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ORIGINAL ARTICLE

Dynamics of von Willebrand factor reactivity in sickle cell disease during vaso-occlusive crisis and steady state

J. W. R. SINS,*† M. SCHIMMEL,* B. M. LUKEN,‡ E. NUR,* S. S. ZEERLEDER,*‡ C. F. J. VAN TUIJN,* D. P. M. BRANDJES,§ W. F. KOPATZ,¶ R. T. URBANUS,** J. C. M. MEIJERS,¶†† B. J. BIEMOND* and K. FIJNVANDRAAT†††

**Department of Hematology, Academic Medical Center, University of Amsterdam; †Department of Pediatric Hematology, Emma Children's Hospital, Academic Medical Center, University of Amsterdam; ‡Department of Immunopathology, Sanquin Research and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam; §Department of Internal Medicine, Slotervaart Hospital; ¶Department of Experimental Vascular Medicine, Academic Medical Center, University of Amsterdam, Amsterdam; **Department of Clinical Chemistry and Hematology, University Medical Center Utrecht, Utrecht; and ††Department of Plasma Proteins, Sanquin Research, Amsterdam, the Netherlands*

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Essentials

- The role of von Willebrand Factor (VWF) in the pathophysiology of sickle cell disease is unclear.
- We assessed markers of VWF during admission for vaso-occlusive crisis (VOC) and steady state.
- VWF reactivity was higher during VOC and was associated with inflammation and neutrophil activation.
- Hyper-adhesive VWF may promote VOC in sickle cell disease.

Summary. *Background:* Endothelial activation plays a central role in the pathophysiology of vaso-occlusion in sickle cell disease (SCD), facilitating adhesive interactions with circulating blood cells. Upon activation, various adhesive molecules are expressed, including von Willebrand factor (VWF). Increased VWF levels have been observed in patients with SCD during steady state. However, the role of VWF in the pathogenesis of SCD vaso-occlusion is unclear. *Objectives:* To longitudinally assess the quantity and reactivity of VWF and its regulating protease ADAMTS-13 during vaso-occlusive crisis (VOC). *Methods:* In this observational study, we obtained sequential blood samples in adult SCD patients during VOC. *Results:* VWF reactivity was significantly higher during VOC (active VWF, VWF glycoprotein Ib-binding activity, and high molecular weight multimers), whereas platelet count and levels of ADAMTS-13 antigen and ADAMTS-13 activity were concomitantly lower than during steady state. Levels of VWF antigen, VWF propeptide (VWF:pp) and ADAMTS-13 specific activity did not change during VOC. VWF reactivity correlated strongly with markers of inflammation and neutrophil activation, and was inversely correlated with the platelet count. In patients who developed acute chest syndrome, levels of VWF, VWF:pp and active, hyperadhesive VWF were significantly higher, whereas ADAMTS-13 activity was lower, than in patients without this complication. *Conclusions:* We provide the first evidence that VOC in SCD is associated with increased reactivity of VWF, without a pronounced ADAMTS-13 deficiency. This hyper-reactivity may be explained by resistance of VWF to proteolysis, secondary to processes such as inflammation and oxidative stress. Hyperadhesive VWF, scavenging blood cells in the microcirculation, may thereby amplify and sustain VOC in SCD.

Keywords: cell adhesion; inflammation; sickle cell disease; vascular endothelium; von Willebrand factor.

Correspondence: Joep Sins, MD, Academic Medical Center, Department of Pediatric Hematology, Room H7-227, Meibergdreef 9, 1105 AZ Amsterdam, the Netherlands.
Tel.: +31 20 566 1693.
E-mail: j.w.sins@amc.nl

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Introduction

Sickle cell disease (SCD) is a recessive hemoglobinopathy. Worldwide, > 300 000 infants with this disease are born each year [1]. SCD is characterized by chronic hemolytic anemia and recurrent vascular occlusion, resulting in frequent episodes of acute pain and secondary organ

damage [2–4]. The pathophysiology of these painful vaso-occlusive crises (VOCs) is complex and involves multiple processes, including inflammation, endothelial activation, increased cellular adhesion, and coagulation activation.

von Willebrand factor (VWF) is a multimeric glycoprotein. Among other adhesive molecules, it has been implicated in the pathophysiology of SCD vaso-occlusion. VWF plays a crucial role in hemostasis by mediating platelet adhesion and thrombus formation upon vascular damage [5]. Upon activation of the endothelium, large, hyperadhesive strands of VWF (ultralarge VWF multimers [ULVWFs]) are released into the bloodstream, that are highly effective in binding platelets, leukocytes, and sickle erythrocytes [6–10]. In addition, when exposed to increased shear stress and flow acceleration, these multimers are able to self-associate and form thick, long strands *in vitro*, thereby obstructing blood flow and adding further to cell adhesion [11]. The main regulator of VWF activity is the protease ADAMTS-13, which cleaves highly adhesive ULVWFs off the surfaces of activated endothelial cells, and cuts these strings into smaller and less adhesive multimers [12,13]. Disturbances in the delicate balance between VWF release and its cleavage by ADAMTS-13 may result in microvascular thrombosis, as observed in patients with thrombotic thrombocytopenic purpura, malaria, malignant hypertension, sepsis, and myocardial infarction [14–18].

Increased plasma levels of VWF have been reported in patients with SCD in steady state, indicative of chronic endothelial activation [19,20]. VWF in its latent, inactive conformation will generally not bind cells. However, in various pathologic conditions, elevated levels of active, hyperadhesive VWF (aVWF) have been observed and associated with thrombotic complications [21]. This aVWF is identified with a sensitive assay, involving specific binding of the A1 domain of VWF. This allows the quantification of all VWF in an active, platelet-binding conformation, thereby also including smaller VWF multimers [22]. aVWF has been identified as an independent predictor of mortality in patients with systemic inflammatory response syndrome [23].

Interestingly, a recent study of SCD patients in steady state demonstrated high levels of aVWF, which strongly correlated with the concentration of lactate dehydrogenase (LDH) [24]. The authors suggested that this increase in VWF reactivity in SCD may be caused by both higher secretion of VWF and reduced susceptibility of VWF to proteolysis. Reactive oxygen species, released by activated neutrophils, have been shown to inhibit VWF proteolysis through oxidation of the ADAMTS-13 cleavage site in VWF [25]. The increased presence of active VWF in SCD may promote adhesion of sickle erythrocytes in the microcirculation, slowing their transit and promoting deoxygenation, polymerization, hemolysis, and, potentially, vaso-occlusion.

On the basis of the available literature, we hypothesized that an increased concentration of active VWF, insufficiently restrained by ADAMTS-13, may contribute to vascular occlusion in SCD and may thereby represent a new therapeutic target. So far, no studies have systematically addressed the dynamics of VWF reactivity during VOC in SCD. In this observational study, we examined parameters of VWF and ADAMTS-13, including the reactivity and multimer composition of VWF, in a cohort of adult SCD patients during the course of admission for VOC. In addition, we evaluated correlations of these parameters with markers of hemolysis, inflammation, and neutrophil activation.

Methods

Study design and population

This was a prospective, observational cohort study. Between October 2012 and November 2013, consecutive, adult patients with SCD (HbSS, HbSβ^0, HbSβ^+, or HbSC) admitted for VOC in two hospitals in Amsterdam, the Netherlands were approached for participation within 24 h after admission. VOC was defined as musculoskeletal pain not otherwise explained and recognized as such by the patient. Acute chest syndrome (ACS) was defined by clinical symptoms including fever, respiratory symptoms, or chest pain, in combination with a new pulmonary infiltrate visible by X-ray imaging. Exclusion criteria for participation were pregnancy, active cancer, chronic HIV infection or blood transfusion in the 3 months prior to admission. Blood samples were obtained on the morning following hospital admission (day 1), and on subsequent mornings on days 2, 3 and 5 of the admission after overnight fasting. In addition, a steady-state blood sample was drawn in the outpatient clinic at least 4 weeks after discharge. Inclusion was limited to a maximum of two separate episodes of VOC per patient. The study was approved by the Medical Ethics Committee of the participating centers, and conducted in agreement with the Helsinki declaration. Written informed consent was obtained from all patients.

VWF and ADAMTS-13 parameters

To assess the reactivity of VWF, both aVWF, VWF glycoprotein (GP) Ib-binding activity (VWF:GPIbM) and the percentage of high molecular weight (HMW) VWF multimers were measured. Levels of aVWF (previously also reported as total active VWF) were measured by ELISA with a llama-derived single-domain antibody (AU/VWFa-11) directed against the A1 domain of VWF, as described previously [23]. The absolute concentration of aVWF was estimated by the use of a calibration curve, obtained by diluting recombinant VWF type 2B (R1306Q) in VWF-deficient plasma. The concentration of aVWF in plasma pooled from at least 250 healthy individuals was determined to be 45 ng mL^{-1}. Additionally, VWF:GPIbM [26] was measured with the INNOVANCE assay (Siemens

Healthcare GmbH, Erlangen, Germany), employing latex particles and gain of function recombinant GPIb to facilitate VWF binding and agglutination [27]. The VWF multimeric pattern was assessed by agarose gel electrophoresis followed by western blotting with a polyclonal VWF antibody [28]. VWF multimers were quantified by densitometric analysis of electrophoresis bands. The proportion of HMW VWF multimers was defined as the area under the curve beyond band 12 as compared with the total area under the curve by the use of IMAGE LAB software (Bio-Rad Laboratories, Hercules, CA, USA). VWF antigen (VWF:ag) was measured by ELISA with commercial antibodies (Dako, Glostrup, Denmark) calibrated to the World Health Organization 07/316 6th International Standard. VWF propeptide (VWF:pp) concentrations were measured by ELISA as described previously [29]. ADAMTS-13 antigen (ADAMTS-13:ag) concentrations were also measured by ELISA as described previously [23,29]. ADAMTS-13 activity (ADAMTS-13:act) was measured by determination of the degree of degradation of purified VWF from plasma, whereby total and residual VWF were quantified as VWF:GPIbM, with the INNOVANCE assay as described above [30]. Pooled, normal plasma was used as a standard for this. ADAMTS-13 specific activity was defined as the ratio between ADAMTS-13:act and ADAMTS-13:ag.

Data on markers of inflammation (C-reactive protein [CRP]) and neutrophil activation (human neutrophil elastase–α1-antitrypsin complexes [HNE–α1-ATs] and calprotectin) on VOC admission day 1 were measured by ELISA as described previously [31–33].

Statistical analysis

Values per time point of the study parameters were presented as medians with interquartile ranges (IQRs). Laboratory values during VOC were compared with steady state by paired, direct comparisons, expressed as relative, percentage change from steady state. Owing to the non-parametric distribution of these data, the median changes with corresponding 95% confidence intervals (CIs) were estimated with the Hodges–Lehmann estimator. For patients with two admissions, only the values of the first admission were included in the paired analysis. Unpaired comparisons between genotype subgroups per time point were assessed with a Mann–Whitney *U*-test. In addition, the median differences in the study parameters between uncomplicated admissions for VOC and admissions complicated by ACS was also estimated with the Hodges-Lehmann estimator with corresponding 95% CIs. Explorative correlation analysis of VWF and ADAMTS-13 parameters with various pathophysiologic markers was performed by means of Spearman's rank test. Time points with missing data were excluded from the analysis. A *P*-value of < 0.05 or a CI not containing 0 was considered to be statistically significant.

Results

Baseline characteristics

The baseline characteristics of this study cohort are summarized in Table 1. In short, a total of 24 patients were included upon admission for VOC. Eight patients were included twice with separate admissions (six HbSS/HbSβ^0 patients and two HbSC/HbSβ$^+$ patients), accounting for 32 VOC admissions in total. The median age of the patients was 27 years (IQR 24–30), and 50% of the patients were female. Six of the 24 patients were receiving hydroxyurea during the study (all HbSS/HbSβ^0 patients; median dose of 15 mg kg^{-1}). The median length of hospitalization over all 32 admissions was 6 days (IQR 3–8). The subset of patients for whom a sample of admission day 5 ($N = 13$) was available presumably had a more severe VOC course, as almost half of the patients had already been discharged at this time point. The median time between the first onset of pain at home and the first blood sample during admission for VOC was 61 h (IQR 40–85), and did not differ between the HbSS/HbSβ^0 and HbSC/HbSβ$^+$ patients ($P = 0.79$). Five patients developed an ACS during admission (four HbSS patients and one HbSβ$^+$ patient).

Longitudinal analysis

Table 2 shows the relative, median changes in the various VWF and ADAMTS-13 parameters from steady state to VOC days 1, 2, and 3 (paired analysis). Most strikingly, markers of VWF reactivity were higher during the course of admission than during steady state. These differences reached significance for aVWF on day 1 (relative change of 33%, 95% CI 5–70), day 2 (30%, 95% CI 9–67), and

Table 1 Baseline characteristics

	N (%) or median (IQR)
Patients – total	24
Admissions – total	32
Age (years)	27 (24–30)
Female sex	12 (50)
Hemoglobin genotype	
HbSS/HbSβ^0	16 (67)
HbSC/HbSβ$^+$	8 (33)
Use of hydroxycarbamide	6 (25)
Steady-state laboratory markers	
Hb (g dL^{-1})*	6.1 (5.3–6.9)
Leukocyte count (× 10^9 L^{-1})*	8.6 (6.3–9.4)
Platelet count (× 10^9 L^{-1})†	306 (235–416)
LDH (U L^{-1})‡	362 (230–433)
Total bilirubin (mg dL^{-1})*	27 (19–44)
CRP (mg L^{-1})	2.9 (1.8–7.1)

CRP, C-reactive protein; IQR, interquartile range; LDH, lactate dehydrogenase. *Steady-state data missing for two patients ($N = 22$). †Steady-state data missing for nine patients ($N = 15$). ‡Steady-state data missing for four patients ($N = 20$).

Table 2 Relative, median changes in von Willebrand factor (VWF) and ADAMTS-13 parameters from steady state to vaso-occlusive crisis (VOC) admission days 1, 2, and 3 (paired comparisons of patients with data available for both steady state and the respective time points)

	Relative change from steady state					
	VOC day 1 (N = 19)		VOC day 2 (N = 20)		VOC day 3 (N = 13)	
	Change (%)	95% CI	Change (%)	95% CI	Change (%)	95% CI
VWF:ag*	− 8	− 16 to 9	− 1	− 8 to 8	2	− 9 to 11
VWF:pp†	9	− 9 to 32	14	− 3 to 30	7	− 14 to 27
VWF:pp/VWF:ag†	11	− 4 to 29	14	− 5 to 31	3	− 20 to 20
aVWF	**33**	**5** to **70**	**30**	**9** to **67**	**35**	**12** to **63**
aVWF/VWF:ag*	**36**	**17** to **64**	**32**	**13** to **66**	**37**	**4** to **62**
VWF:GPIbM	8	− 4 to 20	**13**	**1** to **27**	**18**	**1** to **43**
VWF:GPIbM/VWF:ag*	**11**	**2** to **23**	**9**	**1** to **27**	**17**	**1** to **43**
HMW multimers*	10	− 2 to 28	**16**	**6** to **32**	**10**	**1** to **42**
ADAMTS-13:ag*	− 4	− 15 to 9	− **11**	− **22** to − **1**	− 8	− 23 to 17
ADAMTS-13:ag/VWF:ag‡	3	− 13 to 19	− 11	− 26 to 4	− 7	− 21 to 13
ADAMTS-13:act§	− **7**	− **14** to **0**	− **10**	− **16** to − **2**	− 6	− 20 to 8
ADAMTS-13:act/VWF:GPIbM¶	− **10**	− **21** to **0**	− **19**	− **28** to − **9**	− **18**	− **33** to − **6**
ADAMTS-13 specific activity**	− 1	− 10 to 9	0	− 9 to 12	− 1	− 14 to 24
Platelets††	− **22**	− **53** to − **10**	− 25	− 65 to 55	− **23**	− **80** to − **14**

ADAMTS-13:act, ADAMTS-13 activity; ADAMTS-13:ag, ADAMTS-13 antigen; aVWF, active, hyperadhesive von Willebrand factor; CI, confidence interval; HMW, high molecular weight; VWF:ag, von Willebrand factor antigen; VWF:GPIbM, von Willebrand factor glycoprotein Ib-binding activity; VWF:pp, von Willebrand factor propeptide. Significant values are in bold. The data on VOC day 1 include 13 HbSS/HbSβ0 and six HbSC/HbSβ+ patients, those on VOC day 2 include 12 HbSS/HbSβ0 and eight HbSC/HbSβ+ patients, and those on VOC day 3 include seven HbSS/HbSβ0 and six HbSC/HbSβ+ patients. Data for day 5 are shown in Table S2. *VOC day 1, N = 18; VOC day 2, N = 19. †VOC day 1, N = 17; VOC day 2, N = 17; ‡VOC day 1, N = 17; VOC day 2, N = 18. §VOC day 2, N = 19. ¶VOC day 1, N = 19; VOC day 2, N = 19. **VOC day 1, N = 18; VOC day 2, N = 18. ††VOC day 1, N = 9; VOC day 2, N = 6; VOC day 3, N = 4; VOC day 5, N = 2.

day 3 (35%, 95% CI 12–63), and for HMW multimers on days 2 and 3 (Table 2; relative changes, respectively, of 16% [95% CI 6–32] and 10% [95% CI 1–42]). The change in VWF:GPIbM reached significance on day 2 only (Table 2; relative change of 13%, 95% CI 1–27). Similarly, both the aVWF/VWF:ag ratio and the VWF:GPIbM/VWF:ag ratio were significantly higher on days 1 and 2 of admission than during steady state (Table 2; relative changes, respectively, of 36% [95% CI 17–64] and 32% [95% CI 13–66], and of 11% [95% CI 2–23] and 9% [95% CI 1–27]). In addition, the trend of these parameters from steady state through the course of admission is shown in Fig. 1 (medians per time point).

Inversely, ADAMTS-13:ag, ADAMTS-13:act, the ADAMTS-13:act/VWF:GPIbM ratio and the platelet count were significantly lower during hospital admission than during steady state (Fig. 1D,E,G,H), reaching statistical significance on day 1 for platelets (Table 2; relative change of − 22%, 95% CI − 53 to −10), on day 2 for ADAMTS-13:ag (Table 2; relative change of − 11%, 95% CI − 22 to − 1) and ADAMTS-13:act (Table 2; relative change of − 10%, 95% CI − 16 to − 2), and on days 2 and 3 for the ADAMTS-13:act/VWF:GPIbM ratio (Table 2; relative changes, respectively, of − 19% [95% CI − 28 to − 9) and − 18% [95% CI − 33 to − 6]). Platelet counts were not assessed daily in most patients after the first day of admission, hampering interpretation of this parameter during the further course of admission. However, levels of both ADAMTS-13:ag and ADAMTS-13:act appeared to slowly return to steady-state values after day 3 of admission. Notably, the ADAMTS-13 specific activity and the ADAMTS-13:ag/VWF:ag ratio did not appear to change during the course of admission as compared with steady state (Table 2; Fig. 1F). Values of both VWF:ag and VWF:pp were comparable during steady state and the first days of admission (Table 2; Fig. S1A,B). Finally, the VWF:pp/VWF:ag ratio appeared to be lower on day 5 than during steady state (Table S1 and Fig. 1I; relative change of − 16%, 95% CI − 27 to 1).

Unpaired comparisons between genotype subgroups (HbSS/HbSβ^0 versus HbSC/HbSβ$^+$) demonstrated higher levels of VWF:ag and aVWF both during steady state and during VOC in the HbSS/HbSβ^0 subgroup (Fig. S1A and Fig. 1A; respectively, P = 0.02 and P < 0.01 during steady state, and P < 0.01 and P < 0.01 on day 1 of admission). In contrast, HMW multimer levels were significantly higher in the HbSC/HbSβ$^+$ subgroup on days 1 and 5 of admission (Fig. 1D; P = 0.04 and P = 0.01).

When we compared our study parameters on day 1 of admission between admissions that resulted in ACS and uncomplicated VOC admissions, the levels of VWF:ag, VWF:pp and aVWF were significantly higher (Table 3; respectively, median difference of 136% [95% CI 71–191], 5.5 nM [95% CI 1.0–27.1, and 1593 ng mL^{-1} [95% CI 20–26 501]), whereas the

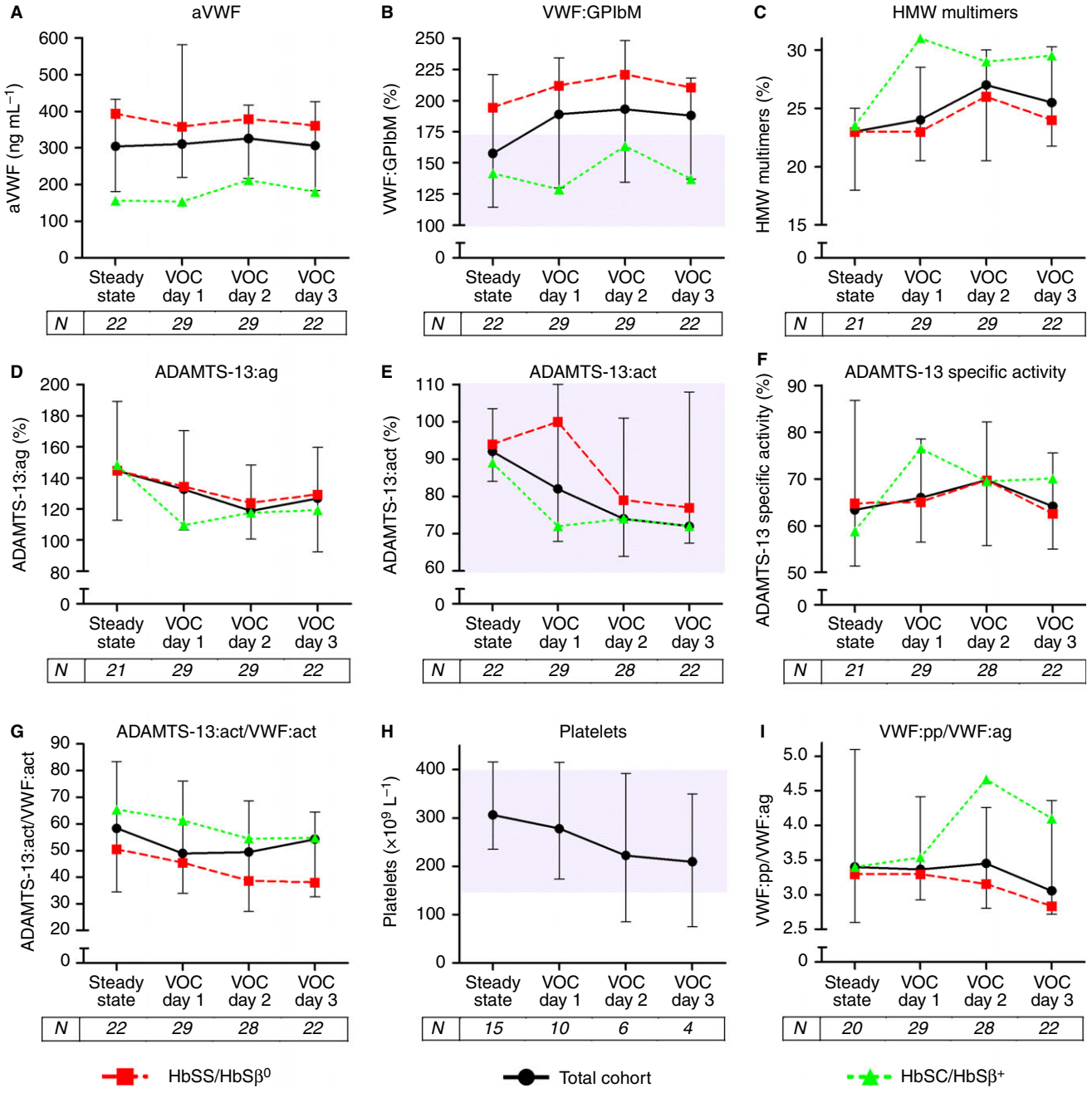

Fig. 1. Median levels of von Willebrand factor (VWF) and ADAMTS-13 parameters during steady state and on vaso-occlusive crisis (VOC) admission days 1, 2 and 3. For the total cohort, medians with interquartile ranges per time point are shown; for genotype subgroups, only medians per time point are shown. The number of patients under observation varies per time point (shown below the *x*-axis). The time point VOC day 5 is excluded, owing to the low number of patients with a fifth day of admission. Platelet count data per genotype subgroup are not shown, owing to the low number of patients with available data. If available, normal ranges are marked in gray (VWF glycoprotein Ib-binding activity [VWF:GPIbM], 48–173%; ADAMTS-13 activity [ADAMTS-13:act], 58–139%; platelet count, 150–400 × 10^9 L^{-1}). The concentration of active, hyperadhesive VWF (aVWF) in plasma pooled from at least 250 healthy individuals was determined to be at 45 ng mL^{-1}. (A) aVWF. (B) VWF:GPIbM. (C) High molecular weight (HMW) multimers. (D) ADAMTS-13 antigen (ADAMTS-13:ag). (E) ADAMTS-13:act. (F) ADAMTS-13 specific activity. (G) ADAMTS-13:act/VWF activity (VWF:act) ratio. (H) Platelets. (I) VWF propeptide (VWF:pp)/VWF antigen (VWF:ag) ratio. [Color figure can be viewed at wileyonlinelibrary.com]

ADAMTS-13:ag/VWF:ag ratio and ADAMTS-13:act were significantly lower in the admissions resulting in ACS (Table 3; respectively, median differences of − 35 [95% CI − 71 to − 17] and − 32% [95% CI − 56 to − 2]).

Correlation analysis

We performed an explorative Spearman correlation analysis on day 1 of admission, correlating our study

Table 3 Median, absolute differences in von Willebrand factor (VWF) and ADAMTS-13 values on day 1 of admission between uncomplicated admissions for vaso-occlusive crisis ($N = 24$; reference group) and admissions complicated by acute chest syndrome (ACS) ($N = 5$)

	No ACS versus ACS*	
	Median difference	95% CI
VWF:ag (%)	136	71 to 191
VWF:pp (nM)	5.5	1.0 to 27.1
VWF:pp/VWF:ag	0.4	− 1.2 to 4.7
aVWF (ng mL^{-1})	1593	20 to 26 501
aVWF/VWF:ag	361	− 89 to 8114
VWF:GPIbM (%)	56	− 75 to 141
VWF:GPIbM/VWF:ag	− 27	− 71 to 3
HMW multimers (%)	− 3	− 11 to 6
ADAMTS-13:ag (%)	− 25	− 68 to 33
ADAMTS-13:ag/VWF:ag	− 35	− 71 to − 17
ADAMTS-13:act (%)	− 32	− 56 to − 2
ADAMTS-13:act/VWF:GPIbM	− 16	− 53 to 23
ADAMTS-13 specific activity (%)	− 11	− 25 to 4
Platelets ($\times 10^9$ L^{-1})†	− 242	− 394 to 104

ADAMTS-13:act, ADAMTS-13 activity; ADAMTS-13:ag, ADAMTS-13 antigen; aVWF, active, hyperadhesive von Willebrand factor; CI, confidence interval; HMW, high molecular weight; VWF:ag, von Willebrand factor antigen; VWF:GPIbM, von Willebrand factor glycoprotein Ib-binding activity; VWF:pp, von Willebrand factor propeptide. *Samples of VOC admission day 1 were missing for three admissions in the 'No ACS' group. †Platelet values were missing for 16 admissions in the 'No ACS' group and three admissions in the 'ACS' group.

parameters with various pathophysiologic markers of disease and the time in hours between the start of the first pain and the drawing of the day 1 sample during admission (Table 4). Levels of VWF and its reactivity correlated positively with indirect markers of hemolysis (LDH and total bilirubin), inflammation (white blood cell count and CRP), and neutrophil activation (HNE–α1-AT and calprotectin), and inversely with hemoglobin levels and platelet count. Figure 2 shows the correlation of these markers with aVWF. In contrast, HMW multimers correlated positively with hemoglobin and inversely with LDH levels and VWF:ag. The time in hours between the start of pain and the drawing of the first sample upon admission was inversely correlated with VWF:ag and VWF:GPIbM levels. A subset analysis limited to HbSS/HbSβ^0 patients demonstrated the consistency of the correlations between aVWF and markers of inflammation and neutrophil activation (Table S2). The correlation with LDH and total bilirubin did not persist in this subset.

Discussion

This is the first study assessing the dynamics and reactivity of VWF in patients with SCD during the course of VOC. We observed significantly higher levels of aVWF, VWF:GPIbM and HMW multimers during the first days of VOC than during steady state, while, concomitantly, platelet counts and plasma levels of ADAMTS-13:ag and ADAMTS-13:act were lower. Higher levels of VWF:ag and aVWF were observed in HbSS/HbSβ^0 patients than in HbSC/HbSβ$^+$ patients, whereas, remarkably, HMW multimer levels were higher in the latter group. In admissions resulting in ACS, the levels of VWF:ag, VWF:pp and aVWF were significantly higher on the first day after admission, whereas the levels of ADAMTS-13:act were lower. The quantity and reactivity of VWF (VWF:ag, aVWF, and VWF:GPIbM) were associated with markers of inflammation and neutrophil activation. Inverse correlations were observed with the hemoglobin concentration and platelet count.

Our results confirm findings from previous studies, showing increased quantities of VWF:ag, VWF:GPIbM and large VWF multimers in steady-state SCD patients [19,24]. However, the dynamics of VWF reactivity in the course of a VOC have not been studied before. Whereas the total concentration of VWF:ag remained unchanged during VOC in comparison with steady-state levels, we demonstrate here that the reactivity of VWF was significantly higher during VOC. Chen *et al.* were first to report the presence of high levels of hyper-reactive VWF in steady-state SCD patients, indicating that an increased amount of VWF was in an elongated, platelet-binding conformation in these patients [24]. Using the same nanobody, we observed even higher levels of aVWF during VOC, in parallel with increases in the levels of HMW multimers and VWF:GPIbM. Furthermore, the platelet count strongly declined during the first days of VOC, and both hemoglobin levels and platelet counts were inversely correlated with VWF reactivity. Previous *in vitro* studies have shown that hyper-reactive VWF potently binds both platelets and sickle erythrocytes [6,8]. Therefore, we hypothesize that this activated VWF scavenges platelets and sickle erythrocytes in the microcirculation of SCD patients, and thereby promotes microvascular occlusion.

Interestingly, increased VWF reactivity and low platelet levels have been observed in various other acute, pathologic conditions with endothelial activation, such as HELLP syndrome, meningococcal sepsis, and malaria [34–36]. Microvascular thrombosis is also a central phenomenon in all of these diseases. In contrast to these conditions, in our study the levels of VWF:ag and VWF:pp were not significantly higher during VOC than during steady state. This observation suggests that additional acute endothelial activation, associated with further increases in VWF:ag and VWF:pp levels, can perhaps not be achieved, owing to exhaustion of the endothelium after

Table 4 Correlation analyses of von Willebrand factor (VWF) and ADAMTS-13 parameters in relation to markers of hemolysis, inflammation, neutrophil activation and duration of pain on vaso-occlusive crisis admission day 1

	VWF:ag		aVWF		VWF:GPIbM		HMW multimers		ADAMTS-13 specific activity	
	R_s	P	R_s	P	R_s	P	R_s	P	R_s	P
Hemoglobin*	− 0.55	0.002	− 0.32	0.092	− 0.41	0.026	0.46	0.011	0.14	0.470
LDH*	0.64	0.001	0.57	0.006	0.48	0.024	− 0.53	0.011	− 0.35	0.112
Total bilirubin*	0.52	0.004	0.56	0.001	0.46	0.012	− 0.24	0.205	− 0.34	0.068
WBC count*	0.39	0.035	0.57	0.001	0.12	0.538	− 0.34	0.070	− 0.08	0.688
Platelets†	− 0.79	0.012	0.0	1.00	− 0.83	0.005	0.17	0.666	0.40	0.286
CRP	0.59	0.002	0.68	< 0.001	0.44	0.024	− 0.37	0.064	0.09	0.674
HNE–α1-AT	0.57	0.001	0.56	0.002	0.31	0.100	− 0.21	0.269	− 0.11	0.560
Calprotectin	0.52	0.004	0.58	0.001	0.18	0.353	− 0.09	0.647	0.16	0.397
ADAMTS-13 specific activity	− 0.31	0.102	− 0.01	0.943	− 0.23	0.229	0.30	0.120		
Time from start of pain to sample in hours	− 0.38	0.049	− 0.08	0.696	− 0.39	0.042	0.25	0.217	0.26	0.186

aVWF, active, hyperadhesive von Willebrand factor; CRP, C-reactive protein; HNE–α1-AT, human neutrophil elastase–α1-antitrypsin complex; HMW, high molecular weight; LDH, lactate dehydrogenase; R_s, Spearman rank correlation coefficient; VWF:ag, von Willebrand factor antigen; VWF:GPIbM, von Willebrand factor glycoprotein Ib-binding activity; WBC, white blood cell. *Hemoglobin, LDH, total bilirubin and WBCs were measured directly upon admission. All other parameters were measured in the same sample drawn on the morning after admission (day 1). †Correlations with platelet levels were only possible in nine patients. Correlations for aVWF are illustrated by scatterplots in Fig. 2.

prolonged chronic endothelial activation. This is supported by the decreasing trend in VWF:pp levels and the VWF:pp/VWF:ag ratio on VOC day 5 as compared with steady state, and the previous observation that VWF stores become depleted upon prolonged endothelial stimulation [37].

Thus, increased secretion of VWF itself most likely does not explain the observed higher VWF reactivity during VOC. Possibly, microvascular occlusion could induce reactivation of VWF. These occlusions may lead to increased shear stress, promoting self-association and reactivation of VWF. Alternatively, insufficient clearance or impaired cleavage by the proteolytic enzyme ADAMTS-13 may be responsible for this VWF hyper-reactivity. We did find significantly lower levels of ADAMTS-13:ag and ADAMTS-13:act during the first days of VOC than during steady state. However, the ADAMTS-13 specific activity remained unchanged. This suggests that there is a quantitative decrease in ADAMTS-13 levels without a decrease in the relative activity of this protease. This decrease may be attributable to increased consumption of ADAMTS-13. Alternatively, the synthesis of ADAMTS-13 could primarily be inhibited, e.g. by inflammatory cytokines [38]. However, despite these reduced levels of ADAMTS-13 during VOC, we did not observe a pronounced ADAMTS-13 deficiency, which is in line with previous findings [19]. These results therefore only partially explain the observed higher VWF reactivity during VOC.

An important, alternative explanation for the higher VWF reactivity may be that the clearance of the hyperadhesive VWF multimers by ADAMTS-13 is hampered by decreased susceptibility of these multimers to proteolysis. Chen *et al.* were the first to demonstrate that reactive oxygen species, released by activated neutrophils, have the potential to inhibit VWF cleavage by ADAMTS-13, through oxidation of its cleavage site [25,39]. Oxidation of VWF increases its resistance to proteolysis, and appears to facilitate VWF self-association [40–42]. In addition, free hemoglobin has been shown to competitively bind to the ADAMTS-13 cleavage site, also blocking proteolysis and promoting VWF-mediated platelet adhesion and microthrombus formation [43,44]. In line with these observations, we have found strong correlations between VWF reactivity and markers of inflammation and neutrophil activation. The correlation with LDH and bilirubin as indirect markers of hemolysis did not persist in the subset of HbSS/HbSβ^0 patients in our cohort. Notably, these parameters have been demonstrated to not correlate well with actual red blood cell survival, and LDH may be more indicative for general tissue damage [45]. These results provide further support for the hypothesis that the higher reactivity of VWF during VOC may be directly associated with both inflammation and oxidative stress, which have been demonstrated to be strongly upregulated during VOC in SCD [46–50]. Further studies are required to assess the role of hemolysis in VWF reactivity.

The later peak and lower plasma levels of HMW multimers that we observed in HbSS/HbSβ^0 patients may also be explained by this increased resistance of VWF to proteolysis. There is a higher level of inflammation in the HbSS/HbSβ^0 genotype than in the HbSC/HbSβ^+ genotype [51]. Thereby, the majority of HMW multimers may be trapped in the microcirculation in these patients, whereas, in HbSC/HbSβ^+ patients, higher concentrations in plasma are reached because of more efficient multimer cleavage. This mechanism would also support the remarkable positive correlation of HMW multimer levels with

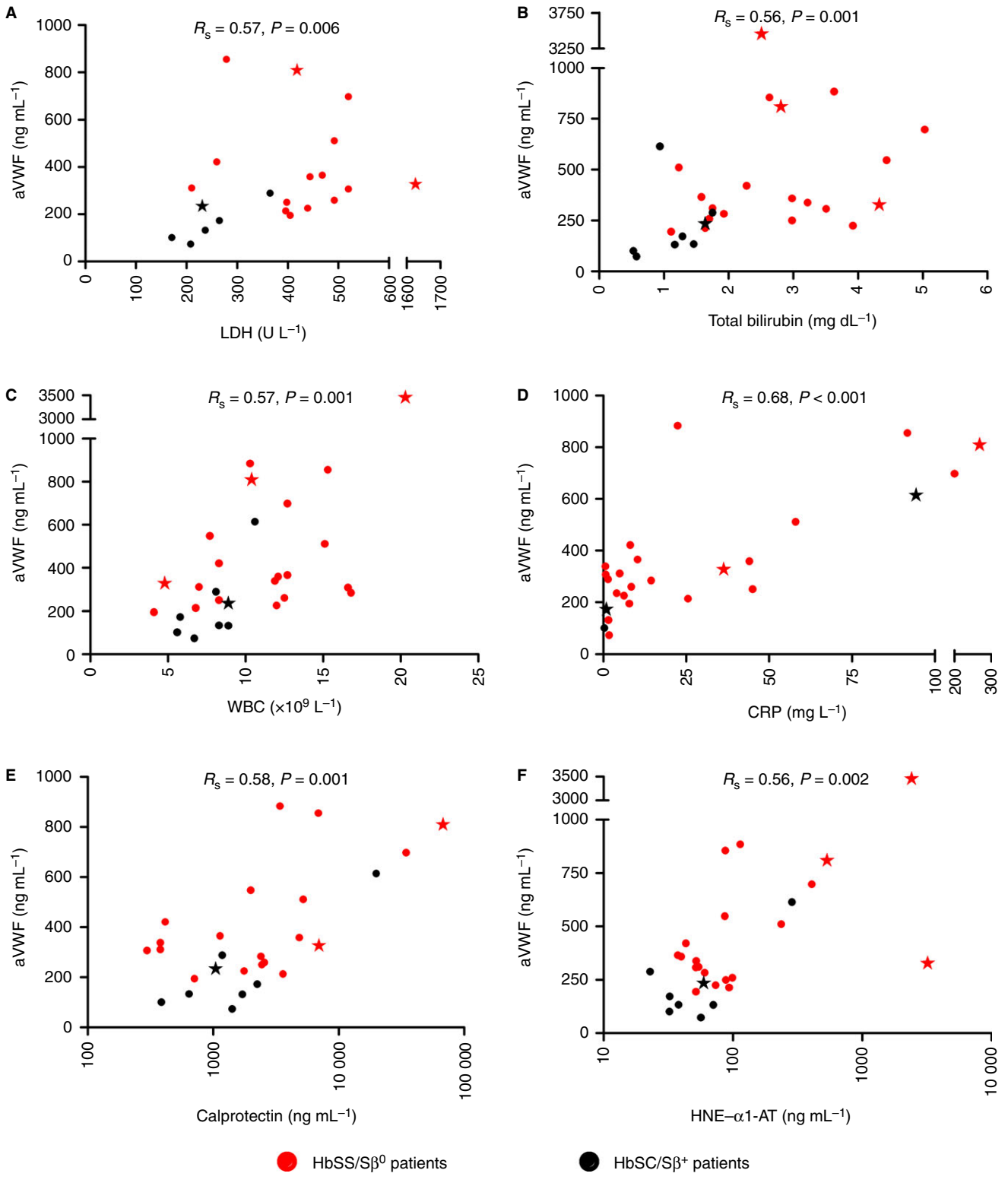

Fig. 2. Plasma levels of active, hyperadhesive von Willebrand factor (aVWF) in relation to various pathophysiological markers on day 1 of admission for vaso-occlusive crisis (VOC): hemolysis (A, B), inflammation (C, D) and neutrophil activation (E, F). Circles depict admissions for simple VOC. Stars depict admissions associated with acute chest syndrome (ACS). The levels of calprotectin (E) and aVWF (A–F) for two distinct HbSS patients with ACS were outside the axis limits of the plots. Additional correlation analysis for aVWF and these markers was performed in a subset of HbSS/HbSβ^0 patients only; see Table S2. R_s, Spearman rank correlation coefficient. CRP, C-reactive protein; HNE–α1-AT, human neutrophil elastase–α1-antitrypsin complex; LDH, lactate dehydrogenase; WBC, white blood cell. [Color figure can be viewed at wileyonlinelibrary.com]

the hemoglobin concentration, and its inverse correlation with LDH levels and inflammatory markers.

A strength of this study is that we performed a standardized blood withdrawal at fixed time points for each patient, allowing reliable evaluation over the course of a VOC. There are also some limitations of our study. Importantly, the observational study design did not allow us to elucidate whether high VWF reactivity during VOC is an actual causal agent in the pathophysiology of VOC in SCD, or rather an effect of the VOC. However, *in vitro* studies have suggested that thick bundles of VWF can obstruct blood flow, binding platelets, leukocytes, and erythrocytes [11]. In addition, increased VWF reactivity has also been observed in other disease models with microvascular thrombosis [34–36].

Second, all samples taken during VOC were drawn during hospital admission. Therefore, we only provide information on the dynamics of the studied parameters during admission. This may not reflect the course of these markers from the initial start of a VOC or during the recovery phase after hospital discharge. Importantly, the presented data on VOC day 5 reflect a more severe subset of patients with a longer length of hospitalization. Moreover, for practical reasons, steady-state samples were drawn after, and not prior to, the VOC under observation. This should be a reliable indication of the patient's baseline values, as we applied a delay of at least 4 weeks, and it is unlikely that steady-state values would differ between different VOCs. Finally, in our analyses we did not take into account the potential effects of hyperhydration during admission. However, hyperhydration is generally a consistent intervention during the first days of admission. Moreover, as we mostly observed increments in our study parameters during VOC, hyperhydration may only have diluted the observed effects.

In conclusion, we provide the first evidence that there is higher VWF reactivity during VOC than during steady state in patients with SCD, in the absence of pronounced ADAMTS-13 deficiency. The higher VWF reactivity is potentially explained by decreased susceptibility of VWF to proteolysis by ADAMTS-13, induced by processes such as inflammation and oxidative stress. In support of this, markers of inflammation and neutrophil activation correlated strongly with markers of VWF reactivity. This hyperadhesive VWF is potent in scavenging platelets and sickle erythrocytes in the microcirculation, and may amplify and sustain microvascular occlusion and symptomatology in SCD. Interventions targeting inflammation or oxidative stress may therefore be effective inhibitors of VWF activation. For example, the antioxidant *N*-acetylcysteine has been demonstrated to reduce the size and activity of VWF multimers in both *in vitro* and *in vivo* models [52]. In addition, administration of recombinant ADAMTS-13 may have a beneficial effect [53], as this could fully restore ADAMTS-13 levels to normal values, optimizing its proteolytic activity. Future studies will have to elucidate whether hyperadhesive VWF is merely a marker of or an actual contributor to the pathophysiology of VOC in SCD, and could thereby represent a new therapeutic target.

Addendum

J. W. R. Sins wrote the manuscript. J. W. R. Sins and M. Schimmel analyzed the data. J. W. R. Sins, M. Schimmel, J. C. M. Meijers, B. J. Biemond, and K. Fijnvandraat interpreted the data. J. W. R. Sins, M. Schimmel, B. J. Biemond, E. Nur, J. C. M. Meijers, B. M. Luken, D. P. M. Brandjes, and K. Fijnvandraat designed the study. M. Schimmel, J. W. R. Sins, C. F. van Tuijn, R. T. Urbanus, B. M. Luken, S. S. Zeerleder, and W. F. Kopatz collected data, assisted with data collection, or performed laboratory analysis. All authors critically revised and approved the final manuscript.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Relative changes in VWF and ADAMTS-13 parameters from steady state to VOC admission day 5 (paired comparisons of patients with data available for both steady state and VOC admission day 5).

Table S2. Subset correlation analysis, in HbSS/HbSβ^0 admissions only, of VWF and ADAMTS-13 parameters in relation to markers of hemolysis, inflammation, neutrophil activation and duration of pain on VOC admission day 1 (admissions $N = 21$).

Fig. S1. Median levels of VWF:ag and VWF:pp in steady state and VOC admission days 1, 2, and 3.

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