneration (i.e. 2h after portal vein ligation) in the liver vein (median PRE OP=138.5%, median liver vein=155.0%; P=0.014, Fig.2B). This increase was accompanied by a significant decrease of circulating platelets in the liver vein (median PRE OP=256x10^3/µl, median liver vein=150x10^3/µl; P=0.001, Fig.2B).

Immediate vWF-Ag Increase is Associated with Platelet Accumulation and Postoperative Liver Dysfunction

Subsequently, we evaluated whether immediate perioperative vWF-Ag dynamics were associated with postoperative liver function recovery. As for the entire cohort, we observed a trend towards elevated preoperative vWF-Ag levels in patients suffering from postoperative LD (median no LD=112.0%, median LD=161.0%; P=0.051, Fig.2C). Intriguingly, this difference was vanished in the liver vein after induction of liver regeneration (median no LD=152.0%, median LD=160.0%; P=0.501, Fig.2C). Of note,

vWF-Ag levels increased significantly after induction of liver regeneration only in patients without postoperative LD (median PRE OP=112.0%, median liver vein=152.0%; P=0.005, Fig.2C). Indeed, when calculating the fold induction of vWF-Ag, the association of the vWF-Ag increase with postoperative liver function recovery was even more evident (median no LD=1.3, median LD=1.0; P=0.043, Fig.2D). Interestingly, the incidence of postoperative LD was significantly elevated in patients without substantial induction of vWF-Ag (cut-off at 1.09-fold acc. Youden index; 0 of 12 [0.0%] in no increase group vs. 8 of 13 [61.5%] in increase group; P=0.002, Fig.2E).

In line with experimental reports suggesting vWF to be crucially involved in platelet accumulation after induction of liver regeneration, we observed that the absolute vWF-Ag level in the liver vein after induction of liver regeneration correlated with the reduction of platelet counts caused by accumulation within the liver (R=-0.580; P=0.030). Furthermore, we observed that patients with an evident reduction of platelets in the liver vein as compared to systemic preoperative levels (cut-off at 80%) had a significant increase in vWF-Ag after induction of liver regeneration, while patients without mentionable platelet accumulation tended to decrease in their vWF-Ag levels (median decrease=1.2, median no decrease=0.9; P=0.042, Fig.2F).

Levels of vWF-Ag are closely related to its functional capacity in the early phase of liver regeneration

As the amount of vWF-Ag does not necessarily correlate with its function, we aimed to characterize the functional capacity of this protein during the early phase of liver regeneration within our intraoperative cohort. As for vWF-Ag, we observed a rapid increase in vWF:RiCo, as a parameter of vWF-Ag function two hours after induction of liver regeneration (median PRE OP=132.0%, median liver vein=164.5%, P<0.001;

Fig.3A), and an additional increase on POD1 (median liver vein=164.5%, median POD1=291.0%, P<0.001; Fig.3A). We did not observe any significant differences in vWF:RiCo between patients with and without LD prior to the surgery or in the liver vein. However, on POD1, patients that developed postoperative LD were found to have significantly elevated vWF:RiCo values (median no LD=241.0%, median LD=395.5%, P=0.001; Fig.3A).

Within our intraoperative cohort we also observed a strong and highly significant correlation of levels of vWF-Ag and its function estimated by vWF:RiCo at each time point (PRE OP: R=0.823, P<0.001; LV: R=0.704, P<0.001; POD1: R=0.691, P=0.006; Fig.3B). We further calculated the ratio of vWF:RiCo to vWF-Ag (Fig.3C). Regarding the perioperative dynamic, we observed a rapid increase already during early liver regeneration in the liver vein (median PRE OP=0.87, median liver vein=1.01, P=0.004; Fig.3C), with a subsequent decrease on POD1 (median liver vein=1.01, median POD1=0.94, P=0.036; Fig.3C). Interestingly, patients with postoperative LD tended to have lower values prior to surgery (median no LD=1.02, median LD=0.77, P=0.072; Fig.3C) and during early liver regeneration in the liver vein (median no LD=1.06, median LD=0.96, P=0.086; Fig.3C).

Ultimately, we assessed ADAMTS13-activity, in this sub-group of patients, and observed similar dynamics for patients with and without LD. Perioperative dynamics showed a significant decrease on POD1 only, with no apparent differences in patients with and without LD (no LD: POD1=62.19%, PRE OP=88.59%, P=0.001; liver vein=95.50%, P=0.001; LD: POD1=58.62%, PRE OP=65.43%, P=0.028; liver vein=79.00%, P=0.0028; Fig.3D).

VWF^{high} Patients Suffer from an Increased Incidence of Postoperative LD and Poor Clinical Performance

We further aimed to quantify the potential of pre- and postoperative vWF-Ag levels to predict postoperative clinical outcome. Therefore, a ROC curve analysis was performed, revealing a significant association of preoperative vWF-Ag levels and postoperative LD with an area under the curve (AUC) of 0.725 (P=0.009, Fig.4A). Subsequently, a cut-off level of vWF-Ag≥182% was chosen to precisely identify patients with postoperative LD with a specificity of 78% and a sensitivity of 69%. To evaluate whether elevated preoperative vWF-Ag values would further translate into poor clinical performance, the incidence of postoperative LD and morbidity was compared between the risk groups. Accordingly, patients exceeding our cut-off level of vWF-Ag≥182% were found to suffer more frequently from postoperative LD (vWF-Ag<182%=5.9%, vWF-Ag≥182%=33.3%, P=0.001; Fig.4A) and morbidity (vWF-Ag<182%=44.1%, vWF-Ag ≥182%=74.1%, P=0.008; Fig.4A).

As patients undergoing major resection are at higher risk to develop postoperative LD, we further performed a subgroup analysis of these patients. Accordingly, we observed a comparable predictive potential with an AUC of 0.7 (P=0.018) and patients exceeding our cut-off were found to suffer from an increased incidence of postoperative LD and morbidity (LD: vWF-Ag<182%=8.8%, vWF-Ag≥182%=50.0%, P=0.002; morbidity: vWF-Ag<182%=58.8%, vWF-Ag≥182%=83.3%, P=0.067).

Additionally, we assessed the predictive potential of postoperative vWF-Ag levels for clinical outcome. A ROC curve analysis, based on vWF-Ag levels measured on POD1, was performed revealing a significant association of postoperative vWF-Ag levels and postoperative LD with an AUC of 0.683 (P=0.035; Fig.4B). Again, the ROC analysis was used to define a suitable cut-off level, which could be identified at vWF-Ag≥315%. In

patients who exceeded the postoperative cut-off level, incidences of LD and morbidity were significantly increased (LD: vWF-Ag<315%=8.3%, vWF-Ag≥315%=25.0%, P=0.029; morbidity: vWF-Ag<315%=40.0%, vWF-Ag≥315%=81.3%, P=0.001), as illustrated in Fig.4B.

Prospective Validation of Preoperative vWF-Ag Levels to Predict Poor Postoperative Performance

Given the clinical relevance to determine patients' risk for adverse postoperative events prior to surgery, we further focused on preoperative vWF-Ag levels to predict clinical outcome after LR. In particular, we aimed to validate our results in a prospective independent validation cohort, in a multicenter/multinational setting (for details of these 133 patients see Table 1). Importantly, we were able to verify, that patients exceeding the preoperative cut-off level of vWF-Ag≥182% suffered from a significantly increased incidence of postoperative LD (vWF-Ag<182%=5.2%, vWF-Ag≥182%=19.6%, P=0.009) and morbidity (vWF-Ag<182%=35.1%, vWF-Ag≥182%=55.4%, P=0.020) which is illustrated in Fig.4C.

We also aimed to validate the results of our subgroup analysis of patients undergoing major LR. Accordingly, we observed an even better predictive potential with an AUC of 0.8 (P=0.005) and patients exceeding our cut-off were found to suffer from an increased incidence of postoperative LD and morbidity (LD: vWF-Ag<182%=7.0%, vWF-Ag≥182%=33.3%, P=0.005; morbidity: vWF-Ag<182%=46.5%, vWF-Ag≥182%=70.0%, P=0.039).

Patients with Elevated Preoperative Levels of vWF-Ag Demonstrate an Increased Incidence of Poor Postoperative Performance, ICU-Stay and Hospitalization: Entire Cohort

To further increase the power of the analysis, we combined the exploration and validation set for additional analyses (N=228, Table 1), demonstrating significantly higher incidences of postoperative LD and morbidity in patients with preoperatively elevated vWF-Ag levels (LD: vWF-Ag<182%=5.5%, vWF-Ag≥182%=24.1%, odds ratio (OR)=5.437, 95%-confidence interval (CI)=2.27-13.01, P=0.001, Fig.5A; morbidity: vWF-Ag<182%=39.3%, vWF-Ag≥182%=61.4%, OR=2.461, 95%-CI=1.42-4.28, P=0.001, Fig.5B). In addition, patients with elevated preoperative vWF-Ag levels were also found to suffer from a prolonged intensive-care-unit (ICU) and hospital stay (hospitalization>10 days: vWF-Ag<182%=31.3%, vWF-Ag≥182%=47.5%, OR=1.99, 95%-CI=1.34-3.49, P=0.016, Fig.5C; ICU stay>2 days: vWF-Ag<182%=9.0%, vWF-Ag≥182%=26.0%, OR=3.538; 95%-CI=1.62-7.73, P=0.001, Fig.5D). Importantly, applying our cut-off, we observed a significantly higher incidence of postoperative mortality in patients with high preoperative vWF-Ag (vWF-Ag<182%=2.1%, vWF-Ag≥182%=7.5%, OR=3.838; 95%-CI=0.93-15.78, P=0.047, Fig.5E).

vWF-Ag Remains an Independent Marker for Postoperative LD and Morbidity upon Multivariable Analysis

As we had observed a significant association of postoperative LD and vWF-Ag prior to LR, we aimed to explore whether vWF-Ag could independently predict postoperative LD. Therefore, MVA was performed on the entire cohort of 228 patients (evaluation and validation cohort). After step-wise forward selection only vWF-Ag, perioperative CTx, extent of resection and INR remained significant. The results from the final model fit are shown in Table 2A.

Similarly, we performed MVA for predictors of postoperative morbidity. After step-wise forward selection vWF-Ag, suffering from CCC and extent of resection remained significant. The results from the final model fit are shown in Table 2B.

Discussion

To date the process of liver regeneration remains poorly understood. Nevertheless, platelets could be shown to play a central role during liver regeneration, most likely via releasing their cargo to promote cell cycle progression and proliferation [16, 17]. Recently, Kirschbaum et al. demonstrated that platelet accumulation within the liver to promote liver regeneration is specifically relevant in the very early phase of liver regeneration after partial hepatectomy in mice [6]. Within this investigation, we report on the first in human data on vWF-dependent platelet accumulation during liver regeneration. Furthermore, we demonstrate that an adequate increase of vWF-Ag after LR seems to be required to allow for sufficient postoperative hepatic regeneration. Additionally, we demonstrate that preoperative vWF-Ag is a valuable predictor for postoperative outcome in patients undergoing LR and validate our results using an independent prospective cohort from 4 different institutions, documenting the reproducibility and clinical relevance of our findings.

Kirschbaum et al. were able to provide solid evidence for a critical relevance of vWF for platelet adhesion within the regenerating liver[6]. Indeed, both blocking vWF via an antibody and knock-out of vWF lead to reduced liver regeneration in affected mice. In line with these findings, we could now document a significant increase of vWF-Ag within the liver vein already two hours after induction of liver regeneration. Moreover, we were able to validate our previous reports on platelet accumulation during liver regeneration in humans using scanning electron microscopy. This association was further displayed by a significant decrease of platelets in blood taken from the liver vein, compared to preoperative systemic platelet levels. Indeed, the extent of platelet decrease could also be shown to be vWF-dependent. Only patients with a significant increase of vWF-Ag demonstrated a mentionable decrease in platelets after passing the

regenerating liver. Furthermore, we observed that this adequate increase of vWF-Ag after LR seemed to be required to allow for sufficient postoperative liver regeneration. In particular, patients that were not able to significantly increase vWF-Ag after ligation of the portal vein were shown to more likely suffer from postoperative LD. Of note, the regenerative vWF-Ag burst was specifically abolished in patients with preoperatively increased vWF-Ag levels, which possibly reflects a state of liver sinusoidal endothelial cell (LSEC) exhaustion. Indeed, the rapid increase of vWF-Ag levels likely reflects its release from a preformed storage pool. As activated LSECs are capable of releasing vWF-Ag from their Weibel-Palade bodies, they might represent a likely source for the increase of circulating vWF-Ag levels after LR. Of note, both in blood samples collected prior to the operation and from the liver vein of the regenerating lobe, a significant correlation of vWF-Ag with E-selectin, a specific marker for endothelial cell activation, could be observed (Supplemental Fig.4). This suggests that the regenerative vWF-Ag burst might predominantly be a result of endothelial cell activation. Indeed, LR was shown to cause a series of pathophysiological changes in the liver sinusoids, including augmented blood flow, increase in portal venous pressure and subsequently LSEC perturbation[15]. Interestingly, the release of vWF from endothelial cells was shown to be associated to augmented shear stress as induced by an increase of portal venous pressure[18]. Further, vWF has been proposed as a marker for disease progression in chronic liver disease and to be elevated in patients suffering from portal hypertension[8, 13]. On the other hand, chronic portal hypertension was shown to lead to endothelial cell dysfunction[19] and hypoactive state of LSECs[20]. With respect to the current literature, this lead us to hypothesize that patients presenting with high preoperative levels of vWF-Ag might suffer from underlying liver disease and/or sub-clinical portal hypertension. This hypothesis is further supported by a highly significant correlation between circulating

levels of vWF-Ag and soluble CD163, another non-invasive marker for portal hypertension, both prior to the operation (Supplemental Fig.6A) and intraoperatively (Supplemental Fig.5B). Importantly, soluble CD163 has been proposed as a marker of Kupffer cell activation, which is known to additionally contribute to portal hypertension[21], and can thus be considered pathomechanistically independent of elevated vWF-Ag levels. Interestingly, we found no significant association between levels of soluble CD163 and development of postoperative LD (Supplemental Fig.5C), which might indicate that the predictive potential of vWF-Ag for postoperative LD and complications is not exclusively due to its association to portal hypertension, but more likely related to the effects of vWF-dynamics on platelet accumulation during the early phase of liver regeneration. While sub-clinical portal hypertension might lead to perpetual activation of LSECs and concomitantly to a continuous release of vWF-Ag culminating in significantly elevated levels of vWF-Ag prior to the operation, these patients seem to be unable to respond with an adequate vWF-Ag burst after LR, leading to diminished platelet accumulation and subsequently to reduced delivery of platelet granule stored molecules required for an adequate induction of liver regeneration (for summary see Fig.6). Indeed, this is also nicely illustrated by our observation that circulating vWF-Ag levels were less predictive for postoperative outcome on POD1 when compared to preoperative values. As patients with normal basal vWF-Ag levels seem to be capable to respond to a certain stressor (such as liver resection) with an adequate vWF-Ag release, they align with levels of patients that display elevated basal vWF-Ag levels after LR. Intriguingly, vasopressin has been shown to stimulate vWF release and our study highlights that the clinical use of similar substances might be an attractive tool to promote liver regeneration. Indeed, this seems of specific interest as Fahrner et al. demonstrated that terlipressin (the long-acting analog of vasopressin) improved liver

regeneration in mice, potentially also by the increase of circulating vWF [15] and a clinical trial evaluating the relevance of this finding in humans is currently ongoing (ClinicalTrials.gov Identifier: NCT01921985).

Of note, the measured amount of vWF-Ag highly significantly correlated with its activity assessed via vWF:RiCo. This specific test examines the capacity of vWF to bind to glycoprotein Ib, a central member of the vWF-receptor on the surface of platelets, and hence allows to quantify the function of vWF-Ag. Indeed, we observed an immediate increase of vWF:RiCo two hours after induction of liver regeneration and a subsequent increase up to POD1, parallel to vWF-Ag and a highly significant correlation of vWF-Ag and its function activity (vWF:RiCo). Interestingly, when normalizing vWF:RiCo to its absolute amount (vWF:RiCo/vWF-Ag) at the respective time points, we observed a mentionable trend towards decreased values in patients that ultimately developed LD. This finding is in line with previous reports on compensatory upregulation of vWF-Ag due to a functional loss in patients with diminished liver reserve[22] and further supports our proposed hypothesis on the disruption of vWF-dependent platelet accumulation within the regenerating liver in patients that will develop LD in the postoperative time course. Regarding the time course of normalized vWF-activity (vWF:RiCo/vWF-Ag), we found a significant increase during early liver regeneration, followed by a decrease on POD1, suggesting that a functional improvement of vWF-Ag takes place during this period of liver regeneration. Interestingly, this transient increase in vWF-activity per molecule supports the hypothesis that the increase in vWF-Ag in the liver vein two hours after ligation of the contralateral portal venous branch indeed arises from newly released compartments, as uncleaved high molecular weight vWF-Ag multimers are known to have a higher activity[23]. Additionally, we did not observe any pre- or intraoperative changes or differences in ADAMTS13-activity, as the main cleavage

protease of vWF, between patients with and without LD, which suggests a minor role of ADAMTS13 during the early phase of liver regeneration.

In the clinical routine, not only promotion of postoperative liver regeneration, but also preoperative risk stratification of patients undergoing LR to avoid postoperative morbidity and mortality represents an issue of critical relevance. While several invasive and non-invasive tests have been developed, only very few have found their way into routine clinical application. Major drawbacks of available predictors are availability, high costs and invasiveness[24]. HVPG, the gold standard to evaluate portal hypertension, has been shown to be of value to predict postoperative clinical outcome in high-risk patients such as patients suffering from HCC[25-27]. Despite several promising exploratory analyses, no non-invasive, easily assessable marker to predict postoperative outcome prior to LR has been identified so far. While the association of vWF-Ag levels and advanced liver disease, and portal hypertension has been established previously[8], its predictive impact in patients undergoing LR has not been evaluated in a large prospective clinical trial. Of note, vWF-Ag is easily assessable in patients' blood and represents a low-cost routine blood parameter (about 6$/analysis) available in most clinical standard laboratories. Accordingly, our results show that preoperative vWF-Ag levels predict postoperative outcome in patients undergoing LR in a routine setting and are of major clinical relevance and applicability. Interestingly, we observed a close relation to markers of liver function and damage, and an association with preexisting liver cirrhosis. However, we were able to show an independency of vWF-Ag for the prediction of both LD and morbidity upon MVA. Importantly, the validation of our results in a multicenter international design, including several standard laboratories and clinical situations further demonstrates the clinical utility of this parameter and suggests that it may be applied in a clinical daily life setting. Using a vWF-Ag cut-off of 182% we

were able to define patients at risk of postoperative complications. Accordingly, vWF-Ag evaluation might help to tailor strategy and extent of surgical intervention to each individual patient and consider a less aggressive surgical approach or alternative treatment options.

To our knowledge, this is the first report that provides evidence for the relevance of vWF during the process of human liver regeneration. We observed an immediate accumulation of platelets after induction of liver regeneration, which was associated with vWF-Ag levels. Importantly, we provided evidence that the immediate vWF burst after induction of liver regeneration has a direct effect on platelet accumulation and postoperative liver function recovery. Ultimately, we documented the predictive potential of vWF-Ag levels for clinical outcome after LR based on a large prospective clinical trial. Applying a cut-off at vWF-Ag≥182%, we were able to demonstrate that elevated preoperative vWF-Ag levels were vital to identify high-risk patients that suffered from an increased incidence of poor postoperative outcome. As vWF-Ag is a non-invasive, cost effective and easily assessable parameter, readily available in most routine laboratories, it represents an attractive clinical marker for distinguishing high and low risk patients prior to LR.

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Figure Legends

Fig.1 Perioperative vWF-Ag Time Course

Von Willebrand Factor (vWF-Ag) was measured prior to surgery (PRE OP), on the first (POD1) and fifth (POD5) postoperative day. The perioperative time course of vWF-Ag (A) is illustrated and shown separately according to the extent of resection (B), liver dysfunction (LD) (C) and postoperative morbidity (D). ISGLS = international study group for liver surgery, *P<0.05, **P<0.005

Fig.2 Dynamics of vWF-Ag and Platelets During Early Liver Regeneration

Platelet (PLT) accumulation within the liver sinusoids was assessed prior to portal vein ligation and from the regenerating liver lobe at the end of liver surgery (A). **Von** Willebrand Factor (WF-Ag) and platelet count were assessed in the liver vein (LV) of the regenerating liver lobe and compared to preoperative levels (PRE OP) (B) VWF-Ag was compared between patients with and without liver dysfunction (LD) (C). Same was done for calculated fold increase of vWF-Ag (D). Incidence of LD is shown for patients with or without significant increase of vWF-Ag after induction of liver regeneration (E) and fold change of vWF-Ag was compared between patients that decreased in their platelet count and patients that did not show a reduction in platelets (F). *P<0.05, **P<0.005

Fig.3 Functional Aspects of vWF-Ag In the Early Phase of Liver Regeneration

Dynamics of ristocetin cofactor (vWF:RiCo) were assessed peri- and intraoperatively (A). Further, vWF:RiCo was compared between patients with and without liver dysfunction (LD) (A). We observed a strong correlation of von Willebrand Factor (vWF-

Ag) and vWF:RiCo (B). VWF:RiCo to vWF-Ag was calculated is shown in the time course and was compared between patients with and without LD (C). Ultimately, also the activity of a disintegrin and metalloproteinase with a thrombospondin type 1 motif member 13 (ADAMTS13) was evaluated and perioperative dynamics are shown for all patients, and separately for patients with and without liver dysfunction (D). PRE OP = preoperative, LV = liver vein, POD = postoperative day, *P<0.05, **P<0.005

Fig.4 VWF-Ag and Prediction of Postoperative Outcome: Exploration-Cohort

A illustrates preoperative receiver operating characteristic (ROC)-curve analysis of von Willebrand Factor (vWF-Ag) to predict postoperative liver dysfunction (LD) and shows the incidence of postoperative LD and of postoperative morbidity according to the defined cut-off value for preoperative (182%) vWF-Ag. B illustrates postoperative ROC-curve analysis of vWF-Ag to predict postoperative liver dysfunction (LD) and shows the incidence of postoperative LD and of postoperative morbidity according to the defined cut-off value for postoperative vWF-Ag (315%). C illustrates the validation of the predictive potential of the preoperative cut-off of 182% vWF-Ag in an independent validation cohort. AUC = area under the curve, ISGLS = international study group for liver surgery, *P<0.05, **P<0.005

Fig.5 Preoperative vWF-Ag and Prediction of Poor Postoperative Performance, ICU-Stay and Hospitalization: Entire Cohort

The incidence of postoperative liver dysfunction (LD) (A), postoperative morbidity (B), prolonged intensive care unit (ICU) (C) and hospital stay (D), and 90 days mortality (D), are shown according to the defined cut-off value for preoperative von Willebrand Factor

(vWF-Ag) (182%). ISGLS = international study group for liver surgery, POD = postoperative day,*P<0.05, **P<0.005

Fig.6 Summary of Proposed Mechanism of Action of vWF during the Early Phase of Liver Regeneration.

LR = liver regeneration, OP = operation, LD=liver dysfunction, PVP = portal venous pressure, PL = platelets, WB = Weibel-Palade bodies, vWF = von Willebrand Factor.

Tables

Table 1. Patient Demographics

Parameter	Collective(N=228) N(%), median (range)	Exploration–Set(N=95) N(%), median (range)	Validation–Set(N=133) N(%), median (range)
Age (years)	61.4(22.0–89.0)	64.4(22.0–89.0)	60.4(24.0–84.0)
Sex			
Male	150(65.8)	65(68.4)	85(63.9)
Female	78(34.2)	30(31.6)	48(36.1)
Neoplastic entity			
mCRC	101(44.3)	53(55.8)	48(36.1)
HCC	52(22.8)	25(26.3)	27(20.3)
CCC	44(19.3)	17(17.9)	27(20.3)
non-neoplastic	10(4.4)	0(0.0)	10(7.5)
others	21(9.2)	0(0.0)	21(15.8)
Preoperative CTx			
yes	97(42.5)	54(56.8)	43(32.3)
Hepatic resection			
major	125(54.8)	52(54.7)	73(54.9)
minor	103(45.2)	43(45.3)	60(45.1)
Postoperative LD			
yes	28(12.3)	13(13.7)	15(11.3)
Morbidity			
yes	108(47.4)	50(52.6)	58(43.6)
90 days mortality			
yes	9(3.9)	2(2.1)	7(5.3)
Preoperative parameters			
vWF-Ag %	163(51-420)	160(51–420)	167(58–420)
SB mg/dl	0.6(0.1–6.9)	0.6(0.2–6.6)	0.6(0.1–6.9)
INR	1.1(0.0–2.2)	0.9(0.7–1.9)	1.1(0.0–2.2)
ALP U/l	89.0(35.0–741.0)	87.5(43.0–423.0)	90.0(35.0–741.0)
GGT U/l	62.5(8.0–2324.0)	62.0(11.0–710.0)	66.0(8.0–2324.0)
AST U/l	30.0(14.0–1035.0)	31.0(17.0–208.0)	29.5(14.0–1035.0)
ALT U/l	30(6–1122)	30(7–196)	30(6–1122)
Albumin g/l	41.9(1.7–54.0)	41.9(30.2–49.6)	42.0(1.7–54.0)
Platelets $x10^3/\mu l$	223.5(66.0–582.0)	221.5(86.0–492.0)	225.5(66.0–582.0)
MELD Score	7(6–15)	7(6–15)	7(6-12)
ICU–days	1(0–39)	1(0–25)	1(0–39)

ALT=alanine aminotransferase, ALP=alkaline phosphatase, AST=aspartate aminotransferase, CCC=cholangiocellular carcinoma, CTx=chemotherapy, GGT=gamma-glutamyl-transferase, HCC=hepatocellular carcinoma, INR=internationalized normalized ratio, LD=liver dysfunction, mCRC=metastatic colorectal cancer, MELD=model of end-stage liver disease, SB=serum bilirubin.

Table 2. Predictors of Postoperative Liver Dysfunction and Morbidity

	univariate logistic regression			multivariable logistic regression		
LD	**OR**	**95 % CI**	**P-value**	**OR**	**95 % CI**	**P-value**
vWF-Ag %	**1.007**	**1.002-1.012**	**0.004**	**1.009**	**1.003-1.014**	**0.003**
Gender	1.112	0.478-2.589	0.81			
Age	1.283	0.408-4.027	0.67			
Neoplastic entity		.				
mCRC	0.299	0.116-0.768	**0.01**			
HCC	2.981	1.307-6.802	**0.009**			
CCC	1.811	0.739-4.436	0.19			
benign	n.a.	n.a.	n.a.			
Others	0.774	0.170-3.528	0.74			
Preoperative CTx	0.255	0.093-0.698	**0.008**	0.244	0.080-0.740	0.01
Extend resection	8.333	2.438-28.488	**0.001**	10.177	2.747-37.703	0.001
Preoperative paramters						
SB mg/dl	0.999	0.589-1.696	0.10			
INR	8.531	1.464-49.712	**0.02**	8.156	1.056-62.996	0.04
ALP U/l	1.003	1.000-1.007	**0.02**			
GGT U/l	1.001	1.000-1.002	0.06			
AST U/l	1.000	0.995-1.005	0.95			
ALT U/l	1.000	0.995-1.004	0.92			
Albumin g/l	0.928	0.879-0.980	**0.007**			
Platelets ($x10^3/\mu l$)	0.997	0.991-1.002	0.24			
MELD Score	1.315	1.059-1.634	**0.013**			
Liver pathoistology						
SOS	0.508	0.059-4.350	0.537			
Steatohepatitis	1.071	0.285-4.029	0.919			
Steatosis	2.129	0.600-7.550	0.242			
Degree of Fibrosis	3.160	0.993-10.057	0.051			
RBC transfusion	1.109	0.356-3.453	0.858			
Anti-platelet drugs	1.311	0.246-6.981	0.751			
Morbidity						
vWF-Ag %	**1.006**	**1.002-1.010**	**0.003**	**1.007**	**1.003-1.011**	**0.001**
Gender	1.260	0.727-2.184	0.41			
Age	1.452	0.647-3.257	0.37			
Neoplastic entity						
mCRC	0.577	0.340-0.982	**0.04**			
HCC	1.282	0.690-2.384	0.43			
CCC	5.995	2.720-13.214	**<0.001**	5.958	2.565-13.837	**<0.001**
benign	0.116	0.014-0.934	**0.04**			
Others	0.252	0.082-0.781	**0.02**			
Preoperative CTx	0.521	0.305-0.890	**0.02**			
Extend resection	3.441	1.984-5.968	**<0.001**	3.359	1.848-6.104	**<0.001**
Preoperative paramters						
SB mg/dl	1.470	0.954-2.266	0.08			
INR	2.418	0.630-9.278	0.20			
ALP U/l	1.004	1.001-1.007	**0.01**			
GGT U/l	1.001	1.000-1.002	0.09			
AST U/l	1.001	0.998-1.004	0.45			
ALT U/l	1.001	0.998-1.005	0.44			
Albumin g/l	0.935	0.876-0.997	**0.04**			
Platelets ($x10^3/\mu l$)	0.999	0.995-1.002	0.55			
MELD Score	1.278	1.060-1.541	**0.01**			
Liver pathohistology						
SOS	0.292	0.074-1.150	0.078			
Steatohepatitis	0.810	0.321-2.043	0.655			
Steatosis	1.083	0.477-2.459	0.849			
Degree of Fibrosis	1.638	0739-3.631	0.224			
RBC transfusion	0.978	0.453-2.113	0.956			
Anti-platelet drugs	1.801	0.483-6.717	0.381			

ALT=alanine aminotransferase, ALP=alkaline phosphatase, AST=aspartate aminotransferase, CCC=cholangiocellular carcinoma, CTx=chemotherapy, GGT=gamma-glutamyltransferase, HCC=hepatocellular carcinoma, INR=international normalized ratio, mCRC=metastatic colorectal cancer, n.a.=not assessable, SB=serum bilirubin, SOS=sinusoidal obstruction syndrome, RBC=red blood cells.

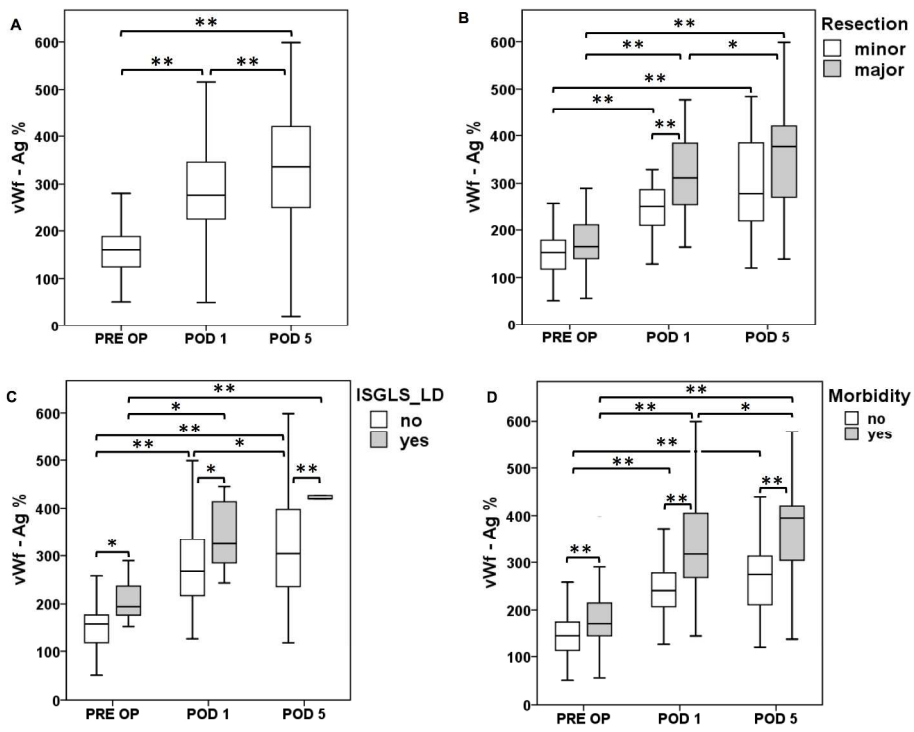

Fig.1 Perioperative vWF-Ag Time Course
Von Willebrand Factor (vWF-Ag) was measured prior to surgery (PRE OP), on the first (POD1) and fifth (POD5) postoperative day. The perioperative time course of vWF-Ag (A) is illustrated and shown separately according to the extent of resection (B), liver dysfunction (LD) (C) and postoperative morbidity (D). ISGLS = international study group for liver surgery, *P<0.05, **P<0.005

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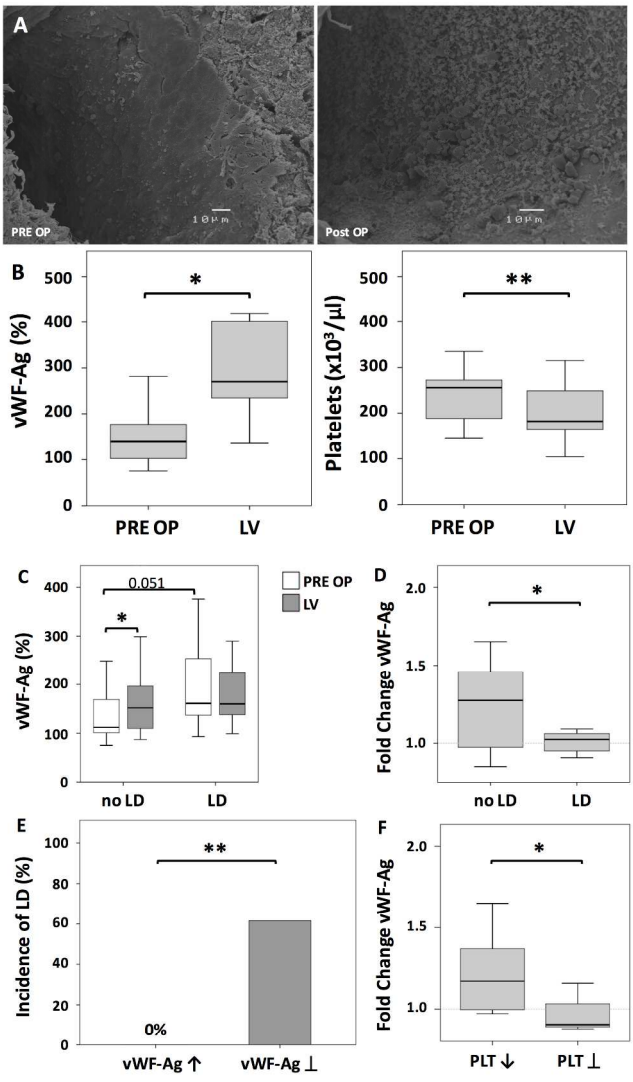

Fig.2 Dynamics of vWF-Ag and Platelets During Early Liver Regeneration
Platelet (PLT) accumulation within the liver sinusoids was assessed prior to portal vein ligation and from the regenerating liver lobe at the end of liver surgery (A). Von Willebrand Factor (WF-Ag) and platelet count were assessed in the liver vein (LV) of the regenerating liver lobe and compared to preoperative levels (PRE OP) (B) VWF-Ag was compared between patients with and without liver dysfunction (LD) (C). Same was done for calculated fold increase of vWF-Ag (D). Incidence of LD is shown for patients with or without significant increase of vWF-Ag after induction of liver regeneration (E) and fold change of vWF-Ag was compared between patients that decreased in their platelet count and patients that did not show a reduction in platelets (F). *P<0.05, **P<0.005

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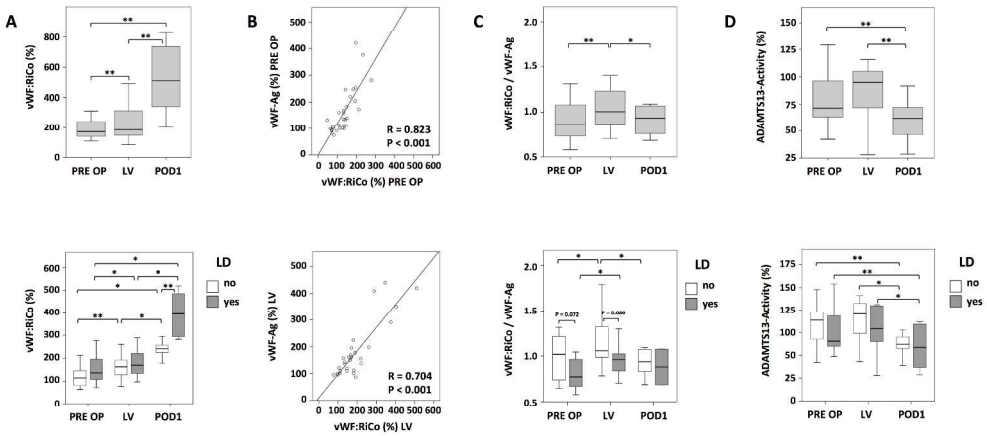

Fig.3 Functional Aspects of vWF-Ag In the Early Phase of Liver Regeneration
Dynamics of ristocetin cofactor (vWF:RiCo) were assessed peri- and intraoperatively (A). Further, vWF:RiCo was compared between patients with and without liver dysfunction (LD) (A). We observed a strong correlation of von Willebrand Factor (vWF-Ag) and vWF:RiCo (B). VWF:RiCo to vWF-Ag was calculated is shown in the time course and was compared between patients with and without LD (C). Ultimately, also the activity of a disintegrin and metalloproteinase with a thrombospondin type 1 motif member 13 (ADAMTS13) was evaluated and perioperative dynamics are shown for all patients, and separately for patients with and without liver dysfunction (D). PRE OP = preoperative, LV = liver vein, POD = postoperative day, *P<0.05, **P<0.005

338x190mm (300 x 300 DPI)

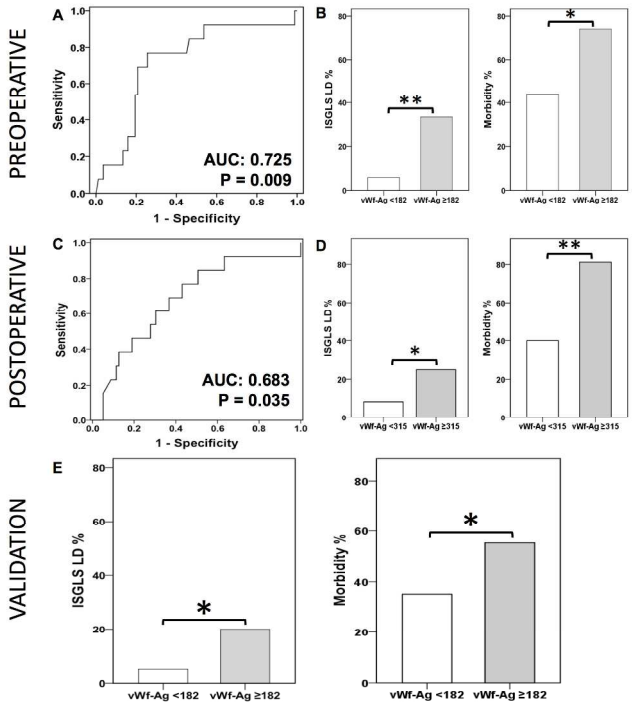

Fig.4 VWF-Ag and Prediction of Postoperative Outcome: Exploration-Cohort
A illustrates preoperative receiver operating characteristic (ROC)-curve analysis of von Willebrand Factor (vWF-Ag) to predict postoperative liver dysfunction (LD) and shows the incidence of postoperative LD and of postoperative morbidity according to the defined cut-off value for preoperative (182%) vWF-Ag. B illustrates postoperative ROC-curve analysis of vWF-Ag to predict postoperative liver dysfunction (LD) and shows the incidence of postoperative LD and of postoperative morbidity according to the defined cut-off value for postoperative vWF-Ag (315%). C illustrates the validation of the predictive potential of the preoperative cut-off of 182% vWF-Ag in an independent validation cohort. AUC = area under the curve, ISGLS = international study group for liver surgery, *P<0.05, **P<0.005

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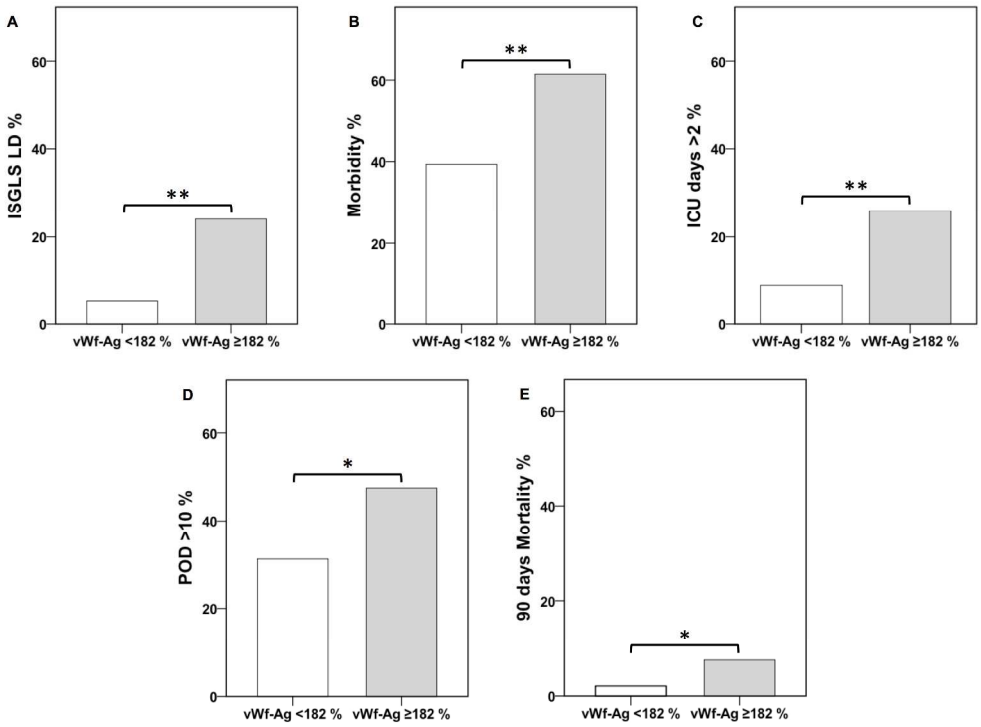

Fig.5 Preoperative vWF-Ag and Prediction of Poor Postoperative Performance, ICU-Stay and Hospitalization: Entire Cohort

The incidence of postoperative liver dysfunction (LD) (A), postoperative morbidity (B), prolonged intensive care unit (ICU) (C) and hospital stay (D), and 90 days mortality (D), are shown according to the defined cut-off value for preoperative von Willebrand Factor (vWF-Ag) (182%). ISGLS = international study group for liver surgery, POD = postoperative day,*P<0.05, **P<0.005

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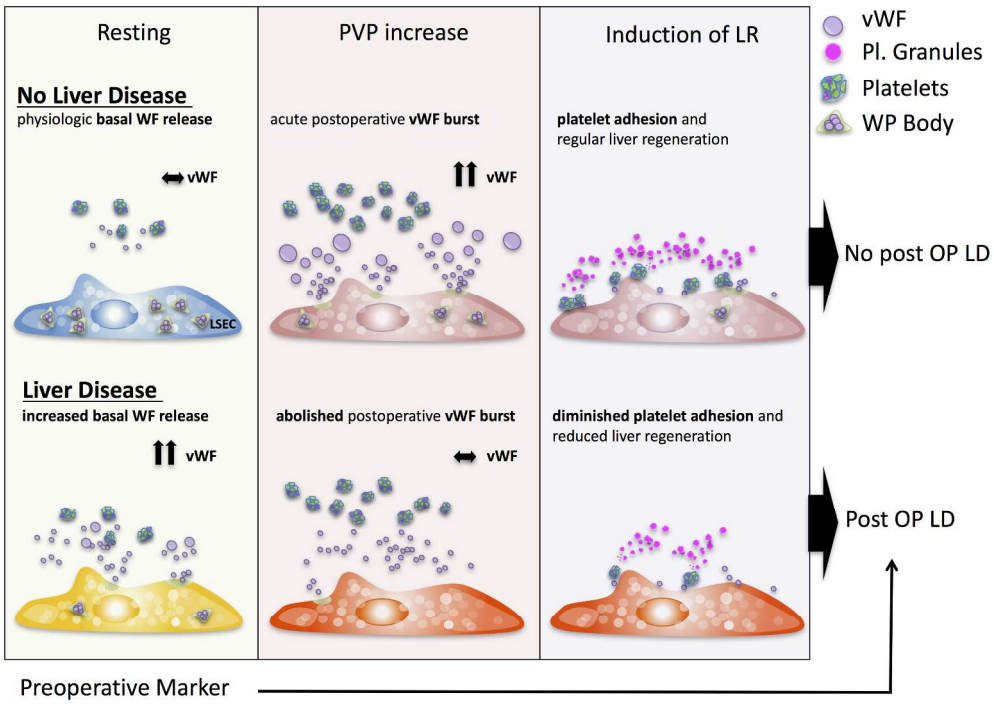

Fig.6 Summary of Proposed Mechanism of Action of vWF during the Early Phase of Liver Regeneration. LR = liver regeneration, OP = operation, LD=liver dysfunction, PVP = portal venous pressure, PL = platelets, WB = Weibel-Palade bodies, vWF = von Willebrand Factor.

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