Sickle cell disease

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Chapter 3

Inflammatory and endothelial markers during vaso-occlusive crisis in sickle cell disease

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Chapter 3

ABSTRACT

Vaso-occlusive crisis (VOC) is the hallmark of sickle cell disease (SCD). The pathophysiology is complex and characterized by inflammation, neutrophil and endothelial activation. In this prospective study, we aimed to determine changes in biomarkers of inflammation, neutrophil and endothelial activation during the course of VOC, to analyze the dynamics of these processes in the pathogenesis of VOC and subsequent complications, like acute chest syndrome (ACS). In consecutive SCD patients, we obtained blood samples on day 1, 2, 3 and 5 of admission for VOC and in steady state, and determined biomarkers of inflammation (C-reactive protein (CRP); pentraxin3 (PTX3), neutrophil activation (elastase-alpha1-antitrypsin-complex (HNE-α1-AT); calprotectin), cell dead (nucleosomes) and endothelial activation (von Willebrand factor antigen (VWF:Ag); VWF propeptide (VWFpp)).

In 32 VOC’s in 24 adult patients (22 HbSS/Sβ0-thalassemia, 10 HbSC/Sβ+-thalassemia), PTX3 and CRP levels peaked on day 2-3 in the course of VOC, while highest levels of nucleosomes and HNE-α1-AT were measured on day 1. Although not significant, a late rise in calprotectin levels was observed. Levels of VWF:Ag were elevated in steady state and remained stable during VOC course while VWFpp levels remained within normal range during steady state and VOC, suggesting chronic endothelial activation. Significant higher levels of HNE-α1-AT and calprotectin levels were observed in patients who developed an ACS.

In conclusion, our results suggest that VOC is an acute inflammatory process with prompt neutrophil activation, on top of chronic endothelial activation in patients with SCD. Neutrophil activation markers HNE-α1-AT and calprotectin may aid the identification of patients at risk for development of ACS.
INTRODUCTION

Sickle cell disease (SCD) is characterized by chronic hemolytic anemia and recurrent vaso-occlusive crisis (VOC).[1, 2] VOC may lead to life-threatening acute complications such as acute chest syndrome (ACS), multi-organ failure and sudden death.[3-5] Pathophysiological factors contributing to VOC include endothelial activation, adhesion of activated sickle red blood cells (sRBCs), leukocytes and platelets, coagulation activation and increased oxidative stress.[6] Even in sickle cell patients without symptoms of VOC (steady state), a chronic inflammatory state is present as reflected by increased levels of circulating C-reactive protein (CRP)[7-9] and inflammatory cytokines like TGF-β-1,[10] TNF-α and IL-8[11] when compared to healthy individuals. Furthermore, neutrophils of patients with SCD in steady state displayed an activated phenotype with increased expression of Mac-1 (CD11b/CD18)[12] and decreased expression of L-selectin (CD62L)[13] when compared to healthy individuals. In addition, increased circulating levels of von Willebrand factor (VWF) antigen were observed in SCD patients in steady state, indicating chronic endothelial activation.[14, 15]

During VOC, close interactions between sickle red blood cells (sRBCs), the endothelium and neutrophils are evident.[16-21] Infusion of the redox active heme in SCD mice, mimicking chronic hemolysis, induced the appearance of VWF on the vessel wall.[16] It was suggested that intravascular hemolysis leading to oxidative damage of endothelial cells, may contribute to the development of VOC as adhesive VWF molecules are able to bind sRBCs.[17] In addition, in another humanized sickle cell murine model, it was shown that upon induction of VOC with TNF-α, sRBCs directly interact with circulating neutrophils, which was accompanied by a reduction in local microcirculatory blood flow and led to a diminished survival of these mice.[18, 19] Inhibition of interactions between sRBCs, neutrophils and endothelial cells resulted in a restored local blood flow in different (murine and human) SCD models of VOC.[19-21] Therefore, it appears that VOC is an acute aggravation of ongoing inflammation with neutrophil and endothelial activation, whereby the exact trigger that initiates VOC, and the sequence of events involved in VOC including the possible progression to further acute complications such as ACS, are still incompletely understood.

In the current study, we aimed to gain insight in the sequence of events during VOC and steady state. Therefore, the dynamics in levels of biomarkers of inflammation,
cell death, neutrophil activation and endothelial activation in patients with SCD during the course of hospitalization for VOC and in steady state were studied.

Levels of the acute phase proteins CRP and pentraxin3 (PTX3) were determined as markers of inflammation. Both CRP and PTX3 are members of the pentraxin protein family,[22] a family of soluble pattern recognition molecules with an important role in pathogen opsonization and complement activation. Levels of the neutrophil derived azurophilic protein elastase in complex with its inhibitor α1-antitrypsin (HNE-α1-AT), and the neutrophil cytosolic protein calprotectin, were determined as markers of neutrophil activation. Circulating nucleosomes, the basic units of DNA organization,[23] were determined as a marker of systemic inflammation and cell death,[24-26] and have been attributed to the formation of neutrophil extracellular traps (NETs) by activated neutrophils.[27-30] Finally, VWF antigen and propeptide (VWF:Ag and VWFpp) were determined as markers of endothelial activation in this study.

**METHODS**

*Patients*

Consecutive adult patients with SCD admitted for VOC to the Academic Medical Center or the Slotervaart Hospital (Amsterdam, The Netherlands) were approached for participation. Inclusion criteria were: age ≥ 18 years with sickle cell anemia (HbSS) or the compound heterozygous states HbSβ0-thalassemia (HbSβ0-thal), HbSβ+-thalassemia (HbSβ+-thal) or sickle-hemoglobin C (HbSC). VOC was defined as musculo-skeletal and/or visceral pain not otherwise explained and recognized as such by the patient. An acute chest syndrome was defined as experiencing clinical symptoms including fever, respiratory symptoms, or chest pain, in combination with a new pulmonary infiltrate visible by X-ray imaging.[31] Exclusion criteria were: pregnancy, active cancer, chronic HIV infection or blood transfusion in the three months prior to admission. Blood samples were obtained after overnight fasting on the morning following hospital admission, and on subsequent mornings (hospitalization days) as indicated. Inclusion was limited to a maximum of two separate episodes of VOC per patient. Patients included in the study were seen at the out-patient clinic at least 4 four weeks after discharge for collection of a steady state blood sample, if at that moment they were not experiencing vaso-occlusive pain. From all patients a written informed consent was obtained. The study
protocol was approved by the Medical Ethical Committee of the participating centers and conducted in agreement with the Helsinki declaration.

**Laboratory analysis**

Blood samples were taken by venipuncture. Blood tubes were centrifuged once or twice (citrated plasma) for 15 minutes at 3000 x g at 4°C to obtain plasma and serum and stored in aliquots at -80°C until further analysis. The following markers were determined only upon admission for VOC: standard blood counts (hemoglobin and white blood cell count) were determined in EDTA-anticoagulated plasma (Cell-Dyn 4000, Abbott, Illinois, USA) and hemolysis parameters (bilirubin and lactate dehydrogenase, LDH) were measured with spectrophotometry in heparinized plasma (P800 Modular, Roche, Switzerland). All markers were determined in a longitudinal approach on the morning upon admission for VOC and on every following day during admission. C-reactive protein (CRP) levels were measured in serum using an automated immunoturbidimetric end point assay with a linearity of 0.3-350 mg/l, on a Roche cobas c702 analyzer. The following markers were measured in EDTA-anticoagulated plasma using previously described ELISA methods: PTX3,[32] extracellular nucleosomes[24, 33] and calprotectin (the heterodimer S100A8/S100A9).[34] Human neutrophil elastase was measured in complex with its inhibitor α1-antitrypsin (HNE-α1-AT), using ELISA as described previously.[35, 36] VWF:Ag levels in citrated plasma were measured by ELISA using commercial antibodies (DAKO, Denmark). Normal human pooled plasma was used as standard and calibrated to the WHO 07/316 6th International Standard for VWF. VWFpp was measured using an in-house commercially available ELISA as previously described.[37] Throat swab samples, taken upon admission, were screened for presence of nucleic acids of Influenza A, Influenza B, adenovirus, Respiratory syncytial virus (RSV) and enterovirus using multiplex real-time polymerase-chain (RT-PCR) on a LightCycler 480 (Roche Life Science) using methods described elsewhere.[38]

**Statistical analysis**

For data analysis a commercial statistical package (IBM SPSS Statistics 21.0, US) was used. Statistical data analysis was done with data of all included patients. An additional analysis was performed dividing patients in two groups: patients with the relatively severe genotypes HbSS and HbSβ⁰-thalassemia were grouped together forming the HbSS/HbSβ⁰-thal group, and patients with the relatively milder HbSC and HbSβ⁺-thalassemia genotypes were gathered together to form group HbSC/HbSβ⁺-thal.[39, 40] Non-normally distributed data are expressed as median with interquartile range (IQR).
Mann-Whitney rank sum test was used to assess differences between groups, or the Kruskal-Wallis one-way analysis of variance when more than two groups were compared. For related sample analysis the related-samples Wilcoxon Signed rank test was used. Correlation analysis was done using Spearman’s rank correlation (Sr). A P-value below 0.05 was considered statistically significant. For multiple comparison testing the Bonferroni correction was used and when applied, the adapted P-value is indicated.

RESULTS

Patients
Twenty-four sickle cell patients admitted for VOC were included in the study, accounting for 32 admissions (22 HbSS/Sβ0-thal and 10 HbSC/Sβ+,-thal). Baseline characteristics are presented in table 1. There were no significant differences in baseline characteristics between patients in VOC or steady state. Median hospital stay was 5.5 (IQR 3-8) days for the whole group, 4 (IQR 3-7) days for the HbSS/Sβ0-thal group and 7 (IQR 6-9) for the HbSC/Sβ+-thal group. Five patients (4 HbSS and 1 HbSβ+-thal patient) developed an ACS during admission. All patients with ACS received antibiotics and

| Table 1. Baseline characteristics. Results are shown as median with interquartile range. |
|----------------------------------------|-------------------------------|-------------------------------|
| **Inclusions**                        | Sickle cell disease           | Sickle cell disease           |
|                                        | Vaso-occlusive crisis         | Steady state                  |
|                                        | First sample after admission  |                               |
| Demographics                          | n = 32                        | n = 24                        |
|                                        | HbSS-HbSβ0 // HbSC-HbSβ+      | 67% // 33%                   |
|                                        | Age (y)                       | 26 (21-30)                    |
|                                        | 26 (22-30)                    |                               |
|                                        | Female/male ratio             | 16/16                         |
|                                        | 10/11                         |                               |
|                                        | On hydroxycarbamide (%)       | 25%                           |
|                                        | 29%                           |                               |
|                                        | Laboratory measurements       |                               |
|                                        | White blood cells (10e9/l)    | 9.9 (7.2-12.7)                |
|                                        | 8.6 (6.1-9.6)                 |                               |
|                                        | Hemoglobin (mmol/l)           | 6.1 (4.7-6.9)                 |
|                                        | 6.0 (5.2-7.0)                 |                               |
|                                        | Bilirubin Total (μmol/l)      | 30 (23-55)                    |
|                                        | 26 (19-40)                    |                               |
|                                        | LDH (U/l)                     | 405 (260-492)                 |
|                                        | 362 (218-435)                 |                               |
|                                        | CRP (mg/l)                    | 3.7 (1.5-10.6)                |
|                                        | 2.1 (0.8-6.2)                 |                               |

*Significant difference between healthy controls and steady state patients with sickle cell disease, P <0.05. ** P <0.001, †Significant difference between steady state patients with sickle cell disease and patients with sickle cell disease in painful crisis P <0.05. ** H P <0.001
two patients (both HbSS) required erythropheresis. The presence of a respiratory viral infection as trigger for VOC was ruled out in all patients that were tested (27 out of 32 admissions) using multiplex RT-PCR.

Longitudinal analysis of the levels of inflammatory markers, nucleosomes, neutrophil activation markers and endothelial activation markers

During hospitalization for VOC the levels of markers of inflammatory response, nucleosomes and markers of neutrophil activation and endothelial activation were followed. The different markers showed clearly distinct temporal patterns (Figure 1). The dynamics of plasma levels of both CRP and PTX3 were largely comparable to each other during the course of admission, with high levels at day 1 of admission when compared to steady state and reaching peak levels at day 2 and 3 for CRP and PTX3, respectively (Figure 1A and 1B). Levels of nucleosomes were highest upon admission and showed a rapid decline on day 2 and further decreased during subsequent days of admission (Figure 1C). Levels of HNE-α1-AT seemed to peak upon admission, but were not significantly different from levels in steady state and normalized from the second day of admission (Figure 1D). Levels of calprotectin showed a more gradual increase during hospitalization reaching peak levels at day 5 of admission, and returned to the normal range in steady state (Figure 1E). Plasma levels of the endothelial activation markers VWF:Ag were comparable to levels in steady state and remained stable during the course of VOC while VWFpp levels remained within the normal range (Figure 1F, G). Levels of all markers, but especially CRP, nucleosomes, HNE-α1-AT and calprotectin, were higher in HbSS/HbSβ0-thal patients compared to levels in HbSC/HbSβ+-thal patients (Figure 1).

Plasma levels of CRP, PTX3 and nucleosomes, but not neutrophil activation markers HNE-α1-AT and calprotectin, were significantly increased at day 1 of admission for VOC when compared to levels of these markers in steady state. Levels of VWF:Ag in steady state were above the normal range but not significantly different from levels at day 1. Levels of VWFpp in steady state were within the normal range (2.8-8.3 nM) [41] and were similar to levels in samples taken during VOC at day 1. (See table 2).
Figure 1. Levels of CRP, PTX3, nucleosomes, HNE-α1-AT, calprotectin, VWF:Ag, and VWFpp, during admission for vaso-occlusive crisis (VOC) and in steady state.

Samples were taken on the first, second, third and fifth morning of VOC. Means with SEM. Levels of all markers were higher in HbSS/HbSβ0-thal patients when compared to levels in HbSC/HbSβ+ thal patients. A and B. Levels of CRP (A) and PTX3 (B) are increased in the first sample taken on admission when compared to steady state levels and show a further increment during respectively the first three (PTX3) and two (CRP) days of admission, thereafter levels tend to return to steady state levels.
Relation of inflammatory markers, nucleosomes, neutrophil and endothelial activation markers to hemolysis

To investigate the contribution of hemolysis and cell death to VOC in this cohort, plasma levels of bilirubin and lactate dehydrogenase (LDH) were measured upon admission for VOC. There was a significant relation between the LDH level and the level of both nucleosomes and HNE-α1-AT, (P=0.028 and P=0.048, Kruskal-Wallis test). CRP (P=0.087), calprotectin (P=0.070), VWF:Ag (=0.079) and VWFpp (P=0.054), but not PTX3 (P=0.289), showed a trend to correlation with this marker. Bilirubin levels were also related to levels of nucleosomes (P = 0.026) and to VWF:Ag (P = 0.002) levels but not to HNE-α1-AT (P = 0.243), calprotectin (P = 0.575), CRP (P = 0.335), PTX3 (P = 0.398), and VWFpp (P = 0.218).

Relation between inflammatory markers, nucleosomes and markers for neutrophil- and endothelial activation

In the steady state, applying Bonferroni correction for multiple correlation testing (P < 0.007 was considered statistically significant), a correlation was observed between levels of CRP and VWF:Ag, Sr = 0.518, P = 0.006. No other correlations were observed between the markers at steady state.

In samples taken the first morning after admission for VOC, strong (Sr ≥0.500) and highly significant correlations became manifest between almost all selected markers for chronic endothelial activation, inflammation, nucleosomes and neutrophil activation. See table 3. Notable exceptions were that VWFpp levels did not correlate with levels of CRP, PTX3, HNE-α1-AT and calprotection. And PTX3 levels did not show any correlation with levels of VWF:Ag and nucleosomes.

--- Figure 1. Levels of CRP, PTX3, nucleosomes, HNE-α1-AT, calprotectin, VWF:Ag, and VWFpp, during admission for vaso-occlusive crisis (VOC) and in steady state. (continued)
C. Levels of nucleosomes were high upon admission and show already upward of day 1 a rapid decline followed by a gradual decrease during subsequent days of admission. D. Levels of HNE-α1-AT appear high upon admission, but levels are not significantly increased, and are already low the second day of admission. E. Levels of calprotectin show a small gradual increment during subsequent days of admission. F and G. Levels of VWF:Ag (F) and VWFpp (G) remain stable during admission for VOC and in steady state.
| Table 2. Levels of CRP, PTX3, nucleosomes, HNE-α1-AT, calprotectin and VWF:Ag and VWFpp in samples of patients with SCD as markers for inflammation, cell death, neutrophil activation endothelial activation.

Values are depicted as median with interquartile range.

* Significant difference between levels in steady state and VOC.
Inflammatory and endothelial markers during vaso-occlusive crisis in sickle cell disease

Table 3.

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Table 3. Correlations between the markers for inflammation, nucleosomes and markers for neutrophil and endothelial activation on first day after admission for VOC. Only significant correlations are depicted here (according Bonferroni correction for multiple comparison a significant value here is when $P \leq 0.007$).

Relation with acute chest syndrome (ACS)

In the five patients that developed an ACS during admission for VOC, levels of nucleosomes, HNE-α1-AT, calprotectin and VWF:Ag in the samples the first morning after admission for VOC were significantly higher compared to levels of these markers in patients without ACS (nucleosomes: 2220.0 (IQR 568.0-19875.0) vs 26.4 (IQR 5.0-50.6) U/ml, $P=0.01$; HNE-α1-AT: 534.0 (IQR 222.4-2805.0) vs 58.7 (IQR 42.4-89.5) ng/ml, $P=0.004$; calprotectin: 9440.0 (IQR 3996.5-191500.0) vs 1875.5 (IQR 691.5-3453.8) ng/ml, $P=0.02$ and VWF:Ag: 315 (IQR 220-365) vs 167 (IQR 129-231) %, $P=0.006$). See Figure 2. Levels of CRP, PTX3 and VWFpp were not different in patients with and those without ACS. (CRP: 88.4 (IQR 20.2-291.1) vs 8.1 (IQR 1.6-44.5) mg/L, $P=0.07$; PTX3: 8.8 (IQR 6.8-18.5) vs 6.8 (IQR 3.3-8.9) ng/mL, $P=0.13$ and VWFpp: 10.6 (IQR 5.7-24.6) vs 6.5 (IQR 4.6-8.2) nM, $P=0.05$).
Figure 2. Levels of CRP, PTX3, nucleosomes, HNE-α1-AT, calprotectin, VWF:Ag and VWFpp in samples of patients during VOC who developed an acute chest syndrome (ACS) during admission for VOC and patients without ACS. Levels of nucleosomes (C), HNE-α1-AT(D), calprotectin(E) and VWF:Ag(F) were significantly elevated in patients who developed an acute chest syndrome during admission compared to levels in patients without acute chest syndrome.
DISCUSSION

Sickle cell disease (SCD) is characterized by recurrent painful vaso-occlusive crises (VOC). The pathophysiology of these VOC is complex and multi-factorial involving hemolysis, an inflammatory response, endothelial activation, neutrophil activation and enhanced cellular adhesion leading to increased cell-cell interactions between sickle erythrocytes, leukocytes, platelets and endothelial cells. In the present study, we describe the dynamics in biomarkers of inflammation, cell death, neutrophil and endothelial activation on consecutive days during the course of VOC and in comparison to steady state.

Levels of CRP and PTX3 were significantly elevated and respectively peaked on day 2 and 3 of the VOC indicating that VOC is accompanied by an acute inflammatory response. Since CRP is considered to be a reflection of systemic inflammation in response to interleukin-6 and PTX3 levels mainly reflect local inflammation, as it is released locally by neutrophils and endothelial cells,[42] both forms of inflammatory response seem to be active in VOC. This observation is different from findings in patients with more local forms of inflammation such as acute myocardial infarction wherein PTX3 levels showed a distinct earlier plasma peak followed by an increase in levels of CRP.[43] Nucleosome levels peaked at day 1 followed by a rapid decline the days thereafter. As nucleosomes represent free DNA due to cell death, we observe a peak of cell death already early in the VOC process. Circulating nucleosomes have also been related to Neutrophil Extracellular Traps (NETs). NETs can be released by activated neutrophils with the capacity to capture and kill pathogens, but NETs can also inflict damage to cells of the host.[30] In the present study, during VOC, levels of neutrophil activation marker HNE-α1-AT mimicked the course of nucleosomes levels, similar to our previous study.[29] And even though we cannot exclude that nucleosomes detected by our ELISA originate from cells other than neutrophils, these findings suggest that neutrophil activation is related to the release of nucleosomes. This is further supported by the strong (Sr≥0.500) and highly significant correlations (P<0.007) that were found between the levels of nucleosomes and levels of neutrophil activation markers HNE-α1-AT and calprotectin during VOC suggesting an increased presence of NETs during VOC.[27, 28] In Berkeley sickle mice, NETs were identified as important players in the pathogenesis of VOC including related lung complications.[51] Increased levels of nucleosomes and HNE- α1-AT, [29] and also PTX3 [50] during VOC and their relation with clinical severity indicate that NETs may...
be involved in the pathogenesis of VOC in sickle cell patients.[29] Our current data indeed support these findings, since the highest levels of nucleosomes, HNE-\(\alpha\)-1-AT and calprotectin were observed in patients with ACS. In our study cohort, 15% of patients admitted with VOC developed an ACS, of which all recovered. Generally, ACS has a high mortality rate.[52] Exchange transfusion early in the onset of ACS is considered the best treatment, but identifying patients at risk for ACS development is challenging.[53] While only a modest elevation of HNE-\(\alpha\)-1-AT and calprotectin levels in our patients with VOC were observed in this study, very high levels were found in the patients developing ACS. It would be interesting to investigate the value of these markers to identify patients at risk for developing ACS in future studies. In addition, the use of anti-inflammatory drugs that reduce neutrophil activation in patients with VOC in an attempt to prevent the development of ACS might be considered. Especially drugs that block the integrin Mac-1 (CD11b/CD18) might be of interest. Mac-1 is upregulated on the surface of neutrophils of patients with SCD,[12] and Mac-1 has been identified as an actor both in neutrophil adhesion to endothelial cells,[54] and in the formation of NETs.[55]

Calprotectin is a damage-associated-molecular-pattern (DAMP) that acts as an endogenous danger signal to support and aggravate an inflammatory response and is also considered to be a marker of neutrophil activation as it is abundant in neutrophil cytosol.[44] Surprisingly and in contrast with the course of nucleosome and HNE-\(\alpha\)-1-AT plasma levels, a gradual, non-significant rise in plasma levels of this biomarker was noticed during admission suggesting that neutrophil activity continues at lower degree during the further course of VOC, possibly due to ischemia-reperfusion induced oxidative stress. However, the calprotectin plasma levels were modest in comparison with observations in patients with sepsis or typhoid fever, in which high plasma levels of calprotectin have demonstrated to correlate with disease severity, indicating less pronounced, or possibly another way of neutrophil activation in VOC than in these conditions.[45, 46].

Whilst biomarkers of inflammation (CRP, PTX3) and nucleosomes were significantly elevated during VOC when compared to levels in steady state, plasma levels of VWF:Ag and VWFpp did not change during admission for VOC as compared to steady state levels. In normal conditions, both VWF:Ag and VWFpp are released upon endothelial cell activation. Since VWF:Ag and VWFpp are released in equimolar amounts[47] but VWFpp has a shorter half-life than VWF:Ag,[48] the ratio between these markers can
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differentiate between acute and chronic endothelial activation.[49] In the present study, we found increased levels of VWF:Ag in the absence of elevated VWFpp indicating chronic endothelial activation, as has been reported previously.[41, 50]

In our study, levels of all biomarkers during admission for VOC, but especially CRP, nucleosomes, HNE-α1-AT and calprotectin levels, were higher in HbSS/HbSβ^0^-thal patients when compared to HbSC/HbSβ^+^-thal patients. In fact, hardly any increase in plasma levels of nucleosomes, HNE-a1-AT and calprotectin was observed in patients with HbSC/HbSβ^+^-thal. This could be explained by the fact that less pronounced hemolysis and its downstream effects such as oxidative damage, inflammatory response and neutrophil activation takes place in these generally milder forms of SCD.

Some limitations of our study have to be mentioned. Firstly, day 1 in our study was defined as the day of admission in hospital while the exact beginning of the complaints may have varied between patients since patients sometimes delay the moment to seek medical help for a VOC. Secondly, our trial included a limited number of VOC which may have resulted in the loss of power to detect correlations or significant elevations of some of the markers.

In conclusion, this study provides increased insight in the sequence of events during VOC. Biomarkers of endothelial activation did not show an acute-on-chronic elevation during VOC, indicating chronically activated, or perhaps exhausted, endothelium in patients with SCD. During VOC, an acute inflammatory response accompanied by neutrophil activation was observed as reflected by elevated CRP, PTX3 and nucleosome levels. Although HNE-α1-AT and calprotectin levels in VOC demonstrated a modest non-significant rise compared to steady state, extremely high levels were observed in patients developing ACS. Neutrophil activation, especially in patients with an ACS, may form an interesting therapeutic target for anti-inflammatory drugs in further studies.
REFERENCES


