Therapeutic targets in sickle cell disease
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Citation for published version (APA):
Sins, J. W. R. (2017). Therapeutic targets in sickle cell disease

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Chapter 3

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


Journal of Thrombosis and Haemostasis 2017: 15; 1-11
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 quantity and reactivity of VWF and its regulating protease ADAMTS13 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 GPIb-binding activity and high-molecular weight multimers), whereas platelet count and levels of ADAMTS13 antigen and ADAMTS13 activity were concomitantly lower when compared to steady state. Levels of VWF antigen, VWF propeptide and ADAMTS13 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 aVWF were significantly higher, while the ADAMTS13:act was lower than in patients without this complication.

Conclusions

We provide first evidence that VOC in SCD is associated with increased reactivity of VWF, without a pronounced ADAMTS13 deficiency. This hyper-reactivity may be explained by resistance of VWF to proteolysis, secondary to processes such as inflammation and oxidative stress. Hyper-adhesive VWF, scavenging blood cells in the microcirculation, may thereby amplify and sustain VOC in SCD.
Introduction

Sickle cell disease (SCD) is a recessive hemoglobinopathy. Worldwide, over 300,000 infants with this disease are born each year. SCD is characterized by chronic hemolytic anemia and recurrent vascular occlusion, resulting in frequent episodes of acute pain and secondary organ damage. The pathophysiology of these vaso-occlusive, painful crises (VOC) 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. Upon activation of the endothelium, large, hyper-adhesive strands of VWF (ultra large VWF multimers [ULVWF]) are released into the bloodstream, highly effective in binding platelets, leukocytes and sickle erythrocytes. 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. The main regulator of VWF activity is the protease ADAMTS13, cleaving highly adhesive ULVWF off the surface of activated endothelial cells, and cutting these strings into smaller and less adhesive multimers. Disturbances in the delicate balance between VWF release and its cleavage by ADAMTS13 may result in microvascular thrombosis, as observed in patients with thrombotic thrombocytopenic purpura, malaria, malignant hypertension, sepsis and myocardial infarction.

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

Interestingly, a recent study of SCD patients in steady state demonstrated high levels of aVWF, which strongly correlated with the concentration of LDH. The authors suggested that this increase in VWF reactivity in SCD may be caused by both higher secretion 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 ADAMTS13 cleavage site in VWF. 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.

Based on the available literature, we hypothesized that an increased concentration of active VWF, insufficiently restrained by ADAMTS13, may contribute to vascular occlusion in SCD and may thereby represent a new therapeutic target. Thus 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 ADAMTS13, 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, HbSβ⁰, HbSβ⁺ or HbSC) admitted for VOC in two hospitals in Amsterdam, The Netherlands, were approached for participation within 24 hours after admission. VOC was defined as musculo-skeletal 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 day 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 2 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 ADAMTS13 parameters
To assess the reactivity of VWF, both aVWF, VWF GPIb-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 using a llama-derived single-domain antibody (AU/VWFa-11) directed against the A1 domain of VWF, as described before.\textsuperscript{23} The absolute concentration of aVWF could be estimated by 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 measured at 45 ng/ml. Additionally, VW:F:GPIbM\textsuperscript{26} was measured using the INNOVANCE assay (Siemens Healthcare GmbH, Erlangen, Germany), employing latex particles and gain of function recombinant glycoprotein Ib to facilitate VWF binding and agglutination.\textsuperscript{27} The VWF multimeric pattern was assessed by agarose gel electrophoresis followed by Western blotting with a polyclonal VWF antibody.\textsuperscript{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 to the total area under the curve using Image Lab software (Bio-Rad Laboratories, Hercules, CA, USA). VWF antigen (VWF:ag) was measured by ELISA using commercial antibodies (DAKO, Glostrup, Denmark) calibrated to the WHO 07/316 6th International Standard. VWF propeptide (VWF:pp) concentrations were measured by ELISA as described before.\textsuperscript{29} ADAMTS13 antigen (ADAMTS13:ag) concentrations were also measured by ELISA as described before.\textsuperscript{23,29} ADAMTS13 activity was measured by determination of the degree of degradation of purified VWF from plasma, whereby total and residual VWF were quantified as VWF:GPIbM, using the INNOVANCE assay as described above.\textsuperscript{30} Pooled, normal plasma was used as a standard here. ADAMTS13 specific activity was defined as the ratio between ADAMTS13:act and ADAMTS13:ag.

Data on markers of inflammation (CRP) and neutrophil activation (human elastase α1-antitrypsin complexes [HNE-α1-AT], calprotectin) on VOC admission day 1 were measured by ELISA as described previously.\textsuperscript{31–33}

**Statistical analysis**

Values per time point of the study parameters were presented as medians with inter-quartile ranges (IQR). Laboratory values during VOC were compared with steady state by paired, direct comparisons, expressed as relative, percentual change from steady state. Due to the non-parametric distribution of these data, the median change with corresponding 95% confidence intervals (CI) was estimated with the Hodges-Lehmann estimator. For patients with 2 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 difference of the study parameters between uncomplicated admissions for VOC and admissions complicated by ACS, was also estimated with the Hodges-Lehmann estimator with corresponding 95% CI. Explorative correlation analysis of VWF and ADAMTS13 parameters with various pathophysiological markers was performed by means of a Spearman’s rank test. Time points with missing data were excluded from the analysis. A P value below .05 or a confidence interval not containing 0 was considered statistically significant.

### Table 1. Baseline characteristics

<table>
<thead>
<tr>
<th>N (%) or median (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patients – total</strong></td>
</tr>
<tr>
<td><strong>Admissions – total</strong></td>
</tr>
<tr>
<td><strong>Age in years</strong></td>
</tr>
<tr>
<td><strong>Female sex</strong></td>
</tr>
<tr>
<td><strong>Hemoglobin genotype</strong></td>
</tr>
<tr>
<td>HbSS / HbSβ⁰</td>
</tr>
<tr>
<td>HbSC / HbSβ⁺</td>
</tr>
<tr>
<td><strong>Use of hydroxycarbamide</strong></td>
</tr>
<tr>
<td>6 (25)</td>
</tr>
<tr>
<td><strong>Steady state laboratory markers</strong></td>
</tr>
<tr>
<td>Hb – mmol/L *</td>
</tr>
<tr>
<td>Leucocyte count – x10⁹/L *</td>
</tr>
<tr>
<td>Platelet count – x10⁹/L ‡</td>
</tr>
<tr>
<td>LDH – U/L †</td>
</tr>
<tr>
<td>Total bilirubin – µmol/L *</td>
</tr>
<tr>
<td>CRP – mg/L ¥</td>
</tr>
</tbody>
</table>

* Steady state data missing in 2 patients (N=22)  
‡ Steady state data missing in 4 patients (N=20)  
† Steady state data missing in 9 patients (N=15)  
¥ Steady state data missing in 11 patients (N=21)

### Results

#### Baseline characteristics

The baseline characteristics of this study cohort are summarized in table 1. In short, a total of 24 patients was included upon admission for VOC. Eight patients were included twice with separate admissions (6 HbSS/HbSβ⁰ and 2 HbSC/HbSβ⁺), accounting for 32 VOC admissions in total. The median age of patients was 27 years (IQR 24 - 30) and 50% of patients were female. Six of the 24 patients were on hydroxyurea during the study (all HbSS/HbSβ⁰ patients, median dose 15 mg/kg). The median length of hospitalization over all 32 admissions was 6 days (IQR 3 - 8). The subset of patients where a sample of admission day 5 (N=13)
## Relative change from steady state

<table>
<thead>
<tr>
<th>Parameter</th>
<th>VOC day 1 (N=19)</th>
<th>VOC day 2 (N=20)</th>
<th>VOC day 3 (N=13)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Change %</td>
<td>95% CI</td>
<td>Change %</td>
</tr>
<tr>
<td>VWF:ag *</td>
<td>-8</td>
<td>-16 – 9</td>
<td>-1</td>
</tr>
<tr>
<td>VWF:pp †</td>
<td>9</td>
<td>-9 – 32</td>
<td>14</td>
</tr>
<tr>
<td>aVWF</td>
<td>33</td>
<td>5 – 70</td>
<td>30</td>
</tr>
<tr>
<td>aVWF / VWF:ag *</td>
<td>36</td>
<td>17 – 64</td>
<td>32</td>
</tr>
<tr>
<td>VWF:GPIbM</td>
<td>8</td>
<td>-4 – 20</td>
<td>13</td>
</tr>
<tr>
<td>VWF:GPIbM / VWF:ag *</td>
<td>11</td>
<td>2 – 23</td>
<td>9</td>
</tr>
<tr>
<td>HMW multimers *</td>
<td>10</td>
<td>-2 – 28</td>
<td>16</td>
</tr>
<tr>
<td>ADAMTS13:ag *</td>
<td>-4</td>
<td>-15 – 9</td>
<td>-11</td>
</tr>
<tr>
<td>ADAMTS13:ag / VWF:ag **</td>
<td>3</td>
<td>-13 – 19</td>
<td>-11</td>
</tr>
<tr>
<td>ADAMTS13:act §</td>
<td>-7</td>
<td>-14 – 0</td>
<td>-10</td>
</tr>
<tr>
<td>ADAMTS13 specific act †</td>
<td>-1</td>
<td>-10 – 9</td>
<td>0</td>
</tr>
</tbody>
</table>

The data on VOC day 1 include 13 HbSS/HbSβ° and 6 HbSC/HbSβ+ patients, on VOC day 2 include 12 HbSS/HbSβ° and 8 HbSC/HbSβ+ patients, and on VOC day 3 include 7 HbSS/HbSβ° and 8 HbSC/HbSβ+ patients. Data for day 5 are shown in supplemental table S2. CI, confidence interval; Significant values are marked bold.

* VOC day 1 N=18, VOC day 2 N=19.
† VOC day 1 N=17, VOC day 2 N=17.
‡ VOC day 1 N=9, VOC day 2 N=6, VOC day 3 N=4, VOC day 5 N=2.
§ VOC day 2 N=19.
¶ VOC day 1 N=18, VOC day 2 N=18.
** VOC day 1 N=17, VOC day 2 N=18.
†† VOC day 1 N=19, VOC day 2 N=19.
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 hours (IQR 40 - 85) and did not differ between the HbSS/HbS\(^{0}\) and HbSC/HbS\(^{+}\) patients \((P=0.79)\). Five patients developed an acute chest syndrome during admission (4 HbSS patients and 1 HbS\(^{+}\) patient).

**Longitudinal analysis**
Table 2 demonstrates the relative, median change of the various VWF and ADAMTS13 parameters from steady state to VOC day 1, 2 and 3 (paired analysis). Most strikingly, markers of VWF reactivity were higher during the course of admission as compared to steady state. These differences reached significance for aVWF on day 1 (relative change 33%, CI 5 – 70 ), day 2 (30%, CI 9 – 67) and day 3 (35%, CI 12 – 63), and for HMW multimers on days 2 and 3 (table 2, relative change respectively 16%, CI 6 – 32, and 10%, CI 1 – 42). The change in VWF:GPIbM reached significance on day 2 only (table 2, relative change 13%, CI 1 – 27). Similarly, the ratios of both aVWF/VWF:ag and VWF:GPIbM/VWF:ag were significantly higher at day 1 and 2 of admission as compared to steady state (table 2, relative change respectively 36%, CI 17 – 64, and 32%, CI 13 – 66, and 11%, CI 2 – 23 and 9%, CI 1 – 27). In addition, the trend of these parameters from steady state through the course of admission is shown in figure 1 (medians per time point).

Inversely, ADAMTS13:ag, ADAMTS13:act, the ratio of ADAMTS13:act/VWF:GPIbM and platelets were significantly lower during hospital admission as compared to steady state (figure 1D,1E,1G,1H), reaching statistical significance on day 1 for platelets (table 2, relative change -22%, CI -53 – -10, on day 2 for ADAMTS13:ag (table 2, relative change -11%, CI -22 – -1) and ADAMTS13:act (table 2, relative change -10%, CI -16 – -2), and on day 2 and 3 for the ratio of ADAMTS13:act/VWF:GPIbM (table 2, relative change respectively -19%, CI -28 – -9); and-18%, CI -33 – -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. Yet, levels of both ADAMTS13:ag and ADAMTS13:act appeared to slowly restore back to steady state values after day 3 of admission. Notably, the ADAMTS13 specific activity and the ratio of ADAMTS13:ag/VWF:ag did not appear to change during the course of admission as compared to steady state (table 2; figure 1F). Values of both VWF:ag and VWF:pp were comparable during steady state and the first days of admission (table 2 and supplemental figure S1A/S1B). Lastly, the ratio of VWF:pp/VWF:ag appeared to be lower on day 5 as compared to steady state (supplemental table S1 and figure 1I, relative change -16%, CI -27 – 1).
Figure 1 – Median levels of VWF and ADAMTS13 parameters in steady state to VOC admission day 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). Time point VOC day 5 is excluded due to the low number of patients with a fifth day of admission. Platelet count data per genotype subgroup are not shown due to the low number of patients with available data. If available, normal ranges are marked in grey (VWF:GPIbM 48-173%, ADAMTS13:act 58-139%, platelet count 150-400 x10^9/L). The concentration of aVWF in plasma pooled from at least 250 healthy individuals was measured at 45 ng/ml.

Unpaired comparisons between genotype subgroups (HbSS/HbS^0 versus HbSC/HbS^+β) demonstrated higher levels of VWF:ag and aVWF both in steady state and during VOC in the HbSS/HbS^0 subgroup (supplemental figure S1A; figure 1A, respectively \( P=0.02 \) and \( P<0.01 \) in 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/HbS^+β subgroup on day 1 and day 5 of admission (figure 1D, \( P=0.04 \) and \( P=0.01 \)).

When comparing our study parameters on day 1 of admission between admissions that resulted in ACS versus uncomplicated VOC admissions, the levels of VWF:ag, VWF:pp and aVWF were significantly higher (table 3, respectively median difference 136%, CI 71 – 191; 5.5 nM, CI 1.0 – 27.1; and 1,593 ng/ml, CI 20 – 26,501) while the ratio of ADAMTS13:ag/VWF:ag and the ADAMTS13:act were significantly lower in the admissions resulting in ACS (table 3, respectively median difference -35, CI -71 – -17; and -32%, CI -56 – -2).
Correlation analysis
We performed an explorative Spearman correlation analysis on day 1 of admission, correlating our study parameters with various pathophysiological 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, total bilirubin), inflammation (white blood cell count, CRP), neutrophil activation (HNE-α1-AT, calprotectin), and inversely with hemoglobin levels and platelet count. Figure 2 illustrates 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 first 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/HbSβ0 patients demonstrated the consistency of the correlations between aVWF and markers of inflammation and neutrophil activation (supplemental table S2). The correlation with LDH and total bilirubin did not persist in this subset.

| Table 3. Median, absolute difference in VWF and ADAMTS13 values on day 1 of admission between uncomplicated admissions for VOC (N=24; reference group) and admissions complicated by ACS (N=5) |
|-------------------------------------------------|---------|-----------|
| No ACS vs ACS *                                 | Median difference | 95% CI     |
| VWF:ag (%)                                      | 136     | 71 – 191  |
| VWF:pp (nM)                                     | 5.5     | 1.0 – 27.1|
| VWF:pp / VWF:ag                                 | 0.4     | -1.2 – 4.7|
| aVWF (ng/ml)                                    | 1,593   | 20 – 26,501|
| aVWF / VWF:ag                                   | 361     | -89 – 8,114|
| VWF:GPIbM (%)                                   | 56      | -75 – 141 |
| VWF:GPIbM / VWF:ag                              | -27     | -71 – 3   |
| HMW multimers (%)                               | -3      | -11 – 6   |
| ADAMTS13:ag (%)                                 | -25     | -68 – 33 |
| ADAMTS13:act / VWF:GPIbM                       | -16     | -53 – 23 |
| ADAMTS13 specific act (%)                       | -11     | -25 – 4   |
| Platelets (x10⁹/L) †                            | -242    | -394 – 104|

CI, confidence interval.
* Samples of VOC admission day 1 were missing in 3 admissions in the ‘No ACS’ group.
† Platelet values missing for 16 admissions in ‘No ACS’ group and 3 admissions in ‘ACS’ group.
Table 4. Correlation analyses of VWF and ADAMTS13 parameters in relation with markers of hemolysis, inflammation, neutrophil activation and duration of pain on VOC admission day 1

<table>
<thead>
<tr>
<th></th>
<th>VWF:ag</th>
<th>aVWF</th>
<th>VWF:GPIbM</th>
<th>HMW multimers</th>
<th>ADAMTS13 spec act</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rs</td>
<td>P</td>
<td>Rs</td>
<td>P</td>
<td>Rs</td>
</tr>
<tr>
<td>Hemoglobin *</td>
<td>-0.55</td>
<td>.002</td>
<td>-0.32</td>
<td>.092</td>
<td>-0.41</td>
</tr>
<tr>
<td>LDH *</td>
<td>0.64</td>
<td>.001</td>
<td>0.57</td>
<td>.006</td>
<td>0.48</td>
</tr>
<tr>
<td>Total bilirubin *</td>
<td>0.52</td>
<td>.004</td>
<td>0.56</td>
<td>.001</td>
<td>0.46</td>
</tr>
<tr>
<td>WBC *</td>
<td>0.39</td>
<td>.035</td>
<td>0.57</td>
<td>.001</td>
<td>0.12</td>
</tr>
<tr>
<td>Platelets †</td>
<td>-0.79</td>
<td>.012</td>
<td>0.0</td>
<td>1.00</td>
<td>-0.83</td>
</tr>
<tr>
<td>CRP</td>
<td>0.59</td>
<td>.002</td>
<td>0.68</td>
<td>&lt;.001</td>
<td>0.44</td>
</tr>
<tr>
<td>HNE-α1-AT</td>
<td>0.57</td>
<td>.001</td>
<td>0.56</td>
<td>.002</td>
<td>0.31</td>
</tr>
<tr>
<td>Calprotectin</td>
<td>0.52</td>
<td>.004</td>
<td>0.58</td>
<td>.001</td>
<td>0.18</td>
</tr>
<tr>
<td>ADAMTS13 spec act</td>
<td>-0.31</td>
<td>.102</td>
<td>-0.01</td>
<td>.943</td>
<td>-0.23</td>
</tr>
<tr>
<td>Time start pain to sample in hours</td>
<td>-0.38</td>
<td>.049</td>
<td>-0.08</td>
<td>.696</td>
<td>-0.39</td>
</tr>
</tbody>
</table>

Correlations for aVWF are illustrated by scatterplots in figure 2. Rs: Spearman rank correlation coefficient.

* Both hemoglobin (Hb), LDH, total bilirubin and white blood cell count (WBC) were measured directly upon admission. All other parameters were measured in the same sample drawn the morning after admission (day 1).
† Correlations with platelet levels were only possible in 9 patients.
Figure 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 (panel A-B), inflammation (panel C-D) and neutrophil activation (panel E-F).

Circles depict admissions for simple VOC. Stars depict admissions associated with an ACS. Rs represents the Spearman rank correlation coefficient with its corresponding P-value. The level of calprotectin (panel E) and aVWF (panel A-F) for 2 distinct HbSS patients with ACS were outside the axis limits of the plots. Additional correlation analysis of aVWF with these markers was performed in a subset of HbSS/HbSβ⁰ patients only, see supplemental table S2.
**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 compared to steady state, while concomitantly platelet counts and plasma levels of ADAMTS13:ag and ADAMTS13:act were lower. Higher levels of VWF:ag and aVWF were observed in HbSS/HbSβ⁰ patients as compared to HbSC/HbSβ⁺ patients, while 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 while the levels of the ADAMTS13:act were lower. The quantity and reactivity of VWF (VWF:ag, aVWF, 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, observing increased quantities of both VWF:ag, VWF:GPIbM and large VWF multimers in steady state SCD patients. However, the dynamics of VWF reactivity in the course of a VOC have not been studied before. While 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 SCD steady state, indicating that an increased amount of VWF was in an elongated, platelet-binding conformation in these patients. Using the same nanobody, we observed even higher levels of aVWF during VOC, parallel with increments in 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 as well as sickle erythrocytes. 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, pathological conditions with endothelial activation, such as HELLP, meningococcal sepsis or malaria. Microvascular thrombosis is also a central phenomenon in all these diseases. In contrast with these conditions, in our study the levels of VWF:ag and VWF:pp were not significantly higher during VOC as compared to steady state. This observation suggests that additional acute endothelial activation, associated with a further increase in VWF:ag and VWF:pp levels, can perhaps
not be achieved due to exhaustion of the endothelium after prolonged chronic endothelial activation. This is supported by the decreasing trend in VWF:pp and VWF:pp/VWF:ag levels on VOC day 5 as compared to steady state and the previous observation that VWF stores become depleted upon prolonged endothelial stimulation.\textsuperscript{37}

Thus, increased secretion of VWF itself most likely does not explain the observed higher VWF reactivity during VOC. Possibly, microvascular occlusion itself 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 ADAMTS13 may be responsible for this VWF hyper-reactivity. We did find a significantly lower level of ADAMTS13:ag and ADAMTS13:act during the first days of VOC as compared to steady state. Yet, the ADAMTS13 specific activity remained unchanged. This suggests that there is a quantitative decrease in ADAMTS13 levels without a decrease in the relative activity of this protease. This decrease may be due to increased consumption of ADAMTS13. Alternatively, the synthesis of ADAMTS13 could primarily be inhibited, for example by inflammatory cytokines.\textsuperscript{38} Yet, despite these reduced levels of ADAMTS13 during VOC, we did not observe a pronounced ADAMTS13 deficiency, which is in line with previous findings.\textsuperscript{19} These results therefore do 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 hyper-adhesive VWF multimers by ADAMTS13 is hampered due to decreased susceptibility of these multimers to proteolysis. Chen et al. were first to demonstrate that reactive oxygen species, released by activated neutrophils, have the potential to inhibit VWF cleavage by ADAMTS13, by oxidation of its cleavage site.\textsuperscript{25,39} Oxidation of VWF increases its resistance to proteolysis and appears to facilitate VWF self-association.\textsuperscript{40–42} In addition, free hemoglobin has been shown to competitively bind to the ADAMTS13 cleavage site, also blocking proteolysis and promoting VWF mediated platelet adhesion and microthrombi formation.\textsuperscript{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/HbS\textsuperscript{β} patients in our cohort. Notably, these parameters have been demonstrated not to correlate well with actual RBC survival and LDH may be more indicative for general tissue damage.\textsuperscript{45} These results provide further support 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.\textsuperscript{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\textsuperscript{0} patients, may also be explained by this increased resistance of VWF to proteolysis. Compared to HbSC/HbSβ\textsuperscript{+}, there is a higher level of inflammation in the HbSS/HbSβ\textsuperscript{0} genotype.\textsuperscript{51} Thereby, the majority of HMW multimers may be trapped in the microcirculation in these patients, while in HbSC/HbSβ\textsuperscript{+} patients higher concentrations in plasma are reached due to more efficient multimer cleavage. This mechanism would also support the remarkable positive correlation of HMW multimer levels with 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 a reliable evaluation over the course of a VOC. There are also some limitations to 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. Yet, in vitro studies have suggested that thick bundles of VWF can obstruct blood flow, binding platelets, leukocytes and erythrocytes.\textsuperscript{51} In addition, increased VWF reactivity has also been observed in other disease models with microvascular thrombosis.\textsuperscript{34–36}

Secondly, all samples during VOC have been 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 VOC. Lastly, in our analyses we did not take into account the potential effects of hyper-hydration during admission. Yet, hyper-hydration generally is a consistent intervention during the first days of admission. Moreover, as we mostly observe increments in our study parameters during VOC, hyper-hydration may only have diluted the observed effects.

In conclusion, we provide first evidence that there is a higher VWF reactivity during VOC as compared to steady state in patients with SCD, in absence of a pronounced ADAMTS13 deficiency. The higher VWF reactivity is potentially explained by de-
creased susceptibility of VWF to proteolysis by ADAMTS13, 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 hyper-adhesive 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. In addition, administration of recombinant ADAMTS13 may be of beneficial effect, as this could fully restore ADAMTS13 levels to normal values, optimizing its proteolytic activity. Future studies will have to elucidate if hyper-adhesive VWF is merely a marker 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, and 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 in data collection or performed laboratory analysis. All authors critically revised and approved the final manuscript.

Acknowledgements

The authors would like to acknowledge the hematology clinical trial office of the Academic Medical Center for their logistical support in this study, and Tesy Merkx of the University Medical Center Utrecht for performing the aVWF and ADAMTS13:ag measurements. In addition, we would like to thank Junmei Chen of the Bloodworks Northwest institute in Seattle for her critical review of the manuscript. This work was supported by a grant from Baxalta US Inc., now part of Shire (K. Fijnvandraat).
References


20. van der Land, V. et al. Markers of endothelial dysfunction differ between subphenotypes in children with


38. Cao, W. J., Niiya, M., Zheng, X. W., Shang, D.


Table S1. Relative change of VWF and ADAMTS13 parameters from steady state to VOC admission day 5 (paired comparisons of patients with data available of both steady state and VOC admission day 5).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>VOC day 5 (N=9)</th>
<th>Change %</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>VWF:ag (%)</td>
<td></td>
<td>7</td>
<td>-6 – 20</td>
</tr>
<tr>
<td>VWF:pp (nM)</td>
<td></td>
<td>-11</td>
<td>-21 – 13</td>
</tr>
<tr>
<td>VWF:pp / VWF:ag</td>
<td></td>
<td>-16</td>
<td>-27 – 1</td>
</tr>
<tr>
<td>aVWF (ng/ml)</td>
<td></td>
<td>35</td>
<td>-20 – 95</td>
</tr>
<tr>
<td>aVWF / VWF:ag</td>
<td></td>
<td>30</td>
<td>-27 – 93</td>
</tr>
<tr>
<td>VWF:GPIbM (%)</td>
<td></td>
<td>24</td>
<td>-4 – 63</td>
</tr>
<tr>
<td>VWF:GPIbM / VWF:ag</td>
<td></td>
<td>23</td>
<td>-3 – 51</td>
</tr>
<tr>
<td>HMW multimers (%)</td>
<td></td>
<td>17</td>
<td>-1 – 67</td>
</tr>
<tr>
<td>ADAMTS13:ag (%)</td>
<td></td>
<td>-3</td>
<td>-35 – 38</td>
</tr>
<tr>
<td>ADAMTS13:ag / VWF:ag</td>
<td></td>
<td>-4</td>
<td>-43 – 35</td>
</tr>
<tr>
<td>ADAMTS13:act (%)</td>
<td></td>
<td>3</td>
<td>-14 – 25</td>
</tr>
<tr>
<td>ADAMTS13:act / VWF:GPIbM</td>
<td></td>
<td>-16</td>
<td>-34 – 5</td>
</tr>
<tr>
<td>ADAMTS13 specific act (%)</td>
<td></td>
<td>12</td>
<td>-14 – 44</td>
</tr>
<tr>
<td>Platelets (x10^9/L)</td>
<td></td>
<td>-31</td>
<td>-62 – -1</td>
</tr>
</tbody>
</table>

The data on VOC day 5 include 4 HbSS/HbSβ⁰ and 5 HbSC/ HbSβ⁺ patients.
IQR, Interquartile Range; 95% CI, 95% confidence interval.
Significant values are marked bold (P<.05)
Table S2. Subset correlation analysis in HbSS/HbSβ⁰ admissions only, of VWF and ADAMTS13 parameters in relation with markers of hemolysis, inflammation, neutrophil activation and duration of pain on VOC admission day 1 (admissions N=21)

<table>
<thead>
<tr>
<th></th>
<th>VWF:ag</th>
<th>aVWF</th>
<th>VWF:GPIbM</th>
<th>HMW multimers</th>
<th>ADAMTS13 spec act</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$R_s$</td>
<td>$P$</td>
<td>$R_s$</td>
<td>$P$</td>
<td>$R_s$</td>
</tr>
<tr>
<td>Hemoglobin *</td>
<td>-0.24</td>
<td>.303</td>
<td>0.18</td>
<td>.448</td>
<td>-0.24</td>
</tr>
<tr>
<td>LDH *</td>
<td>0.43</td>
<td>.100</td>
<td>0.22</td>
<td>.411</td>
<td>0.18</td>
</tr>
<tr>
<td>Total bilirubin *</td>
<td>0.20</td>
<td>.396</td>
<td>0.33</td>
<td>.147</td>
<td>0.31</td>
</tr>
<tr>
<td>WBC *</td>
<td>0.13</td>
<td>.574</td>
<td>0.41</td>
<td>.067</td>
<td>-0.23</td>
</tr>
<tr>
<td>Platelets †</td>
<td>-0.89</td>
<td>.007</td>
<td>-0.07</td>
<td>.879</td>
<td>-0.86</td>
</tr>
<tr>
<td>CRP</td>
<td>0.33</td>
<td>.173</td>
<td>0.55</td>
<td>.015</td>
<td>0.09</td>
</tr>
<tr>
<td>HNE-α1-AT</td>
<td>0.49</td>
<td>.024</td>
<td>0.41</td>
<td>.067</td>
<td>0.13</td>
</tr>
<tr>
<td>Calprotectin</td>
<td>0.51</td>
<td>.018</td>
<td>0.56</td>
<td>.008</td>
<td>0.02</td>
</tr>
<tr>
<td>ADAMTS13 spec act</td>
<td>-0.47</td>
<td>.033</td>
<td>-0.02</td>
<td>.920</td>
<td>-0.30</td>
</tr>
<tr>
<td>Time start pain to sample in hours</td>
<td>-0.30</td>
<td>.198</td>
<td>0.17</td>
<td>.481</td>
<td>-0.30</td>
</tr>
</tbody>
</table>

Rs: Spearman rank correlation coefficient. Significant values are marked bold ($P<.05$).

* Both hemoglobin (Hb), LDH, total bilirubin and white blood cell count (WBC) were measured directly upon admission. All other parameters were measured in the same sample drawn the morning after admission (day 1).

† Correlations with platelet levels were only possible in 7 patients.
Figure S1 – Median levels of VWF:ag and VWF:pp in steady state to VOC admission day 1, 2 and 3. For the total cohort medians with interquartile ranges per timepoint 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). Time point VOC day 5 is excluded due to the low number of patients with a fifth day of admission. Normal ranges are marked in grey (VWF:ag 50-150%, VWF:pp 2.8-8.3 nM).