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Ischaemia–reperfusion injury with Pringle's maneuver induces unusually large von Willebrand factor multimers after hepatectomy

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ABSTRACT

Introduction: von Willebrand factor (VWF) is synthesised in vascular endothelial cells and released into the plasma as unusually large VWF multimers (UL-VWFMs). Sinusoidal endothelial cells are a major target of ischaemia–reperfusion injury due to liver surgery. This study aimed to clarify the effect of hepatectomy on UL-VWFMs.

Materials and methods: Thirty-five patients who underwent hepatectomy were eligible for the study. Plasma ADAMTS13 activity and VWF antigen levels were measured by enzyme-linked immunosorbent assay and multimer analysis of plasma VWF was performed according to Ruggeri and Zimmerman's method. For analyses, patients were categorised according to UL-VWFM positivity after hepatectomy.

Results: Plasma ADAMTS13 activity significantly decreased from 61.0% (27.7%–126.2%) before operation to 37.4% (20.2%–71.4%) on postoperative day 7 ($p < 0.001$). Plasma VWF antigen levels significantly increased from 172.1% (80.5%–412.8%) before operation to 361.0% (154.7%–745.8%) on postoperative day 2, which remained high until postoperative day 7 ($p < 0.001$). Seven patients remained UL-VWFMs-negative and 22 patients became UL-VWFMs-positive after operation. Pringle's maneuver duration was significantly longer and blood loss volume was significantly higher in the UL-VWFMs-positive group ($p = 0.001$ and $p = 0.003$, respectively). By multivariable analysis, Pringle's maneuver duration [odds ratio 1.049, 95% confidence interval (CI) 1.001–1.098; $p = 0.043$] was significantly associated with increased UL-VWFMs level after hepatectomy. UL-VWFMs index was significantly correlated with Pringle's maneuver duration ($r = 0.444$, $p = 0.017$).

Conclusions: Plasma UL-VWFMs levels increased after hepatectomy due to ischaemia–reperfusion injury with Pringle's maneuver.

1. Introduction

von Willebrand factor (VWF) is exclusively synthesised in the vascular endothelial cells and is released into the plasma as unusually large VWF multimers (UL-VWFMs) by various stimuli, such as inflammation, exercise, infection and pregnancy [1]. Disintegrin and metalloproteinase with thrombospondin motifs-13 (ADAMTS13) is a metalloproteinase that is primarily produced in the hepatic stellate cells and specifically cleaves VWF-5 [2–5]. Because UL-VWFMs elicit strong platelet aggregation, an increase in UL-VWFMs leads to platelet clumping and/or thrombus formation, thereby resulting in microcirculatory disturbance [6,7].

Liver resection is performed as a curative treatment for patients with hepatocellular carcinoma (HCC). Recent developments in surgical techniques and perioperative care have dramatically improved postoperative outcomes in these patients. However, postoperative morbidity rates remain high (38.4%45%) [8–11]. Clamping of the portal pedicle, *i.e.* Pringle's maneuver, is used to reduce bleeding during liver transection [12,13]; however, the liver can suffer from ischaemia–reperfusion injury using this maneuver [14,15]. Hepatic

Abbreviations: ADAMTS13, a disintegrin and metalloproteinase with thrombospondin motifs-13; CI, confidence interval; CRLM, colorectal liver metastases; ELISA, enzyme-linked immunosorbent assay; OR, odds ratios; SDS, sodium dodecyl sulphate; FFP, fresh frozen plasma; HCC, hepatocellular carcinoma; UL-VWFMs, unusually large VWF multimers; VWF, von Willebrand factor

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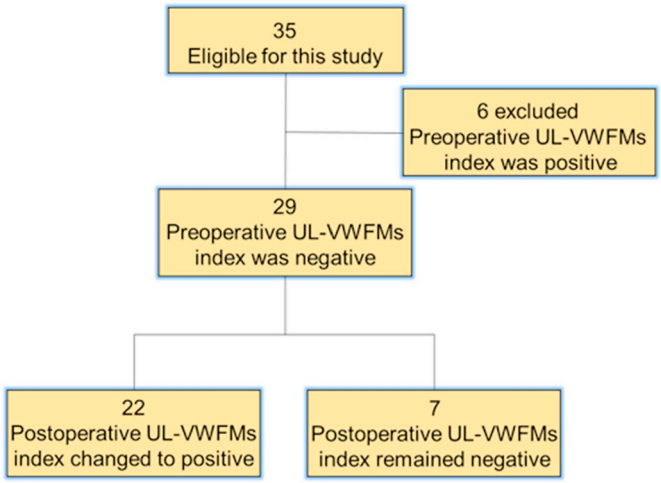

Fig. 1. Flow chart of patient categorisation.

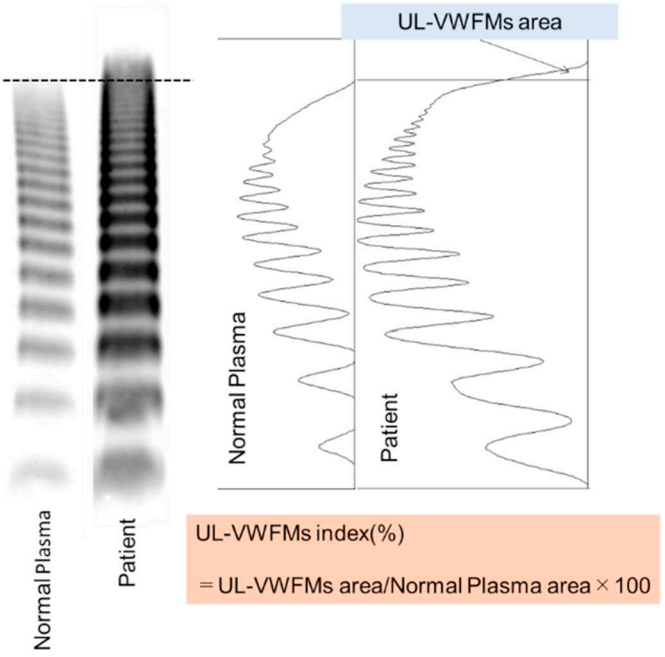

Fig. 2. Quantification method for unusually large von Willebrand factor (VWF) multimers using images of VWF multimer analysis by the NIH ImageJ software. UL-VWFMs index was calculated using the equation described in the figure.

ischaemia–reperfusion injury is a major cause of liver dysfunction and can lead to serious complications during liver surgery. One prominent feature of this injury is an excessive inflammatory response that is characterised by the release of inflammatory cytokines and chemokines that recruit circulating leukocytes, mainly neutrophils, into the ischaemic tissues [16]. This inflammation may affect the release of UL-VWFMs from the endothelial cell in addition to direct effect of ischaemia–reperfusion injury to the endothelial cells. However, the precise mechanism of liver injury due to ischaemia–reperfusion injury remains unclear.

Numerous studies indicate sinusoidal endothelial cells as a major target of ischaemia–reperfusion injury of the liver [17–19]. Stimulation of sinusoidal endothelial cells can lead to marked increases in UL-VWFMs, which is proposed to play a role in the mechanism underlying microcirculatory disturbances of the liver, such as thrombotic thrombocytopenic purpura. Although such a microcirculatory disturbance is proposed to cause liver damage due to ischaemia–reperfusion injury, the relationship of ischaemia–reperfusion injury due to Pringle's maneuver with VWF and ADAMTS13 and particularly the accumulation of UL-VWFMs after hepatectomy has not yet been investigated. The aim of this study is to clarify the effect of hepatectomy on UL-VWFMs levels.

2. Material and methods

2.1. Patients

This prospective cohort study included 35 patients who underwent curative hepatic resection between January 2015 and December 2017 at the Department of Surgery of Nara Medical University. Underlying diseases were primary HCC in 22 patients; colorectal liver metastases (CRLM) in 10 patients and gallbladder cancer, intrahepatic cholangiocarcinoma and ovarian liver metastases in three patients. As shown in Fig. 1, six patients had UL-VWFM before operation and were excluded from further analysis. This study was performed after the approval from the ethics committee of Nara Medical University and complied with the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from all patients.

2.2. Blood sampling

Blood samples were obtained for analysis at the following time points: the day before surgery, intraoperative period and on postoperative days 1, 2, 3, 5 and 7. During the intraoperative period, blood samples were collected after each Pringle's maneuver, which was typically performed in one cycle by clamping the portal pedicle for 15 min followed by the release of the pedicle for 5 min. Blood was collected in plastic tubes containing 3.8% sodium citrate (1:10 volume). Plasma was separated by centrifugation at 3000*g* for 15 min at 4 °C. Aliquots were stored at −80 °C until use.

2.3. Measurements

Plasma VWF antigen levels were measured by sandwich enzyme-linked immunosorbent assay (ELISA) using two rabbit polyclonal anti-human VWF antiserums (DAKO, Denmark) [20]. A previous study using this assay reported that the mean plasma VWF antigen levels in normal individuals (n = 20) was 102% ± 33% [20]. Plasma ADAMTS13 activity was determined using a commercially available chromogenic act-ELISA kit (Kainos Laboratories, Japan) [21]. Using this assay, ADAMTS13 activity in normal individuals (n = 55) was reported as 99% ± 22% [21]. The VWF antigen level and the ADAMTS13 activity in the pooled normal human plasma samples were set at 100%. In the current study, the VWF antigen/ADAMTS13 activity ratio was used to evaluate the relationship between VWF level and ADAMTS13 activity. Plasma VWF ristocetin cofactor activity was measured in sodium citrated plasma using BC von Willebrand reagent (Siemens Healthcare Diagnostics, Marburg, Germany) by the automated coagulation analyser, CS-2000i™ (Sysmex, Kobe, Japan). This method was performed as an agglutination assay and standard human plasma (Siemens Healthcare Diagnostics) was used for calibration. The assay had approximately 10% of sensitivity limit and low levels of inter-assay imprecision using normal plasma (coefficient of variation < 4.0%) [22].

2.4. VWF multimer analysis

Multimer analysis of plasma VWF was performed according to the

Table 1
Univariate and multivariate analyses of factors associated with UL-VWFMs.

Variables	Univariate analysis			Multivariate analysis	
	UL-VWFMs positive (n = 22)	UL-VWFMs negative (n = 7)	*p*-Value	OR (95% CI)	*p*-Value
	n (%)				
Age, median (range)	73.5 (45–82)	72 (58–84)	0.858		
Sex male	18 (81.8)	6 (85.7)	0.653		
Disease					
HCC	14 (63.6)	4 (57.1)	0.547		
CRLM	5 (22.7)	3 (42.9)	0.282		
Other	3 (13.6)	0 (0.0)	0.421		
Preoperative laboratory data, median (range)					
Platelet count, $\times 10^3/\mu L$	20.3 (11.1–37.6)	17.1 (13.3–46.0)	0.919		
Albumin, g/dL	4.2 (3.5–4.8)	4.2 (3.3–4.7)	0.858		
Total bilirubin, mg/dL	0.7 (0.3–1.3)	0.7 (0.5–1.2)	0.643		
Prothrombin time, %	90.0 (38.0–114.0)	81.0 (28.0–101.0)	0.185		
ICG retention rate at 15 min	13.5 (2.8–27.8)	13.2 (3.5–31.5)	0.541		
AST, U/L	27.5 (9.0–68.0)	24.0 (18.0–39.0)	0.628		
ALT, U/L	22.5 (7.0–74.0)	20.0 (9.0–40.0)	0.540		
ADAMTS13 activity, %	62.9 (37.0–94.6)	42.6 (30.4–126.2)	0.139		
VWF antigen, %	170.5 (85.7–412.8)	148.2 (80.5–278.7)	0.221		
VWF/ADAMTS13	2.8 (1.5–7.8)	3.0 (0.9–7.0)	0.919		
Surgery related factor					
Major resection	3 (13.6)	0 (0.0)	0.421		
Open liver resection	16 (72.7)	5 (71.4)	0.647		
Duration of Pringle's maneuver, min median (range)	111.5 (31–216)	68 (30–83)	0.001	1.049 (1.001–1.098)	0.043
Number of Pringle's maneuver, median (range)	7 (2 − 12)	5 (2–5)	0.002		
Blood loss, ml median (range)	513 (50–3735)	140 (80–485)	0.022	1.003 (0.996–1.010)	0.371
RCC transfusion	3 (13.6)	1(14.2)	0.692		
FFP transfusion	10 (45.4)	1 (14.2)	0.151		

method by Ruggeri and Zimmerman [23], with modifications as reported by Warren et al. [24]. In brief, the lower platelet gel comprised 1% agarose (SeaKem® Gold Agarose, Lonza, Rockland, ME, USA) and 10% glycerol dissolved in 50 mmol/L phosphate buffer (pH 8.8) with 0.1% sodium dodecyl sulphate (SDS). The upper gel was prepared with 0.8% agarose (SeaKem® HGT Agarose, Cambrex, Rockland) dissolved in 370 mmol/L phosphate buffer (pH 6.8) with 0.1% SDS. The electrophoresis buffer comprised 50 mmol/L Tris-glycine buffer (pH 8.3) containing 0.1% SDS. The experimental conditions, including western blotting with luminographic detection, were followed as per the protocol of Budde et al. [25].

High-molecular-weight bands that were not detected in normal plasma were defined as UL-VWFMs (Fig. 2). UL-VWFMs were quantified using images of VWF multimer analysis by NIH ImageJ software (National Institute of Health, Bethesda, MD, USA). UL-VWFMs index in the current study was defined as the ratio of the UL-VWFM area to that of the normal plasma analysed in the same gel (Fig. 2). All UL-VWFM indices of 40 healthy volunteers were < 1.00%. Therefore, UL-VWFM positivity was defined as a UL-VWFM index of > 1.00%. The patients were categorised and analysed according to their UL-VWFM positivity after hepatectomy.

2.5. Statistical analysis

Data were expressed as medians with ranges. Statistical analyses were performed using the Mann–Whitney *U* and χ^2 tests to compare differences between two groups. The Kruskal–Wallis test was performed to compare differences between more than two groups, and paired comparisons among different time points in each group were performed using Wilcoxon signed-rank test. A p-value < 0.05 was considered as statistically significant. Correlations between two parameters were evaluated by calculating Pearson's correlation coefficients. Multivariate analysis was performed to identify factors associated with UL-VWFMs level. The results were expressed as adjusted odds ratios (ORs) with 95% confidence intervals (CIs), and p values were calculated using the likelihood ratio test. All statistical analyses were performed using SPSS for Windows version 18.0 (IBM, Armonk, New York, USA).

3. Results

3.1. Intraoperative changes of ADAMTS13 and VWF

All patients underwent two or more Pringle's maneuvers (Table 1), with one patient receiving 12 Pringle's maneuvers. Intraoperative plasma samples could be obtained from 30 patients. Of these, only two patients (patients 4 and 29) received fresh frozen plasma (FFP). The plasma samples obtained after each Pringle's maneuver were analysed for ADAMTS13 activity and VWF antigen levels, which revealed that neither was significantly changed during the intraoperative period (Fig. 3A and B).

We performed VWF multimer analysis using intraoperative plasma samples of 10 representative patients. Of these, the results in samples from four patients are shown in Fig. 4. The VWF multimer analysis of patient number 4 (Fig. 4A) who received four units of FFP during the operation revealed that there were no UL-VWFMs present in the plasma sample collected during the operation. In contrast, in patient number 29 (Fig. 4D) who also received four units of FFP, the UL-VWFMs were detectable after the second, fourth and fifth Pringle's maneuver, whereas the UL-VWFMs were not found in the plasma sample obtained immediately after the sixth Pringle's maneuver in this patient. In summary, we detected the presence of UL-VWFMs in the intraoperative samples of six of the 10 representative patients. The UL-VWFMs indices derived from the VWFM antigen levels in these 10 patients did not change significantly during the operation (Fig. 3C). The results of both the VWF antigen levels in all patients and the UL-VWFMs indices in these 10 representative patients indicated that the UL-VWFMs were not significantly increased during hepatectomy.

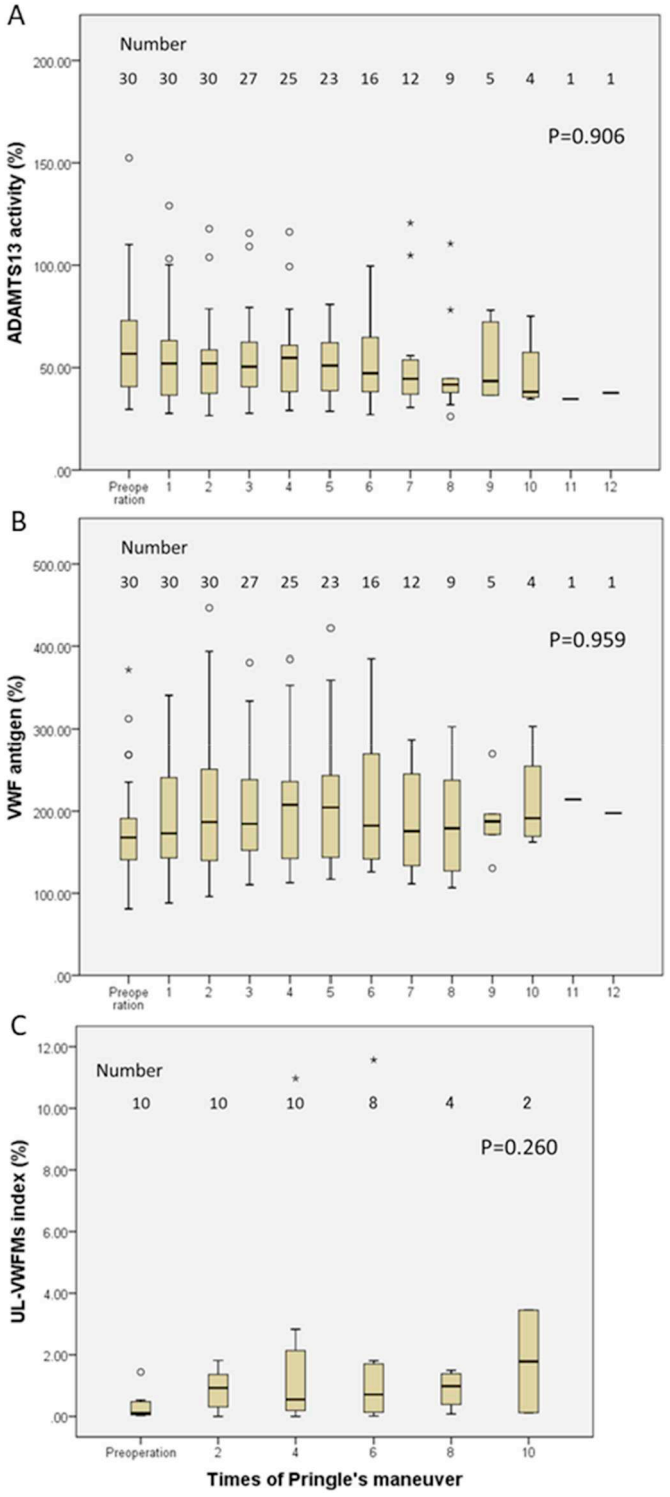

Fig. 3. Intraoperative changes in (A) a disintegrin and metalloprotease with a thrombospondin type 1 motifs-13 (ADAMTS13) activity, (B) plasma von Willebrand factor (VWF) antigen levels and (C) unusually large VWF multimer (UL-VWFMs) index. In (A) and (B), 30 patients whose intraoperative samples could be obtained were analysed. In (C), 10 representative patients were analysed. Boxes show medians with 25–75 percentile values. The Kruskal–Wallis test was performed to compare differences among the groups.

3.2. Postoperative changes in ADAMTS13 activity and VWF antigen levels

During the postoperative period, plasma VWF antigen levels and ADAMTS13 activity were analysed in all 35 patients at five time points on days 1, 2, 3, 5 and 7. Plasma ADAMTS13 activity gradually decreased over the postoperative course (Fig. 5A), with that on postoperative day 7 [37.4% (20.2%–71.4%)] significantly lower than that before the operation [61.0% (27.7%–126.2%)] ($p < 0.001$). In contrast, plasma VWF antigen levels gradually increased until postoperative day 2 and remained stable until postoperative day 7 (Fig. 5B). Plasma VWF antigen levels on postoperative day 2 [361.0% (154.7%–745.8%)] and postoperative day 7 [351.1% (193.1%–711.4%)] were significantly higher than that before the operation [172.1% [(80.5%–412.8%)] ($p < 0.001$). Moreover, the VWF/ADAMTS13 ratio were gradually increased over the postoperative course (Fig. 5C) compared with that on postoperative day 7 [9.2 (3.1–20.7)], which was significantly higher than that before the operation [3.1 (0.9–7.8)] ($p < 0.001$).

VWF ristocetin cofactor activity and VWF multimer were also analysed using plasma samples obtained on days 1, 3, 5 and 7 after hepatectomy in all 35 patients. Plasma levels of VWF ristocetin cofactor activity were gradually increased until postoperative day 3 and remained stable until postoperative day 7 (Fig. 5D). VWF ristocetin cofactor activity levels on postoperative day 3 (257.6% [63.8%–415.2%]) and postoperative day 7 (237.2% [138.6%–426.8%]) were significantly higher than that before the operation (123.8% [71.3%–258.0%]) ($p < 0.001$). As shown in Fig. 5E, the UL-VWFMs indices of these samples were compared with the indices of samples collected before the operation, which revealed that the UL-VWFMs indices were significantly increased from 0.2% (0.0%–7.8%) before the operation to 4.2% (0.1%–16.3%)on postoperative day seven after the operation ($p < 0.001$). These results indicated that the UL-VWFMs were significantly increased on day one after, but not during, the operation.

3.3. The effect of hepatectomy on UL-VWFMs

Six patients had detectable UL-VWFMs even before the operation, as shown in Fig. 6A. The remaining 29 patients who did not have detectable UL-VWFMs before the operation were divided into two groups based on the UL-VWFMs analysis conducted after the operation, as shown in Fig. 1. Accordingly, seven patients were UL-VWFMs-negative after the operation (Fig. 6B; UL-VWFMs-negative group). In contrast, 22 patients converted from UL-VWFMs-negative status before the operation to UL-VWFMs-positive status after the operation (Fig. 6C and D; UL-VWFMs-positive group).

These two groups were compared using univariate analysis, as shown in Table 1. There were no significant differences in the patient characteristics or the laboratory data of the before the operation. The plasma levels of both the VWF antigen and the ADAMTS13 activity in the UL-VWFMs-positive group tended to be higher than those in the UL-VWFMs-negative group, albeit without significance.

Among the operation-related factors, the duration of Pringle's maneuver in the UL-VWFMs-positive group was significantly longer than that in the UL-VWFMs-negative group ($p = 0.001$), and the number of Pringle's maneuvers performed was significantly higher in the UL-VWFMs-positive group than in the UL-VWFMs-negative group ($p = 0.002$). In addition, the volume of blood loss was larger in the UL-VWFMs-positive group than in the UL-VWFMs-negative group ($p = 0.003$). Multivariable analysis revealed that the duration of Pringle's maneuver (OR, 1.049, 95% CI, 1.001–1.098, $p = 0.043$) was significantly associated with UL-VWFMs

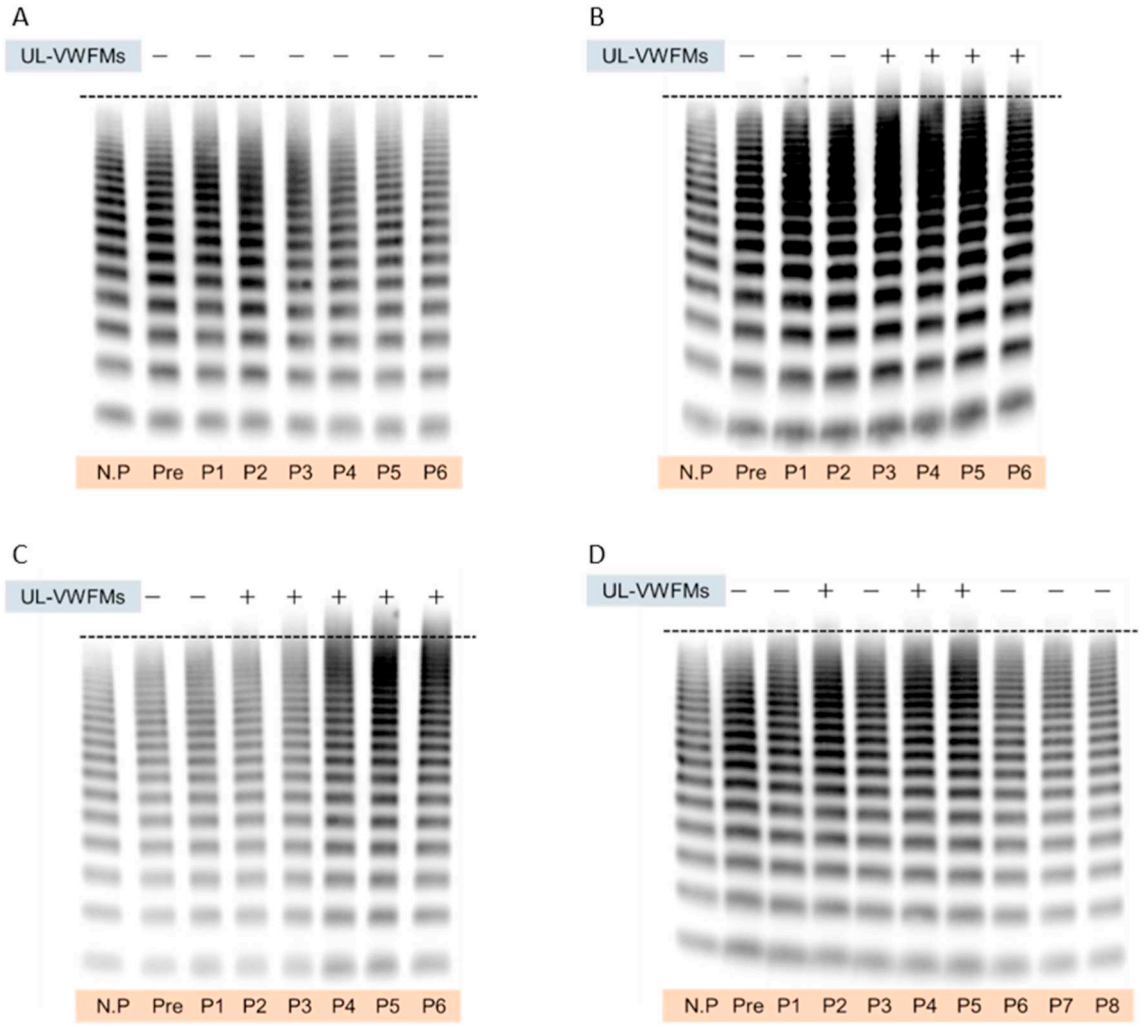

Fig. 4. Intraoperative VWF multimer analysis of samples from four representative patients. UL-VWFMs were not found during operation of the samples obtained from patient A depicted in (A). UL-VWFMs were found during operation of B, C and D. Only two cases (A and D) received fresh frozen plasma (FFP) during operation.

after hepatectomy (Table 1).

Transfusion of FFP might affect the plasma VWF levels. In the UL-VWFMs-positive group, 10 patients (45.4%) received FFP transfusions whereas only one patient (14.2%) in the UL-VWFMs-negative group received FFP transfusions; this difference was not significant.

3.4. Relationship between the duration of Pringle's maneuver and UL-VWFMs index

Based on the results of the multivariate analysis in Table 1, we compared the relationship between the UL-VWFMs index and the duration of Pringle's maneuver. As shown in Fig. 7, the UL-VWFMs index was significantly correlated with the duration of Pringle's maneuver ($r = 0.444$; $p = 0.017$).

4. Discussion

Our analyses in the current study revealed three major results. First, plasma ADAMTS13 activity levels were significantly decreased after hepatectomy. Second, plasma VWF antigen levels were significantly increased after hepatectomy. Third, the UL-VWFMs index was significantly correlated with the duration of Pringle's maneuver. To the best of our knowledge, the association between the duration of Pringle's maneuver and UL-VWFMs level after hepatectomy was not previously reported.

This study showed an imbalance between the platelet adhesive protein VWF and its regulatory protease ADAMTS13 for up to 7 days after hepatectomy. The imbalance between VWF levels and ADAMST13 activity in patients after hepatectomy was previously reported [26,27]. Moreover, previous studies showed a correlation between high VWF/ADAMTS13 ratio and thrombotic diseases, such as myocardial infarction [28], cerebral infarctions [29] and thrombotic microangiopathies [30]. It is likely that patients who underwent hepatectomy had increased susceptibility for developing thrombosis, including portal thrombosis. Actually, we observed four patients with thrombotic complications who had UL-VWFMs after hepatectomy in this study. These results may suggest association between thrombotic complication and UL-VWFMs after hepatectomy.

Sinusoidal endothelial cells are the primary site of hepatic ischaemia–reperfusion injury, and apoptosis of sinusoidal endothelial cells is a

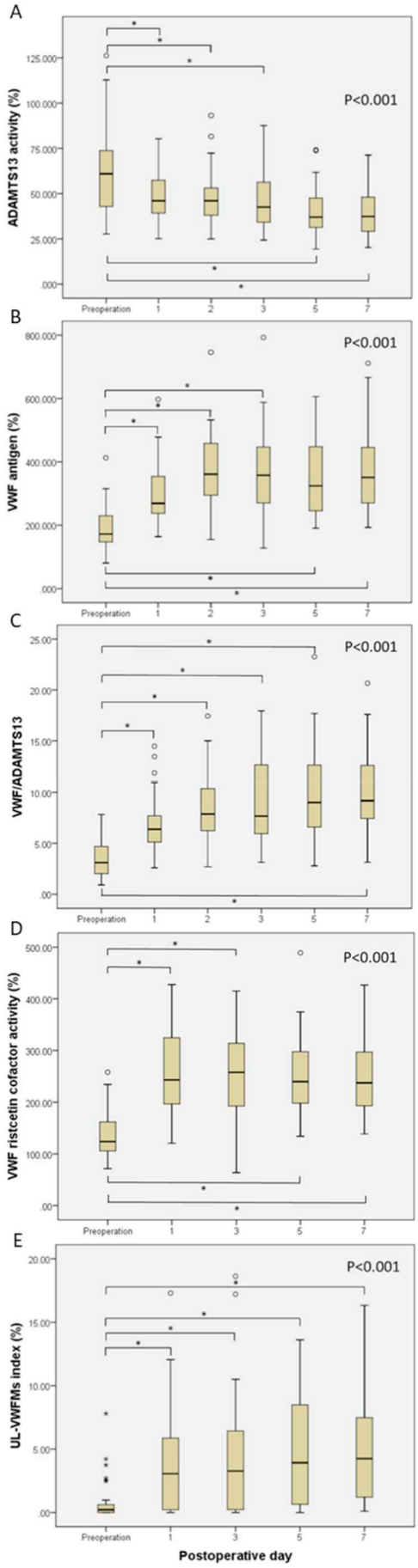

Fig. 5. Postoperative changes in (A) ADAMTS13 activity, (B) plasma VWF antigen levels, (C) VWF/ADAMTS13 ratio, (D) VWF ristocetin cofactor activity and (E) UL-VWFMs index. Analyses were performed in 35 patients. Boxes show medians with 25–75 percentile values. The Kruskal–Wallis test was performed to compare differences among the groups, and paired comparisons of different time points with the preoperative values were performed using Wilcoxon signed-rank test. $^{*}p < 0.001$.

pivotal mechanism of hepatic ischaemia–reperfusion injury [31]. Severe endothelial cell damage results in the release of UL-VWFMs from the endothelial cells [32]. In the current study, we demonstrated that plasma UL-VWFMs levels were increased in association with Pringle's maneuver after hepatectomy. UL-VWFMs also appear to be released after major abdominal surgery by a general postoperative stress response. Our results suggested that ischaemia–reperfusion injury with Pringle's maneuver might induce the release of UL-VWFMs from the endothelial cells. Therefore, we believe that UL-VWFMs were increased, particularly in hepatectomy compared with general abdominal surgery. Moreover, UL-VWFMs are a strong activator of platelet aggregation, and an increase in UL-VWFMs leads to platelet clumping and/or thrombus formation, resulting in microcirculatory disturbance. Platelet recruitment to the regenerating liver has been well established in experimental animal models [33], and it is conceivable that excessive platelet accumulation may have detrimental effects mediated by micro ischaemia. Therefore, intrahepatic microcirculatory disturbance may also lead to postoperative liver failure, which could potentially result in the increase of UL-VWFMs with Pringle's maneuver. In this context, postoperative aspartate transaminase and alanine transaminase levels were significantly higher in the UL-VWFMs-positive group compared with the UL-VWFMs-negative group in the current study.

ADAMTS13 cleaves UL-VWFMs into smaller, less active forms and prevents the formation of thrombi [34]. Therefore, maintaining a balance between VWF and ADAMTS13 levels appears to be crucial in preserving microcirculation and macrocirculation, particularly under stressful conditions [35]. The findings of the present study suggest that treatment of patients with ADAMTS13 concentrates to normalise UL-VWFMs in circulation may decrease postoperative liver failure following hepatectomy. Such concentrates, however, are still under development and not yet available in the clinical setting. FFP is the currently used, unique source for ADAMTS13 replacement therapy that might improve both thrombosis and liver dysfunction after hepatectomy. In the current study, our results demonstrated that the number of FFP transfusions was not significantly different between the two groups. However, we believe that a larger sample size can potentially reveal that FFP may significantly reduce postoperative UL-VWFMs levels after hepatectomy.

Plasma infusion therapy, albeit generally effective, is frequently complicated by volume overload as well as allergic and anaphylactic reactions [36]. In addition, plasma infusions carry a certain risk of contracting infection of blood-borne pathogens, whereas plasma infusions performed at hospitals or outpatient settings are burdensome and time-consuming, and therefore, are a significant source of stress for younger patients. Therefore, novel recombinant ADAMTS-13 products can represent potential new therapeutic options to improve the current standard of care. First-in-human, phase 1 study indicated that recombinant ADAMTS-13 was safe and non-immunogenic; it was also well tolerated in congenital thrombotic thrombocytopenic purpura [37]. Recombinant Therefore, ADAMTS13 can be a novel option for the treatment of liver failure after hepatectomy and can eliminate many of the risks associated with products derived from human plasma to facilitate the introduction of more precise and individualised dosing regimens [36,38,39].

There are several limitations in the present study. First, the study comprised a relatively small number of patients, which limited clear conclusions. Studies with larger sample sizes are necessary to validate the

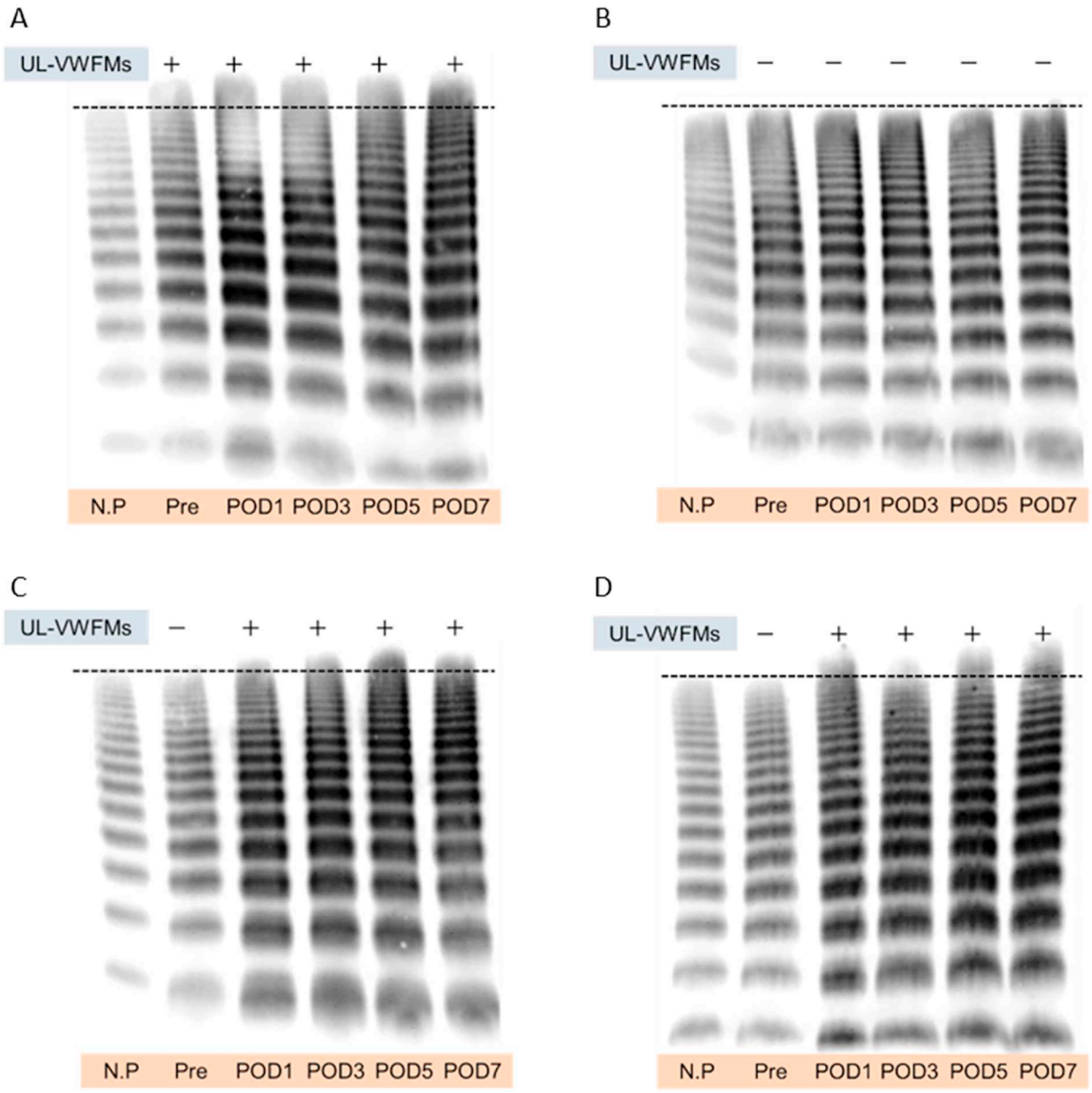

Fig. 6. Postoperative VWF multimer analysis of four representative patients. UL-VWFMs were present even before hepatectomy in the patient presented in (A). UL-VWFMs levels remained negative after operation in the patient presented in (B). UL-VWFMs levels changed from negative before the operation to positive after the operation in the patients presented in (C) and (D).

current findings. Second, the residual liver could not be histopathologically evaluated. We should find VWF-rich platelet thrombi in the residual liver to indicate that the microcirculatory disturbance was induced by UL-VWFMs [40]. However, it is difficult to perform the biopsy of the residual liver due to ethical concerns. Third, we did not show a direct evidence of the relationship between microcirculatory disturbance and UL-VWFMs, which requires further analysis in animal models.

5. Conclusion

In conclusion, we have demonstrated that the appearance of plasma UL-VWFMs after hepatectomy was associated with ischaemia–reperfusion

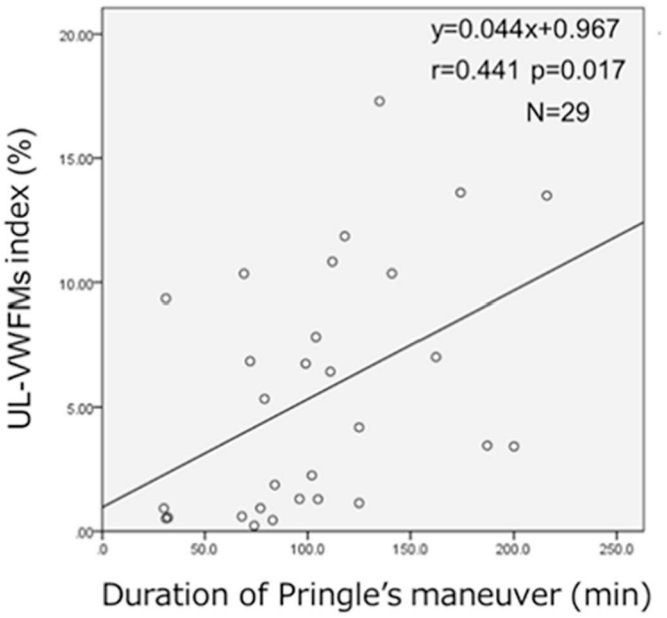

Fig. 7. Association between duration of Pringle's maneuver and UL-VWFMs index.

injury with Pringle's maneuver.

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Conflict of interest

All authors state that there are no conflicts of interest to declare.

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