

# Variations in the ratio between von Willebrand factor and its cleaving protease during systemic inflammation and association with severity and prognosis of organ failure

Ralf A. Claus<sup>1</sup>; Clemens L. Bockmeyer<sup>1\*</sup>; Ulrich Budde<sup>2</sup>; Karim Kentouche<sup>3</sup>; Maik Sossdorf<sup>1,4</sup>; Thomas Hilberg<sup>4</sup>; Reinhart Schneppenheim<sup>5</sup>; Konrad Reinhart<sup>1</sup>; Michael Bauer<sup>1</sup>; Frank M. Brunkhorst<sup>1</sup>; Wolfgang Lösche<sup>1</sup>

<sup>1</sup>Department of Anaesthesiology and Intensive Care Medicine, University Hospital, Friedrich-Schiller-University, Jena, Germany; <sup>2</sup>AescuLabor Hamburg, Institute for Laboratory Medicine, Hamburg, Germany; <sup>3</sup>Department of Pediatrics, University Hospital, Friedrich-Schiller-University, Jena, Germany; <sup>4</sup>Department of Sports Medicine, Friedrich-Schiller-University Jena, Germany; <sup>5</sup>Children's University Hospital Hamburg-Eppendorf, Department of Pediatric Hematology and Oncology, Germany

## Summary

Von Willebrand factor (VWF) and related parameters as well as the protease activity regulating its biological activity were measured in plasma of healthy controls and patients with different cause and severity of systemic inflammation to examine the efficacy of the measures to detect highly prothrombotic states including thrombotic microangiopathy (TMA), one of the sequelae of sepsis. Plasma levels of VWF increased with increasing severity of systemic inflammation, probably due to activation of the endothelium. In parallel, the proteolytic activity of VWF inactivating protease, ADAMTS13, stepwise declined with the severity of inflammation, emphasizing the role of VWF-triggered

platelet aggregation on the endothelium subsequently followed by development of TMA. As a consequence, the ratio of VWF antigen level and ADAMTS13 activity was significantly higher in patients with inflammation and sepsis, suggesting that this ratio might be more useful for the diagnosis of highly prothrombotic states including TMA than VWF multimer analysis alone. These findings suggest that ADAMTS13, VWF and related parameters, even in a combined approach, might be useful for the diagnosis and the therapeutic monitoring of patients with sepsis associated thrombotic microangiopathy.

## Keywords

ADAMTS13, sepsis, systemic inflammation, organ failure, platelets, thrombotic microangiopathy, TMA-index

Thromb Haemost 2009; 101: 239–247

## Introduction

Systemic dysregulation of inflammatory response resulting from a wide variety of causes, such as infection, trauma, burns and hypoxic states, is accompanied by a generalised activation of the endothelium with a shift of the haemostatic balance, characterised by the activation of procoagulant pathways and an attenuation of anti-coagulant activity. These pathways are involved in the development

of disseminated intravascular coagulation and microvascular thrombosis, ultimately leading to multiple organ failure and death (1, 2). Sepsis is termed the culmination of complex interactions between the infecting microorganism and the host immune, inflammatory, and coagulation responses (3, 4), as soon as an infectious agent switches the balance to a procoagulant and proinflammatory status, respectively. Septic shock is the commonest cause of death in intensive care units (5–7), and its incidence is increasing.

## Correspondence to:

Ralf A. Claus, PhD

Dept. of Anaesthesiology and Intensive Care Medicine

Friedrich-Schiller-University

Erlanger Allee 101, D-07747 Jena, Germany

Tel.: +49 3641 932 58 60, Fax: +49 3641 932 58 62

E-mail: ralf.claus@med.uni-jena.de

\*Present address: Institute for Pathology, Hannover Medical School, Germany.

## Footnote:

The study was presented at the 3<sup>rd</sup> Workshop on Thrombotic Microangiopathies Jena/Germany, October 6<sup>th</sup>, 2007.

## Financial support:

The study was supported in part by a grant from the Thuringian Ministry of Science and Arts (TMWFK, project B-309-00014), by the Centre for Clinical Research Jena (IZKF), subproject 4.8 (Thrombotic Microangiopathy in sepsis) and by Deutsche Forschungsgemeinschaft (CL 173/4-1). CLB has received financial support from the 'Förderverein' Friedrich-Schiller University Jena (Loder-Grant for young investigators).

Received: March 13, 2008

Accepted after major revision: November 25, 2008

Prepublished online: January 15, 2009

doi:10.1160/TH08-03-0161

Several lines of evidence support the concept, that an altered biological function of the acute phase protein von Willebrand factor (VWF) is crucially involved in the pathophysiology of sepsis (8, 9). VWF is a multimeric glycoprotein circulating in plasma as multimers up to 20,000 kDa in size and functions as an adapter protein mediating platelet adhesion and aggregation to subendothelial collagen (10). It is released from the activated endothelium, whereas a portion is secreted as ultra-large VWF multimers (ULVWF). Secreted ULVWF multimers spontaneously activate and bind platelets with high affinity and are thought to be extraordinarily prothrombotic (11). The thrombogenic potential of VWF is regulated by a protease secreted from hepatic stellate and endothelial cells (12, 13), termed ADAMTS13 (A Disintegrin-like and metalloprotease with thrombospondin type 1 motif), which reduces VWF's agglutinative properties by limited proteolysis. In addition to the pioneering observations that severe deficiency in ADAMTS13 activity and the resulting appearance of ULVWF multimers in plasma are the pathophysiological basis for primary thrombotic microangiopathy (TMA) (14, 15), several studies indicated that also other factors may contribute to clinical manifestation of TMA and an altered VWF multimer pattern (16). It became evident that variations in the VWF multimer pattern and of the ADAMTS13/VWF balance are not only restricted to primary thrombotic microangiopathies such as thrombotic thrombocytopenic purpura (TTP), but are also observed in liver cirrhosis (17) as well as in the course of an inflammatory response such as physical stress (18), postoperative systemic inflammatory response syndrome (SIRS) (19, 20) and sepsis (20–22).

TMA as consequence of ADAMTS13 deficiency and ULVWF appearance in plasma is associated with a higher platelet thrombogenicity followed by consumption, disappearance of platelets with subsequent occlusion of microvessels, thrombocytopenia and aggravation of organ dysfunction (23). Among other factors that may at least partially compensate for ADAMTS13 deficiency is thrombospondin that have been shown to degrade VWF multimers by reducing disulfide bridges linking the monomers (24).

At present, the gold standard for detection of ULVWF is immunological visualization after electrophoretic separation, blotting and visualization of VWF multimers with a video system (25–27). However, this diagnostic tool displays various disadvantages such as the need of specialised equipment, time consuming analysis as well as an insufficient reproducibility and comparability between laboratories making it unfeasible for real-time diagnosis and therapeutically monitoring. Otherwise, conventional parameters describing the function and activity of the VWF multimer pattern, such as VWF:ristocetin cofactor activity (VWF:RCo) and VWF:collagen binding activity (VWF:CB) are well established (28). In normal plasma, the degree of VWF-activity can be estimated by calculating the VWF:RCo as well as VWF:CB/VWF:Ag ratio, which is by definition close to one (28). In recent studies the ratio VWF:Ag/ADAMTS activity was found markedly enhanced in patients with TMA, suggesting that this value might be more useful for the differential diagnosis than the determination of ADAMTS13 alone. These findings suggest that ADAMTS13 related markers are useful for the diagnosis and analysis of TTP and TMA (29).

The aim of our study was to determine qualitative and quantitative variations of VWF and related parameters in a large group of patients with inflammation of varying severity and origins to evaluate the diagnostic value of the biological VWF activity and its multimer pattern. Therefore, groups of patients with uncomplicated SIRS following physical or surgical stress, organ failure and sepsis were studied. Parameters examined were VWF:Ag levels, VWF:RCo as a measure for the ability of the protein to interact with platelets, the VWF collagen binding activity, the activity of the VWF inactivating protease ADAMTS13 as well as the multimeric structure of VWF to add further evidence of a possible role of a dysbalanced ratio during the inflammatory response and of an association with the severity of the disease.

The findings of our study suggest that combined interpretation of the assays provide more insights to what extent elevated VWF levels in patients with inflammation contribute to the development of thrombotic microangiopathy.

## Study design and methods

After institutional ethical approval and written informed consent, healthy individuals after strenuous physical exercise and three groups of ICU-patients with SIRS were studied:

- 1) For standardised strenuous and long-term physical stress, six healthy, moderately trained volunteers were consecutively enrolled, plasma samples were acquired before and after a standardised exercise test on a treadmill ergometer with 90% of the individual anaerobic threshold for 90 minutes (min) by venipuncture from an antecubital vein under controlled venous stasis after 30 min rest (baseline) and immediately, two, six and 24 hours (h) after exercise.
- 2) Twenty-four patients who underwent *elective* cardiac surgery with low risk to develop post-operative organ dysfunction as indicated by the EURO-score (European System for Cardiac Operative Risk Evaluation [30]) <6 and a maximum post-operative ICU (intensive care unit) stay of two days (92% with one day). Plasma samples were taken within the first 24 h after ICU admission (prior to surgery/*pre-op*, immediately after surgery/*post-op* and finally at the first day on ICU/*late post-op*) and normalised for haemodilution to overcome dilution effects due to the regular use of priming solutions during cardio-pulmonary bypass (CPB). Normalised values were obtained by calculation as the change from *pre-op* haematocrit to the post-operative one. Data from this group were used as ICU reference values.
- 3) Twenty-two patients after *non-elective* on-pump cardiac surgery with high risk to develop organ dysfunction (EURO-score  $\geq 6$ ). Plasma samples were taken on five consecutive post-operative days (no post-operative deaths) and normalised for haemodilution.
- 4) Eleven patients who met the criteria for severe sepsis or septic shock according to the ACCP/SCCM Consensus Conference (31). Plasma samples were obtained on a daily basis until discharge or death. A total number of 133 patient days were evaluated. Five patients survived the observation period (length of stay on ICU 19 days [12/27] vs. 14.5 [11/28] of non-survivors).

For blood sampling, a 20-gauge canula and 10 ml monovettes containing 1 ml of 0.13 molL<sup>-1</sup> sodium citrate were used and plasma was obtained by centrifugation (1,500xg, 20 min, 4°C within 30 min after venipuncture), divided into aliquots and stored at -80° until assayed.

ADAMTS13 activity was determined by the collagen-binding method using recombinant VWF as substrate (32). Intra- and inter-assay coefficients of variation were 12% on the basis of six replicates and 16% in six different runs, respectively. The assay used had provided highly reliable results in multicenter studies (33). Auto-inhibitory factors of ADAMTS13 were excluded by dilution experiments.

VWF:Ag was measured by an enzyme-linked immuno-sorbent assay (25). VWF multimers were separated by gel electrophoresis (60V, 16°C, 15 h) using 1.2% LGT-Agarose (Sigma-Aldrich, Seelze, Germany), detected by a VWF-antibody conjugated with horseradish peroxidase (Dako Hamburg, Germany) and visualised by an enhanced chemiluminescence (ECL detection kit; Amersham, Freiburg, Germany). The gels were recorded by a CCD camera and evaluated by AIDA software (26).

The ISTH-score for overt disseminated intravascular coagulation (DIC) (34) was calculated with the following modifications: fibrinogen concentration was not included as it is often increased in systemic inflammation; for D-dimer concentration (Technozym D-Dimer ELISA, Technoclone, Vienna/Austria) the following cut-off values were used: 0–0.25 µg/ml = 0 points, 0.25–2.5 µg/ml = 2 points, > 2.5 µg/ml = 3 points (35).

Normal values for VWF:Ag [median 0.93 (IQR 0.58) U/mL], VWF:RCo [1.54 (0.31) IU/mL], VWF:CB [1.05 (0.42) IU/mL], ADAMTS13 activity [0.75 (0.48) U/mL] and D-Dimer concentration [below detection limit of the assay] were evaluated in 20 age-matched healthy volunteers. The normal range of VWF:CB/VWF:Ag ratio as well as the VWF:RCo/VWF:Ag ranged up to 1.5. By definition, SOFA values received from healthy individuals undergoing physical exercise were set to zero.

### Statistical analysis

The non-parametric Mann-Whitney-U test and ANOVA were applied for comparison between patient groups as well as between survivors and non-survivors. P-values <0.05 were considered significant. The results are presented as medians and 1<sup>st</sup>/3<sup>rd</sup> quartile.

### Results

The conventional diagnostic process specifically describing VWF activity and function typically include VWF:antigen (VWF:Ag) assay and functional activity assays using the VWF:ristocetin cofactor (VWF:RCo). Additional tests such as the collagen binding activity assay (VWF:CB) or VWF:multimer analysis may be used as supplementary assays. Hereby, the ratio of VWF:RCo and VWF:CB with regard to the antigen content is established for semiquantitative analysis of the biological function of VWF (36). Moreover, in patients with primary TMA such as TTP the ratio is associated with ADAMTS13 activity.

As shown in Figure 1, VWF:Ag levels were found stepwise elevated in plasma from healthy individuals with moderate SIRS to sepsis. In detail, strenuous physical exercise as a model for

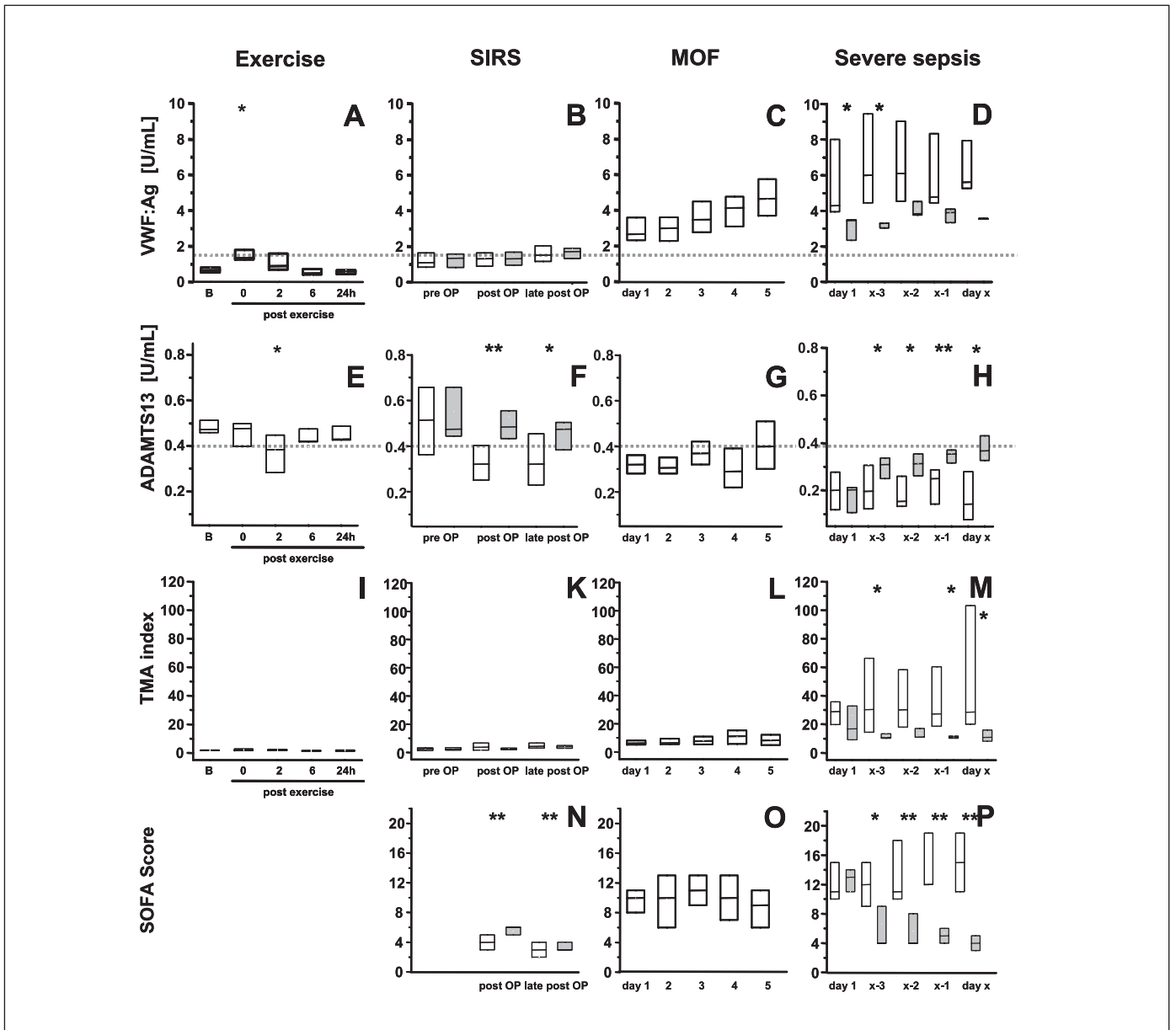
moderate SIRS resulted in overriding of the upper limit of normal (ULN [37]) ( $p < 0.05$ ) directly after physical stress with a rapid reversion to baseline values within six hours (Fig. 1A). In patients with uncomplicated postoperative inflammatory state after cardiac surgery we observed a moderate increase from baseline in a 24 h time course without reaching significance in both ON-pump and OFF-pump surgical technique (Fig. 1B). However, in patients at risk for development of organ failure after surgery we found substantially elevated levels at the first and all following observation days compared to the ICU-reference group ( $p < 0.05$ ; Fig. 1C). Patients with sepsis exhibited enormously elevated VWF:Ag levels compared to ICU reference. When patients were classified according to outcome, we found only a tendency to discriminate between survivors (3.53 [3.53/3.56] U/ml at day of discharge from ICU; n=5) and of non-survivors (5.80 [5.25/7.95] U/ml; n=6) by the use of VWF:Ag levels (Fig. 1D).

In our patients with different states of inflammatory disease the ratio of VWF:CB/VWF:Ag were in the normal range (1.2 [1.0/1.5] at the first observation day, 0.93 [0.71/1.26] at the last observation day). Similar results were obtained in the basis of VWF:RCo (data not shown). Thus, the ratios were not sufficient to detect differences in the biological function of VWF as well as in the inflammatory state.

However, a stepwise decrease in ADAMTS13 activity dependent on the inflammatory state was shown in our setting: In strenuous exercise, a nadir in proteolytic activity was found 2 h after physical stress in comparison to baseline levels (0.40 [0.28/0.45],  $p < 0.05$ , Fig. 1E). Starting from similar baseline levels, proteolytic activity declined towards values below the LLN of 0.40 U/ml (37) in the group of patients who underwent elective ON-pump surgery, the decline was delayed and attenuated in the group undergoing elective OFF-pump surgery ( $p < 0.05$ ; Fig. 1F). Activity values clearly below the LLN were found in cardiac surgery patients with organ failure at the day of enrolment ( $p < 0.05$ ) followed by a tendency to approach physiological levels at day 5 (Fig. 1G). As shown in Figure 1H, ADAMTS13 activity was found substantially decreased in patients with sepsis at enrolment in the study compared with the ICU reference group ( $p < 0.001$ ). However, there is an increase in patients surviving sepsis reaching LLN levels at the day of discharge. Contrary, values persisted at dramatically decreased levels in non survivors (0.37 [0.33/0.43] vs. 0.20 [0.08/0.28] U/ml,  $p < 0.05$ ).

Comparing a set of clinical parameters at days with lowest and highest ADAMTS13 activity during the time course of patients with sepsis (mean  $0.12 \pm 0.07$  vs.  $0.37 \pm 0.07$  U/ml,  $p < 0.005$ ) we found significant differences in the plasma concentration for interleukin-6 (IL-6) and procalcitonin as markers of disease severity as well as for SOFA score and in albumin levels as a parameter for liver function (mean  $14.4 \pm 5.0$  mg/ml vs.  $18.8 \pm 4.5$  mg/ml,  $p < 0.05$ ). Data are given in Table 1.

Besides many prior attempts to reach more clarification which needs to be taken into account for the clinical interpretation of results and for the purpose of reliable, therapeutically monitoring, in the present study we compared the values of the negative acute phase protein ADAMTS13 and of its substrate, the positive acute phase protein VWF, in one parameter termed TMA index, which is defined as the ratio of VWF:Ag and



**Figure 1: Laboratory and clinical findings.** VWF antigen (VWF:Ag), ADAMTS13 activity and TMA index according to the degrees of severity of the disease as indicated by SOFA score after strenuous physical exercise as well as in the course of patients with uncomplicated postoperative SIRS (used as ICU-reference group), with multiple organ failure and developing severe sepsis/septic shock. The boxes indicate the interquartile range, divided by the median (central horizontal line) expressed as a percentage of a reference plasma (plasma pool of 45 healthy volunteers). In patients with uncomplicated SIRS, OFF-pump surgery is indicated by

filled boxes, surviving patients with severe sepsis or septic shock are also indicated by filled boxes. Physiological ranges (lower limit of normal, upper limit of normal; LLN, ULN, respectively) are shown by dotted horizontal lines. No data with respect to organ failure (SOFA score) were obtained from individuals undergoing strenuous physical exercise. Levels of significance during the course of strenuous exercise (A, E), between used surgical technique (B, F, N) as well as between survivors and non-survivors in patients with sepsis (D, H, M, P) are indicated by asterisks: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

ADAMTS13 activity. In Figure 1I-M we illustrate the dramatic shift of the parameter in patients devolving into severe sepsis or septic shock: In healthy individuals with moderate SIRS or patients with uncomplicated postoperative course, TMA index ranged up to 5 (Fig. 1I, K), however, there is an increase with persistent values in patients with organ failure at all observation days (maximal median at day four: 11.2 [5.6/15.3],  $p < 0.05$  vs. ICU controls; Fig. 1L). A profound increase was observed in pa-

tients with sepsis at the first observation day, with similar course over time in survivors (decrease over time, 10.9 [8.2/16.0] at the day of discharge) in comparison to that of non-survivors (increase over time, 35.3 [20.2/103.0] at the day of death;  $p < 0.05$ ; Fig. 1M).

SOFA score ranged between 2 to 4 points during uncomplicated ICU stay (Fig. 1N), to values ranging between 6 to 13 points in patients with organ failure (Fig. 1O) and the highest in

**Table 1: Comparison of laboratory and clinical parameters at days with extremely elevated or diminished ADAMTS13 activity.** Comparison of IL-6, procalcitonin, C-reactive protein (CRP), and albumin levels as well as SOFA score at days with minimum versus maximum ADAMTS13 activity in the course of patients with sepsis. Mann-Whitney U-Test, ‡ p < 0.05, †† p < 0.005; ††† p < 0.0005.

	Minimum ADAMTS13 activity (0.12 U/ml ± 0.07)	Maximum ADAMTS13 activity (0.37 U/ml ± 0.07)†††
IL-6 [pg/ml]‡	3,547 ± 5,611	259 ± 137
procalcitonin [ng/ml]‡	22.1 ± 30.8	2.7 ± 1.9
CRP [mg/ml]	134 ± 59	114 ± 64
albumin [mg/mL]‡	14.4 ± 5.0	18.8 ± 4.5
SOFA score [pts.]††	14.0 ± 3.6	9.5 ± 4.5

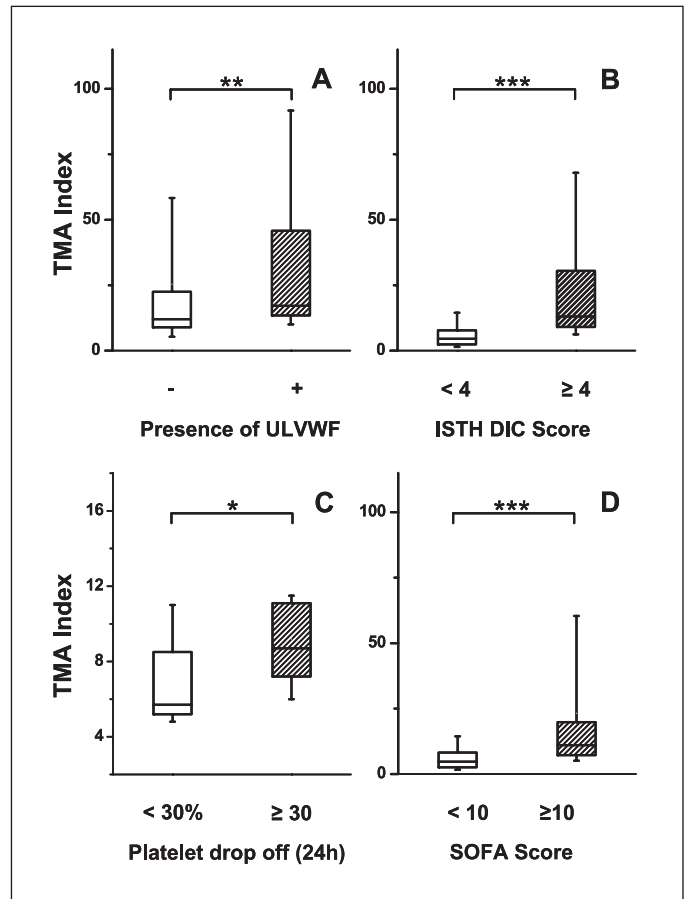
septic patients with diametrically opposed run in survivors (decrease) and non-survivors (increase) (Fig. 1P).

The plasma appearance of ULVWF is one condition for severe organ dysfunction in TTP. Presence of ULVWF was found in 75% of analysed patients with SIRS-associated organ dysfunction and in all septic patients (38). ULVWF disappeared in patients surviving sepsis, but persisted in non-survivors. The disappearance of ULVWF was accompanied by a substantial increase of ADAMTS13 activity over time in survivors to LLN while it successively decreased in non-survivors with a final drop to non-detectable levels (< 0.05 U/ml) two days prior to death (data not shown).

The appearance of ULVWF multimers was associated with an about two-fold increase in the TMA index (Fig. 2A). An association between an elevated TMA index and changes in haemostasis were also obvious. As shown in Figure 2B and C, a high ISTH-DIC score (> 4) or a drop of platelet count by more than 30 % within 24 h were associated with significantly higher TMA indices compared to situations when these criteria did not apply. Finally, TMA index increased with the severity of organ failure. Significantly 1.5-fold higher values were found when the SOFA score was above 10 compared to lower SOFA scores (Fig. 2D).

Furthermore, in a subgroup analysis ADAMTS13 activity was significantly diminished and TMA index was increased when (i) ULVWF multimers were present in plasma, when (ii) platelet count was < 100 Gpt/, when (iii) patients developed organ failure as indicated by elevated SOFA score or when (iv) DIC became obvious indicated by an increased ISTH-DIC score ≥4 points. A similar power to discriminate between the subgroups as mentioned above was observed for VWF:Ag content platelet count and ISTH-DIC score (Table 2).

In contrast, the differences between the subgroups were much less pronounced or even lacking when test that are established to describe functional activities of VWF were performed. The differences in VWF:CB/VWF:Ag ratio did not exceed a level of 20% in either subgroup and the level of significance was much lower when compared to ADAMTS13 activity or TMA index. For comparison, the differences in TMA index in the subgroups amounted to up two-fold. The VWF:RCV/VWF:Ag ratio completely failed to discriminate between the subgroups (Table 2).



**Figure 2: TMA index, hypercoagulopathy and organ failure.** In patients with organ failure or severe sepsis elevated values for TMA index were associated with the presence of ULVWF (A), increased ISTH-DIC Score (B), a drop in platelet count ≥30% within 24 h in comparison to a drop off < 30 % or an increase (C) as well as the severity of the disease as determined by SOFA Score (D). Levels of significance are indicated by asterisks: \*, p < 0.05; \*\*, p < 0.01.

Overall, ADAMTS13 activity was not correlated with VWF:Ag concentration without consideration of association into subgroups (Fig. 3). Surprisingly, correlation analysis found positive correlation between ADAMTS13 and VWF:Ag content (r<sup>2</sup>=0.61, slope 0.893) in the subgroup of healthy individuals who underwent strenuous physical exercise with subsequent moderate systemic inflammation, which is in contrast to results analysed in patients with a more pronounced inflammatory state obtained in the present study or from other groups (21, 37).

When all measurements of ADAMTS activity and VWF:Ag concentration were considered together there was a negative correlation in patients with decreased ADAMTS13 activity, i.e. in patients with sepsis or post-operative SIRS and organ failure. In contrast, in healthy patients, who underwent strenuous physical exercise there was a highly significant positive correlation between ADAMTS13 activity and VWF:Ag concentration (Fig. 3).

The value of TMA index as an indicator for the plasma presence of ULVWF was further evaluated by logistic regression analysis including all parameters listed in Table 2 with the addition of D-dimer concentration. In a model of stepwise reduction

	A) ULVWF		B) Platelet count	
	No	Yes	> 100Gpt/l	< 100 Gpt/l
	n=31	n=27	n=175	n=145
ADAMTS 13 (U/ml)	0.27 ± 0.11	0.17 ± 0.08**	0.49 ± 0.40	0.26 ± 0.12**
VWF:Ag (U/ml)	4.10 ± 2.03	4.83 ± 2.58**	2.72 ± 2.09	4.43 ± 2.29**
VWF:CB/VWF:Ag	0.90 ± 0.32	1.06 ± 0.33*	1.13 ± 0.51	0.94 ± 0.33*
VWF:Rco/VWF:Ag	0.93 ± 0.21	0.97 ± 0.17	1.02 ± 0.35	0.93 ± 0.46
TMA index	25.3 ± 29.1	37.2 ± 38.4**	11.9 ± 18.8	26.5 ± 29.6**
Platelet count (Gpt/l)	108 ± 36	75.6 ± 42.3**	n.d.	n.d.
ISTH DIC score	3.71 ± 0.82	4.52 ± 1.19**	3.09 ± 0.95	4.49 ± 0.92**
	C) SOFA score		D) ISTH DIC	
	< 10	≥10	< 4	≥4
	n=136	n=121	n=128	n=111
ADAMTS 13 (U/ml)	0.37 ± 0.14	0.26 ± 0.12**	0.36 ± 0.16	0.26 ± 0.11**
VWF:Ag (U/ml)	2.71 ± 1.42	4.41 ± 2.47**	2.83 ± 2.17	4.38 ± 2.13**
VWF:CB/VWF:Ag	1.18 ± 0.52	0.99 ± 0.36*	1.17 ± 0.52	0.98 ± 0.38*
VWF:Rco/VWF:Ag	1.05 ± 0.34	0.96 ± 0.43	1.01 ± 0.34	0.95 ± 0.45
TMA index	10.2 ± 12.6	26.4 ± 29.4**	13.0 ± 19.7	25.3 ± 28.0**
Platelet count (Gpt/l)	189 ± 140	105 ± 69**	n.d.	n.d.
ISTH DIC score	3.29 ± 1.19	4.04 ± 1.01**	n.d.	n.d.

**Table 2: Comparison of laboratory and clinical parameters at different clinical situations (discriminators).** A) Presence or absence of ULVWF; B) Thrombocytopenia, cut-off of platelet count 100 Gpt/l; C) Severity of clinical course as determined by the use of SOFA score, cut-off value 10 pts.; D) Manifestation of overt DIC by the use of an modified ISTH DIC Score, cut-off value 4 pts. Data are given as mean ± SD. Levels of significance were determined using t-test and indicated by asterisks: \*  $p < 0.0001$ , \*\*  $p < 0.000001$ . Abbreviations: VWF:Ag, VWF antigen; VWF:CB, VWF collagen binding activity; VWF:Rco, VWF Ristocetin cofactor activity; TMA index, index for thrombotic microangiopathy, defined as the ratio of VWF:Ag and ADAMTS13 activity; ISTH DIC score, score for diagnosis of disseminated intravascular coagulation as published by the International Society on Thrombosis and Haemostasis (ISTH).

of variables (conditional backwards) a significant impact on the presence of ULVWF was found for ADAMTS13 ( $p=0.013$ ), ISTH-DIC- score and D-dimers ( $p=0.021$ ) as well as TMA index ( $p=0.038$ ). The association between TMA index and organ failure became also evident from a linear stepwise regression analysis: with the parameters listed in Table 2 as well as D-dimer concentration as independent variables only platelet count and TMA index were proved to be significantly correlated with SOFA score ( $p<0.00001$  for both).

## Discussion

In a side-by-side approach, we have performed a study on VWF and related parameters in a comprehensive cohort of individuals with systemic inflammation of varying severity and origin. Highly elevated levels of VWF were found, which were strongly associated with the severity of the disease. The elevated levels possibly reflect endothelial dysfunction, which has been shown to be relevant in the course of the disease (9, 39). Postoperative VWF levels were significantly enhanced to similar extent in both surgery groups, ON- as well as OFF-pump surgery, which is in accordance with previous results of Lo et al. (40). These results are also consistent with previous data from our group obtained in a model of systemic inflammatory response subsequent to extracorporeal circulation (41).

VWF is secreted as ultralarge multimers from endothelial cells and anchored to the surface as extraordinarily long string-forming structures under the conditions of shear stress (42). The proteolysis of VWF by ADAMTS13 diminishes the size of plasma VWF to a series of multimers with limited functional activity.

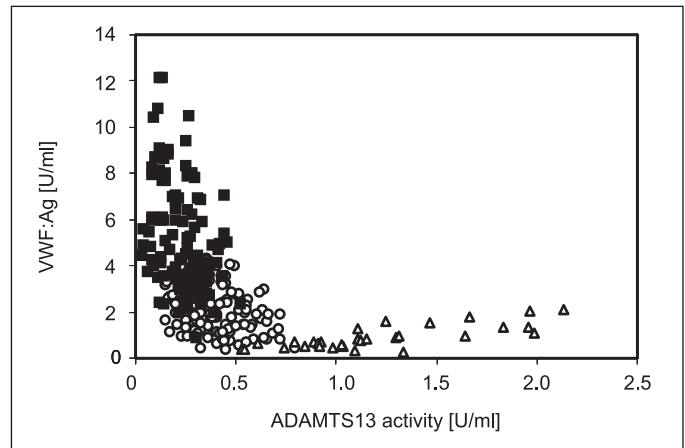
Severe deficiency of ADAMTS13 with subsequent increase in VWF-multimeric size is considered as a trigger of TMA in TTP patients (14, 29, 43). TMA, i.e. formation of platelet-rich thrombi in the microcirculation is believed to contribute to the development of organ failure in patients with severe inflammation, especially with sepsis (20–22). Studying patients and healthy volunteers with varying degrees of systemic inflammation and inflammation associated organ dysfunction we observed a positive association between VWF:Ag concentration and the severity of inflammation which is probably caused by endothelial activation and dysfunction (9, 39, 44). We could also show that ADAMTS13 activity decreases with increasing severity of inflammation and an extreme imbalance between ADAMTS13 activity and VWF:Ag concentration as found in some patients with severe sepsis/septic shock or SIRS with organ failure was associated with the presence of ULVWF in plasma. This observation is in accordance with studies indicating an inverse correlation between ADAMTS13 activity and VWF:Ag concentration or functional activity after administration of endotoxin or DDAVP to healthy volunteers (45, 46). The discrepant data about a positive correlation between VWF:Ag level and ADAMTS13 activity in healthy individuals may be explained by a yet unknown compensatory mechanism which is only active in healthy individuals undergoing a very moderate systemic inflammatory insult. Otherwise, a postoperative decrease in ADAMTS13 independent of cardiac surgical procedures was also observed, whereas VWF:Ag and VWF:CB were both increased (19). In line with other studies, it has become clear that the functional capacity of VWF in these patients is enhanced.

Various mechanisms can be discussed as cause of the decrease in ADAMTS13 activity in systemic inflammation. ADAMTS13 is expressed and secreted from hepatic stellate cells as well as endothelial cells (12, 13, 47). Severe systemic inflammation is known to be associated with dysfunction of both endothelium and liver (48, 49), and both cytokinemia and cellular damage may contribute to decreased ADAMTS13 activity (50, 51). It has been also shown that thrombin, one principal factor in coagulation as well as IL-6, one of the key mediators of inflammation, are capable to degrade ADAMTS13 protein (52–54). Finally, there is also evidence that ADAMTS13 is an enzyme that becomes inactive after cleaving its substrate (55). In critically ill patients, hepatic dysfunction may be one cause of decreased ADAMTS13 activity in plasma, which is reflected in our study by correlation with a commonly used variable (albumin) to monitor liver function. The increased VWF levels and the diminished proteolytic activity are in accordance with common complications in patients with advanced liver dysfunction, i.e. thrombocytopenia and a hypercoagulopathic state with thrombogenesis in portal and hepatic veins (56) corresponding to ULVWF appearance in plasma (17).

Severe thrombocytopenia or an acute drop in platelet count are often present in patients with sepsis and are associated with poor prognosis (57). Not only the presence, but also the biological activity of ULVWF is held responsible as one pathophysiological mechanism for this phenomenon (11). Elevated levels of VWF in sepsis may be a consequence of endothelial perturbation caused by bacterial infection and shear stress; however, little is known on the functional capacity of the protein in these patients.

There are certain limitations in measuring VWF levels and related parameters (i.e. VWF:CB and VWF:RCo) to detect highly active forms of VWF in patients with severe inflammation, especially in sepsis. The tests were validated to measure the biological activity of VWF under conditions of decreased or normal VWF:Ag levels as in von Willebrand disease or TTP. As VWF is an acute phase protein, its plasma level is tremendously increased in systemic inflammation, and plasma samples have to be diluted for measurement of both the VWF:Ag and VWF activity, i.e. VWF:CB or VWF:RCo. In particular when calculating activity/antigen ratios one has to consider a very high bias. Accordingly, in our study the ratio showed no or only weak association with the presence of ULVWF, DIC or organ failure.

Although ADAMTS13 controls the size of VWF, an enzyme deficiency must not necessarily result in TMA that may develop not only in TTP but also in other clinical conditions including severe systemic inflammation (16). As the detection of ULVWF is rather laborious one has to look for other markers for TMA development. As a marked dysbalance between VWF:Ag concentration and ADAMTS13 activity may cause and even predict the presence of ULVWF, we proposed to calculate a TMA development index (TMA index) defined as the ratio VWF:Ag/ADAMTS13. Determination of the TMA-index is less time consuming in relation to electrophoretic visualization of VWF. At least in in-vitro studies, a good and nearly linear correlation between ULVWF content and specific activities as determined by VWF:RCo has been shown (36). Table 3 lists TMA indices that are calculated from data described in the literature for various clinical conditions. In healthy controls TMA indices amounted



**Figure 3: Analysis of ADAMTS13 activity and VWF:Ag level in plasma of patients with systemic inflammation.** ADAMTS13 activity and VWF:antigen concentration in the plasma of healthy individuals subsequent to strenuous physical exercise (open triangles; normalised to basal values of 45 healthy individuals), in patients with post-operative SIRS with and without organ failure (open circles) or patients with severe sepsis/septic shock (closed rectangles) were determined as described in *Study design and methods*. Correlation of proteolytic activity and substrate level ( $r^2=0.61$ , slope 0.893) was determined in individuals who underwent physical exercise by Pearson testing.

**Table 3: TMA Indices in different entities of disease.** Data are extracted from cited references with regard to given standard deviations. TTP (INH<sup>+</sup>): inhibitor positive thrombotic thrombocytopenic purpura. Calculations on the basis of the given 1<sup>st</sup>/3<sup>rd</sup> quartiles are indicated by a symbol (‡).

Diagnosis	Patients	Controls	Reference
Systemic lupus erythematosus	0.8 – 4.3‡	0.5 – 1.9‡	[60]
1 hour after DDAVP infusion	4.2 – 6.1‡	0.5 – 0.9‡	[45]
HELLP-Syndrome	5.0 – 35.2	0.3 – 3.9	[61]
4 days after abdominal surgery	0.7 – 16.7	0.3 – 4.3	[37]
Chronic renal insufficiency	0.5 – 33.6	0.3 – 4.3	[37]
Acute infection	2.1 – 40.7	0.3 – 4.3	[37]
Liver cirrhosis	1.6 – 293.3	0.3 – 4.3	[17, 37]
Organ failure	2.1 – 5.1‡	0.5 – 1.2‡	[22]
Sepsis	3.2 – 9.9‡	0.5 – 1.2‡	[22]
TTP (INH <sup>+</sup> )	> 15	0.6 – 3.6	[43]

between 0.3 and 4.3. For TTP with manifested TMA, a TMA index > 15 has been calculated (43). In our patients with circulating ULVWF, a platelet count <100 Gpt/l, organ failure (SOFA score ≥10) or ISTH-DIC score ≥4 exhibited a mean TMA index > 15. However, as the variation coefficients of the indices in the various subgroups are rather high, future work is needed to define a cut-off level of TMA index that may be more precisely indicative for development of TMA and organ failure in systemic inflammation.

Surveying clinical conditions with altered TMA index, extremely elevated values were confirmed in patients with organ

failure and sepsis, at the presence of ULVWF, *prior* to platelet consumption, and during overt DIC, suggesting that an elevated TMA index might indicate ULVWF triggered microthrombus formation. According to our findings, TMA index might be more useful for diagnosis and therapeutically monitoring than VWF multimer analysis, requiring specially equipped laboratories for its determination. Best correlational data with regards to ULVWF abundance, and both the extent of organ failure or coagulopathy were obtained by use of variables describing VWF plasma concentration, ADAMTS13 activity and platelet count.

ADAMTS13 is the only enzyme held responsible for proteolytic degradation of VWF. The resulting steady-state distribution of multimers reflects equilibrium between the inflammation-triggered secretion of ULVWF and their proteolysis into smaller, rather inactive multimers. Beyond the unchanged VWF:RCO/VWF:Ag ratio, the TMA index as well as the increased portion of circulating ULVWF support the concept that the reciprocal behaviour of VWF function and ADAMTS13 activity specifies a discriminating value regarding disease severity and organ involvement (20, 21, 58). Plasma levels of ADAMTS13 do not regularly coincide with those of the immuno-reactive protease (59) assuming an circulating, proteolytic inactive amount of the enzyme. Therefore we refrained from analyzing a crude ADAMTS13 antigen content without any information on functional activity.

Summarising, it could be shown that systemic inflammation results in a dysbalance between plasma concentration of VWF and its degrading protease ADAMTS13. This imbalance increases the risk of the persistence of ULVWF as well as the risk of developing TMA and organ failure. We propose that the calculation of a 'TMA development index' defined as the ratio between ADAMTS13 activity and VWF:Ag may function as a helpful tool to identify patients with high risk for TMA and organ failure due to systemic inflammation or other conditions with an imbalance between VWF and ADAMTS13. The methods used in this combined approach are more sensitive determining artefacts than the most widely used assays analyzing the functional activity and are not restricted to specialised laboratories for the presentation of multimeric composition. The dysbalanced VWF/ADAMTS13 system is definitively assumed to play a crucial pathophysiological factor in the aetiology of sepsis-associated TMA; however, the predictive value of the proposed TMA index as a sensitive marker of TMA development remains to be determined in well controlled prospective studies.

### Acknowledgements

The authors would like to thank Edith Walther and Brigitte Specht (Jena) for expert technical assistance.

### References

- Aird WC The role of the endothelium in severe sepsis and multiple organ dysfunction syndrome. *Blood* 2003; 101: 3765–3777.
- Aird WC Endothelium as a therapeutic target in sepsis. *Curr Drug Targets* 2007; 8: 501–507.
- Hotchkiss RS, Karl IE The pathophysiology and treatment of sepsis. *N Engl J Med* 2003; 348: 138–150.
- Russell JA Management of sepsis. *N Engl J Med* 2006; 355: 1699–1713.
- Angus DC, Linde-Zwirble WT, Lidicker J, et al. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. *Crit Care Med* 2001; 29: 1303–1310.
- Angus DC, Pereira CA, Silva E Epidemiology of severe sepsis around the world. *Endocr Metab Immune Disord Drug Targets* 2006; 6: 207–212.
- Anname D, Aegerter P, Jars-Guincestre MC, et al. Current epidemiology of septic shock: the CUB-Rea Network. *Am J Respir Crit Care Med* 2003; 168: 165–172.
- Ware LB, Eisner MD, Thompson BT, et al. Significance of von Willebrand factor in septic and nonseptic patients with acute lung injury. *Am J Respir Crit Care Med* 2004; 170: 766–772.
- Kayal S, Jais JP, Aguin N, et al. Elevated circulating E-selectin, intercellular adhesion molecule 1, and von Willebrand factor in patients with severe infection. *Am J Respir Crit Care Med* 1998; 157: 776–784.
- Sadler JE Biochemistry and genetics of von Willebrand factor. *Annu Rev Biochem* 1998; 67: 395–424.
- Arya M, Anvari B, Romo GM, et al. Ultralarge multimers of von Willebrand factor form spontaneous high-strength bonds with the platelet glycoprotein Ib-IX complex: studies using optical tweezers. *Blood* 2002; 99: 3971–3977.
- Turner N, Nolasco L, Tao Z, et al. Human endothelial cells synthesize and release ADAMTS-13. *J Thromb Haemost* 2006; 4: 1396–1404.
- Uemura M, Tatsumi K, Matsumoto M, et al. Localization of ADAMTS13 to the stellate cells of human liver. *Blood* 2005; 106: 922–924.
- Levy GG, Nichols WC, Lian EC, et al. Mutations in a member of the ADAMTS gene family cause thrombotic thrombocytopenic purpura. *Nature* 2001; 413: 488–494.
- Ruggeri ZM Von Willebrand factor, platelets and endothelial cell interactions. *J Thromb Haemost* 2003; 1: 1335–1342.
- George JN Clinical insights from observations on ADAMTS13 deficiency in liver cirrhosis. *Thromb Haemost* 2008; 99: 987–988.
- Uemura M, Fujimura Y, Matsumoto M, et al. Comprehensive analysis of ADAMTS13 in patients with liver cirrhosis. *Thromb Haemost* 2008; 99: 1019–1029.
- Claus RA, Bockmeyer CL, Sossdorf M, et al. Physical stress as a model to study variations in ADAMTS-13 activity, von Willebrand factor level and platelet activation. *J Thromb Haemost* 2006; 4: 902–905.
- Mannucci PM, Parolari A, Canciani MT, et al. Opposite changes of ADAMTS-13 and von Willebrand factor after cardiac surgery. *J Thromb Haemost* 2005; 3: 397–399.
- Bockmeyer CL, Claus RA, Budde U, et al. Inflammation-associated ADAMTS13 deficiency promotes formation of ultra-large von Willebrand factor. *Haematologica* 2008; 93: 137–140.
- Kremer Hovinga JA, Zeerleder S, Kessler P, et al. ADAMTS-13, von Willebrand factor and related parameters in severe sepsis and septic shock. *J Thromb Haemost* 2007; 5: 2284–2290.
- Martin K, Borgel D, Lerolle N, et al. Decreased ADAMTS-13 (A disintegrin-like and metalloprotease with thrombospondin type 1 repeats) is associated with a poor prognosis in sepsis-induced organ failure. *Crit Care Med* 2007; 35: 2375–2382.
- Tsai HM Molecular mechanisms in thrombotic thrombocytopenic purpura. *Semin Thromb Hemost* 2004; 30: 549–557.
- Xie L, Chesterman CN, Hogg PJ Control of von Willebrand factor multimer size by thrombospondin-1. *J Exp Med* 2001; 193: 1341–1349.
- Ruggeri ZM, Zimmerman TS The complex multimeric composition of factor VIII/von Willebrand factor. *Blood* 1981; 57: 1140–1143.
- Budde U, Schneppenheim R, Plendl H, et al. Luminographic detection of von Willebrand factor multimers in agarose gels and on nitrocellulose membranes. *Thromb Haemost* 1990; 63: 312–315.
- Schneppenheim R, Plendl H, Budde U Luminography--an alternative assay for detection of von Willebrand factor multimers. *Thromb Haemost* 1988; 60: 133–136.
- Adcock DM, Bethel M, Valcour A Diagnosing von Willebrand disease: a large reference laboratory's perspective. *Semin Thromb Hemost* 2006; 32: 472–479.
- Franchini M, Montagnana M, Targher G, et al. Reduced von Willebrand factor-cleaving protease levels in secondary thrombotic microangiopathies and other diseases. *Semin Thromb Hemost* 2007; 33: 787–797.
- Roques F, Michel P, Goldstone AR, et al. The logistic EuroSCORE. *Eur Heart J* 2003; 24: 881–882.
- American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference: definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. *Crit Care Med* 1992; 20: 864–874.
- Schneppenheim R, Budde U, Oyen F, et al. von Willebrand factor cleaving protease and ADAMTS13 mutations in childhood TTP. *Blood* 2003; 101: 1845–1850.
- Tripodi A, Chantarangkul V, Bohm M, et al. Measurement of von Willebrand factor cleaving pro-

- tease (ADAMTS-13): results of an international collaborative study involving 11 methods testing the same set of coded plasmas. *J Thromb Haemost* 2004; 2: 1601–1609.
34. Taylor FB, Jr., Toh CH, Hoots WK, et al. Towards definition, clinical and laboratory criteria, and a scoring system for disseminated intravascular coagulation. *Thromb Haemost* 2001; 86: 1327–1330.
35. Bernard GR, Vincent JL, Laterre PF, et al. Efficacy and safety of recombinant human activated protein C for severe sepsis. *N Engl J Med* 2001; 344: 699–709.
36. Budde U, Metzner HJ, Muller HG Comparative analysis and classification of von Willebrand factor/factor VIII concentrates: impact on treatment of patients with von Willebrand disease. *Semin Thromb Hemost* 2006; 32: 626–635.
37. Mannucci PM, Canciani MT, Forza I, et al. Changes in health and disease of the metalloprotease that cleaves von Willebrand factor. *Blood* 2001; 98: 2730–2735.
38. Moake JL Thrombotic microangiopathies. *N Engl J Med* 2002; 347: 589–600.
39. Wada H, Mori Y, Shimura M, et al. Poor outcome in disseminated intravascular coagulation or thrombotic thrombocytopenic purpura patients with severe vascular endothelial cell injuries. *Am J Hematol* 1998; 58: 189–194.
40. Lo B, Nierich AP, Kalkman CJ, et al. Relatively increased von Willebrand factor activity after off-pump coronary artery bypass graft surgery. *Thromb Haemost* 2007; 97: 21–26.
41. Tomic V, Russwurm S, Moller E, et al. Transcriptomic and proteomic patterns of systemic inflammation in on-pump and off-pump coronary artery bypass grafting. *Circulation* 2005; 112: 2912–2920.
42. Dong JF Cleavage of ultra-large von Willebrand factor by ADAMTS-13 under flow conditions. *J Thromb Haemost* 2005; 3: 1710–1716.
43. Kobayashi T, Wada H, Nishioka N, et al. ADAMTS13 related markers and von Willebrand factor in plasma from patients with thrombotic microangiopathy (TMA). *Thromb Res* 2008; 121: 849–854.
44. Rondaj MG, Bierings R, Kragt A, et al. Dynamics and plasticity of Weibel-Palade bodies in endothelial cells. *Arterioscler Thromb Vasc Biol* 2006; 26: 1002–1007.
45. Reiter RA, Knobl P, Varadi K, et al. Changes in von Willebrand factor-cleaving protease (ADAMTS13) activity after infusion of desmopressin. *Blood* 2003; 101: 946–948.
46. Reiter RA, Varadi K, Turecek PL, et al. Changes in ADAMTS13 (von-Willebrand-factor-cleaving protease) activity after induced release of von Willebrand factor during acute systemic inflammation. *Thromb Haemost* 2005; 93: 554–558.
47. Zhou W, Inada M, Lee TP, et al. ADAMTS13 is expressed in hepatic stellate cells. *Lab Invest* 2005; 85: 780–788.
48. Schouten M, Wiersinga WJ, Levi M, et al. Inflammation, endothelium, and coagulation in sepsis. *J Leukoc Biol* 2008; 83: 536–545.
49. Jarrar D, Chaudry IH, Wang P Organ dysfunction following hemorrhage and sepsis: mechanisms and therapeutic approaches (Review). *Int J Mol Med* 1999; 4: 575–583.
50. Kume Y, Ikeda H, Inoue M, et al. Hepatic stellate cell damage may lead to decreased plasma ADAMTS13 activity in rats. *FEBS Lett* 2007; 581: 1631–1634.
51. Cao WJ, Niiya M, Zheng XW, et al. Inflammatory cytokines inhibit ADAMTS13 synthesis in hepatic stellate cells and endothelial cells. *J Thromb Haemost* 2008; 6: 1233–1235.
52. Bernardo A, Ball C, Nolasco L, et al. Effects of inflammatory cytokines on the release and cleavage of the endothelial cell-derived ultralarge von Willebrand factor multimers under flow. *Blood* 2004; 104: 100–106.
53. Lam JK, Chion CK, Zanardelli S, et al. Further characterization of ADAMTS-13 inactivation by thrombin. *J Thromb Haemost* 2007; 5: 1010–1018.
54. Crawley JT, Lam JK, Rance JB, et al. Proteolytic inactivation of ADAMTS13 by thrombin and plasmin. *Blood* 2005; 105: 1085–1093.
55. Mannucci PM, Capoferri C, Canciani MT Plasma levels of von Willebrand factor regulate ADAMTS-13, its major cleaving protease. *Br J Haematol* 2004; 126: 213–218.
56. Amitrano L, Guardascione MA, Brancaccio V, et al. Risk factors and clinical presentation of portal vein thrombosis in patients with liver cirrhosis. *J Hepatol* 2004; 40: 736–741.
57. Vanderschueren S, De Weerd A, Malbrain M, et al. Thrombocytopenia and prognosis in intensive care. *Crit Care Med* 2000; 28: 1871–1876.
58. Ono T, Mimuro J, Madoiwa S, et al. Severe secondary deficiency of von Willebrand factor-cleaving protease (ADAMTS13) in patients with sepsis-induced disseminated intravascular coagulation: its correlation with development of renal failure. *Blood* 2006; 107: 528–534.
59. Feys HB, Canciani MT, Peyvandi F, et al. ADAMTS13 activity to antigen ratio in physiological and pathological conditions associated with an increased risk of thrombosis. *Br J Haematol* 2007; 138: 534–540.
60. Mannucci PM, Vanoli M, Forza I, et al. Von Willebrand factor cleaving protease (ADAMTS-13) in 123 patients with connective tissue diseases (systemic lupus erythematosus and systemic sclerosis). *Haematologica* 2003; 88: 914–918.
61. Lattuada A, Rossi E, Calzarossa C, et al. Mild to moderate reduction of a von Willebrand factor cleaving protease (ADAMTS-13) in pregnant women with HELLP microangiopathic syndrome. *Haematologica* 2003; 88: 1029–1034.