Sickle cell disease
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Chapter 2

Nucleosomes and neutrophil activation in sickle cell disease painful crisis

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ACTIVATED POLYMORPHONUCLEAR NEUTROPHILS PLAY AN IMPORTANT ROLE IN THE PATHOGENESIS OF VASO-OCCULSIVE PAINFUL SICKLE CELL CRISIS. UPON ACTIVATION POLYMORPHONUCLEAR NEUTROPHILS CAN FORM NEUTROPHIL EXTRACELLULAR TRAPS. NEUTROPHIL EXTRACELLULAR TRAPS CONSIST OF A MESHWORK OF EXTRACELLULAR DNA, NUCLEOSOMES, HISTONES AND NEUTROPHIL PROTEASES. NEUTROPHIL EXTRACELLULAR TRAPS HAVE BEEN DEMONSTRATED TO BE TOXIC TO ENDOTHELIAL AND PARENCHYMAL CELLS. THIS PROSPECTIVE COHORT STUDY WAS CONDUCTED TO DETERMINE NEUTROPHIL EXTRACELLULAR TRAP FORMATION IN SICKLE CELL PATIENTS DURING STEADY STATE AND PAINFUL CRISIS. AS A MEASURE OF NEUTROPHIL EXTRACELLULAR TRAPS, PLASMA NUCLEOSOMES LEVELS WERE DETERMINED AND POLYMORPHONUCLEAR NEUTROPHIL ACTIVATION WAS ASSESSED MEASURING PLASMA LEVELS OF ELASTASE-\(\alpha_1\)-ANTITRYPSIN COMPLEXES IN 74 PATIENTS IN STEADY STATE, 70 PATIENTS DURING PAINFUL CRISIS, AND 24 RACE-MATCHED CONTROLS USING ENZYME LINKED IMMUNOSORBENT ASSAY. NUCLEOSOME LEVELS IN STEADY STATE SICKLE CELL PATIENTS WERE SIGNIFICANTLY HIGHER THAN LEVELS IN CONTROLS. DURING PAINFUL CRISIS LEVELS OF BOTH NUCLEOSOMES AND ELASTASE-\(\alpha_1\)-ANTITRYPSIN COMPLEXES INCREASED SIGNIFICANTLY. LEVELS OF NUCLEOSOMES CORRELATED SIGNIFICANTLY TO ELASTASE-\(\alpha_1\)-ANTITRYPSIN COMPLEX LEVELS DURING PAINFUL CRISIS, (\(Sr = 0.654, P < 0.001\)). THIS WAS SEEN IN BOTH HBSS/HB\(\beta^0\)-THALASSEMIA (\(Sr=0.55, P<0.001\)) AND HBSC/HB\(\beta^+\)-THALASSEMIA PATIENTS (\(Sr=0.90, P<=0.001\)) DURING PAINFUL CRISIS. LEVELS OF NUCLEOSOMES SHOWED A CORRELATION WITH LENGTH OF HOSPITAL STAY AND WERE HIGHEST IN PATIENTS WITH ACUTE CHEST SYNDROME. THESE DATA SUPPORT THE CONCEPT THAT NEUTROPHIL EXTRACELLULAR TRAP FORMATION AND NEUTROPHIL ACTIVATION MAY PLAY A ROLE IN THE PATHOGENESIS OF PAINFUL SICKLE CELL CRISIS AND ACUTE CHEST SYNDROME.
INTRODUCTION

Sickle cell disease (SCD) is characterized by recurrent acute painful vaso-occlusive crisis (VOC), accounting for the vast majority of SCD related hospital admissions [1-3]. VOC related complications, such as acute chest syndrome, stroke and multi-organ failure are associated with high morbidity and mortality [4]. The exact pathogenesis of acute painful VOC remains to be elucidated. Alongside the crucial role for sickle erythrocytes in this, it encompasses an inflammatory response as evidenced by endothelial activation, coagulation activation and enhanced cellular adhesion, finally all contributing to microvascular occlusion.

Leukocytes play an important role in the development of microvascular obstruction and sickle cell disease related complications. In steady state sickle cell patients, leukocytosis is associated with severity of disease [5]. Clinical studies show that leukocytosis is a risk factor for major sickle cell related complications such as stroke [6], acute chest syndrome [7] and early death [8]. Additionally, the clinical benefit of hydroxyurea in sickle cell patients has partly been attributed to a reduction in polymorphonuclear neutrophil (PMN) cell count [9] and reduced PMN adhesion [10]. In vitro studies have demonstrated that PMN isolated from sickle cell patients are primed as evidenced by an increased expression of adhesion molecules [11-13], rendering them more susceptible for inflammatory stimuli as compared to PMN from healthy controls [14]. Moreover, activation of PMN, e.g. upon interaction with red blood cells [15], leads to the production of toxic reactive oxygen species (ROS), contributing to oxidative stress [16]. In-vitro studies as well as in-vivo studies in SCD mice models demonstrate P- and E-selectin interactions with integrins [17-19] to be crucial for the adherence of leukocytes to endothelial and sickle red blood cells, contributing to the complex process of vaso-occlusion [20, 21]. This identifies PMN activation and adhesion as important processes in the pathogenesis of vaso-occlusion in SCD.

Recently, activated PMN have been demonstrated to form neutrophil extracellular traps (NET) [22]. During NET formation, DNA and DNA-binding proteins are extruded from the neutrophils exposing a mesh consisting of nucleosomes, histones and neutrophil proteases such as elastase. These NET are regarded to be part of the innate immune response system [23]. However, their function is considered to be a double-edged sword. On one hand, NET formation is an efficient strategy to kill invading micro-organisms, like bacteria and fungi. On the other hand, NET can become harm-
ful for the host since its exposed compounds (e.g. the mesh of DNA, histones and neutrophil proteases) are toxic to endothelial cells and parenchymal tissue [24-26]. NET formation has been reported to be pro-coagulant in inflammatory models and is thought to contribute to the development of disseminated intravascular coagulation, and hence to morbidity and mortality in sepsis [27-29]. Circulating nucleosomes and markers of neutrophil activation have been reported to be suitable markers for NET formation in plasma in baboons and humans [29-31]. Nucleosomes consist of a core octamer of two copies each holding the histones H2A, H2B, H3 and H4, around which a segment of helical DNA of 146 base pairs is wrapped [32]. Nucleosomes can be actively released into the circulation from dead cells as a result of the activity of factor-VII activating protease (FSAP) [33]. Circulating cell-free DNA in form of nucleosomes has been reported to correlate with organ dysfunction, disease severity and mortality in sepsis patients and children suffering from meningococcal sepsis [34-36].

So far, no data are available on NET formation in sickle cell patients. Since white blood cell counts have been shown to correlate with morbidity of sickle cell patients and since PMN activation seems to play an important role in the development of sickle cell painful vaso-occlusive crisis we hypothesized that NET formation may be involved in these processes. The aim of this prospective cohort study therefore was to measure plasma levels of circulating nucleosomes and PMN activation as evidenced by human neutrophil elastase-α₁-antitrypsin (EA) complexes as a measure of NET formation in plasma in sickle cell patients both during steady state and painful VOC and to evaluate their correlation with crisis severity.

METHODS

Patients
This study followed a prospective design in which patients with sickle cell anemia (HbSS) and compound heterozygous states HbSβ⁰-thalassemia, HbSβ⁺-thalassemia and sickle-hemoglobin C (HbSC) patients were eligible for inclusion. Diagnosis of hemoglobinopathy was confirmed by means of high performance liquid chromatography in combination with measurement of erythrocyte mean corpuscular volume. Consecutive sickle cell patients, ≥ 18 years of age, attending the outpatient clinic (steady state) or being admitted for a painful crisis to the Academic Medical Center or the Slotervaart Hospital, Amsterdam, The Netherlands, were approached for partici-
A painful crisis was defined as musculo-skeletal pain not otherwise explained and recognized as such by the patient and requiring medical treatment. Samples during painful crisis were obtained within the first 24 hours of admission. Patients with painful crisis within four weeks and/or blood transfusion within three months prior to the evaluation for the present study were excluded from inclusion. Other exclusion criteria were; pregnancy, inflammatory autoimmune disease or any acute infection within 3 months prior to study participation. Information concerning complications during admission was collected from medical records. An acute chest syndrome was defined as a new infiltrate (on admission or during hospitalization) on chest x-ray associated with one or more new symptoms of chest pain, fever, tachypnea, wheezing, cough or hypoxemia [37]. Samples from race-matched volunteers were taken for control reference measurements. Written informed consent was obtained from all participants before any study procedure was performed. The study protocol was approved by the local medical ethical committee and conducted in agreement with the Helsinki declaration of 1975, as revised in 2008.

**Blood sample collection and laboratory analysis**

Blood samples were taken by venipuncture. Blood vials were centrifuged at 4°C for 15 minutes at 3000 rpm and serum and plasma was stored in small aliquots at -80°C until further analysis.

Hematology parameters, nucleosome and EA levels were measured in EDTA-anticoagulated plasma. Soluble vascular adhesion molecule1 (sVCAM1) levels were determined in serum (R&D Systems; Minneapolis, USA). Lactate hydrogenase (LDH) and bilirubin levels were measured with spectrophotometry in heparinized plasma (P800 Modular, Roche, Switzerland). Plasma levels of the long pentraxin-3 (PTX3) were determined using sandwich ELISA [38]. Antigen levels of von Willebrand factor (vWFag) were assessed by ELISA using antibodies from Dako (Glostrup, Denmark). Nucleosome levels were measured using ELISA as described previously [35, 39]. Neutrophil activation in form of elastase-α1-antitrypsin (EA) complexes was measured by an ELISA as previously described [30, 40].

**Statistical analysis**

For statistical analysis patients were divided primarily in two groups; patients with the relatively severe genotypes HbSS and HbSβ0-thalassemia grouped together (HbSS/ HbSβ0-thal) and patients with the relatively milder HbSC and HbSβ+-thalassemia
genotypes gathered in the other group (HbSC/HbS\textsuperscript{β\textsuperscript{-}thal}) \cite{41, 42}. We used a commercial statistical package (IBM SPSS Statistics 19, SPSS Inc, Hong Kong, PRC) for data analysis. Since results were not normally distributed, they are expressed as median with interquartile range. Unless stated otherwise, \(P<0.05\) was considered statistically significant. Bonferroni’s correction was applied for multiple testing (\(P<0.004\) was considered statistically significant).

**RESULTS**

**Patients**
Seventy-four patients in steady state (49 HbSS/HbS\textsuperscript{β\textsuperscript{0}-thal} and 25 HbSC/HbS\textsuperscript{β\textsuperscript{-}thal}), 70 patients during painful crisis (53 HbSS/HbS\textsuperscript{β\textsuperscript{0}-thal} and 17 HbSC/HbS\textsuperscript{β\textsuperscript{-}thal}) and 24 healthy race-matched controls (HbAA) were included in the study. Patients’ characteristics are summarized in Table 1. Twenty-four percent of sickle cell patients during steady state and a similar percentage (23%) of patients during painful crisis were on hydroxycarbamide treatment. Of the patients included with painful crisis, 1 (HbSS/HbS\textsuperscript{β\textsuperscript{0}-thal}) patient was admitted with acute chest syndrome (ACS) and 5 (4 HbSS/HbS\textsuperscript{β\textsuperscript{0}-thal} and 1 HbSC/HbS\textsuperscript{β\textsuperscript{-}thal}) patients developed an ACS between 48 and 60 hours after admission. No infection was reported for any of the patients with an acute chest syndrome.

**Nucleosomes and neutrophil activation**
Plasma levels of nucleosomes were significantly higher during painful crisis (20.0 U/ml; IQR 7.9-107.3) as compared to those in steady state (6.4 U/ml; 3.5-9.7, \(P<0.001\)) (Figure 1A). This was seen in both HbSS/HbS\textsuperscript{β\textsuperscript{0}-thal} (20.2 U/ml; 8.9-129.0 vs 6.0 U/ml; 3.0-9.8, \(P<0.001\)) and HbSC/HbS\textsuperscript{β\textsuperscript{-}thal} (11.7 U/ml; 5.1-67.7 vs 7.1 U/ml; 4.6-9.6, \(P=0.045\)) patients (Figure 1B). Plasma levels of nucleosomes in healthy controls were just above the detection limit of the assay (5.0 U/ml; 3.0-6.5). In steady state sickle cell patients plasma levels of nucleosomes were significantly higher compared to levels in healthy controls (\(P=0.031\)). In the analysis for the two genotype groups separately, the same was seen for HbSC/HbS\textsuperscript{β\textsuperscript{-}thal} patients in steady state (\(P=0.020\)) while plasma nucleosome levels in HbSS/HbS\textsuperscript{β\textsuperscript{0}-thal} patients in steady state were comparable with those in healthy controls (\(P=0.089\)).
Table 1. Baseline characteristics

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Asymptomatic state</th>
<th>Painful crisis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HbAA (n=24)</td>
<td>HbSS/HbSβ0 (n=49)</td>
<td>HbSC/HbSβ+ (n=25)</td>
</tr>
<tr>
<td>Age (y)</td>
<td>37 (29-46)</td>
<td>26 (22-40)</td>
<td>30 (24-42)</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>9/15</td>
<td>19/30</td>
<td>7/18</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>12.7 (11.9-14.0)</td>
<td>9.0 (8.1-10.0)</td>
<td>11.1 (10.5-11.6)</td>
</tr>
<tr>
<td>Leukocytes (10⁹/l)</td>
<td>5.6 (4.5-7.0)</td>
<td>9.9 (7.5-10.9)</td>
<td>6.4 (5.0-8.4)</td>
</tr>
<tr>
<td>Neutrophils (10⁹/l)</td>
<td>2.7 (1.7-3.4)</td>
<td>4.7 (3.4-6.0)</td>
<td>3.7 (2.8-4.3)</td>
</tr>
<tr>
<td>LDH (U/l)</td>
<td>181 (152-205)</td>
<td>385 (301-483)</td>
<td>228 (178-254)</td>
</tr>
<tr>
<td>Total bilirubin (mg/dl)</td>
<td>0.5 (0.4-0.8)</td>
<td>3.1 (1.8-4.9)</td>
<td>1.2 (0.8-1.5)</td>
</tr>
<tr>
<td>Hospitalization (days)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Numbers are medians with interquartile range (IQR)
Hb hemoglobin; LDH lactate dehydrogenase; NA Not applicable
a Significantly different as compared to HbAA controls (P<0.05).
† Significantly different as compared to asymptomatic state (P<0.05).
‡ Significant difference between HbSS/HbSβ0-thalassemia and HbSC/HbSβ+-thalassemia patients within steady state or painful crisis (P<0.05).

Plasma levels of EA were significantly higher during painful crisis (73.6 ng/ml; 54.9-100.8) as compared to those in steady state (46.2 ng/ml; 34.3-65.6, P < 0.001) (Figure 1C). This was seen in HbSS/HbSβ0-thal patients (75.1 ng/ml; 56.5-102.4 vs. 45.7 ng/ml; 34.7-59.7, P < 0.001), while in HbSC/HbSβ+-thal patients, the increment did not reach statistical significance (62.0 ng/ml; 48.0-96.7 vs 50.2 ng/ml; 33.3-67.7, P = 0.051) (Figure 1D). Plasma levels of EA in healthy controls (39.9 ng/ml; 31.5-62.2) were comparable to those in steady state sickle cell patients (P = 0.330).

During painful crisis, levels of nucleosomes correlated significantly with levels of EA (Sr = 0.654, P < 0.001). This was seen in both HbSS/HbSβ0-thal (Sr = 0.55, P < 0.001) as well as in HbSC/HbSβ+-thal patients (Sr = 0.90, P < 0.001). During steady state the correlation between levels of nucleosomes and EA was significant but weak (Sr = 0.236, P = 0.043). The correlation in HbSC/HbSβ+-thal patients in steady state remained significant (Sr = 0.63, P = 0.001), while no correlation was found between levels of nucleosomes and EA in HbSS/HbSβ0-thal patients in steady state (Sr = 0.043, P = 0.77).
Figure 1. Levels of nucleosomes (A and B) and elastase-α1-antitrypsin complexes (EA) (C and D) in healthy controls and in sickle cell patients in steady state and during painful crisis. Results are shown for all patients (A and C) and for the subgroups containing HbSS/HbSβ0-thalassemia and HbSC/HbSβ+—thalassemia patients (B and D). The number of patients in each group is indicated above the x-axis. Patients who developed an acute chest syndrome during admission were among those with the highest nucleosome and EA levels. The corresponding levels are indicated in black. Results are indicated as median with interquartile range. Comparison of the groups was performed by means of Mann Whitney Rank Sum test. A P-value < 0.05 was considered as statistical significant.

There was no difference in levels of nucleosomes and EA between patients with and patients without documented infection during painful crisis (data not shown). The use of hydroxycarbamide had no effect on levels of nucleosomes or EA in sickle cell patients whether in steady state or during painful crisis (data not shown).
In a paired analysis of 25 patients, accounting for 36 painful crises, significant increments were observed during painful crisis in plasma levels of both nucleosomes (from 5.0 U/ml; 3.0-10.8 to 20.2 U/ml; 6.8-94.3, \( P < 0.001 \)) and EA (47.9 ng/ml; 36.0-67.6 to 70.6 ng/ml; 55.9-101.4, \( P < 0.001 \)) as compared to those in steady state (Figure 2A and 2B).

![Graph of nucleosomes and elastase-α1-antitrypsin complexes](image)

**Figure 2.** Paired analysis of levels of nucleosomes (A) and elastase-α1-antitrypsin complexes (EA) (B) in 36 painful crisis in 25 patients included both in steady state and in painful crisis. For comparison between related samples the Wilcoxon Signed Rank Test was used. A \( P \)-value < 0.05 has been considered as statistically significant. Results are indicated as median with interquartile range.

**Nucleosomes and EA in association with markers of endothelial activation, hemolysis and inflammation**

While nucleosome levels in steady state HbSS/HbSβ^0^-thal patients correlated significantly with vWF:Ag (\( Sr = 0.452, P = 0.001 \)) and sVCAM-1 (\( Sr = 0.421, P = 0.003 \)) they only correlated significantly with PTX3 (\( Sr = 0.623, P = 0.001 \)) during painful crisis. In the same patient group during painful crisis EA levels just failed to reach a statistical significant correlation with PTX3 levels (\( Sr = 0.529, P=0.008 \)).

Leukocyte counts did not correlate with levels of nucleosomes or EA. In addition, neutrophil count did not correlate with levels of nucleosomes or levels of EA, neither when results of patients were pooled, nor when they were evaluated separately in
the different subgroups. No association was found between markers of hemolysis (hemoglobin, LDH and bilirubin) and levels of nucleosomes or EA.

**Association with acute chest syndrome and duration of hospitalization**

*Acute chest syndrome*

The 6 patients who developed an ACS were among those with the highest nucleosome (359, 274.8, 190, 130, 128 and 100 U/ml, respectively) and EA levels (549.9, 120.8, 91.8, 86.7, 75.1, and 63.9 ng/ml, respectively) (Figure 1A-1D). In these 6 sickle cell patients with ACS, nucleosome levels were significantly higher than those in patients during painful crisis without ACS (n= 64; 160.0 U/ml; 121.0-295.9 vs 20.07 U/ml; 7.9-107.3, \( P = 0.002 \)).

*Hospitalization duration*

Nucleosome levels, but not EA levels, correlated significantly with duration of hospital stay in all sickle cell patients during painful crisis (Sr = 0.441, \( P < 0.001 \)). Excluding the patients with acute chest syndrome the correlation remained statistically significant (Sr = 0.385, \( P = 0.002 \)). When analyzing the correlation for HbSS/HbS\( \beta^{0}\)-thal patients the correlation between nucleosome levels and duration of hospital stay was stronger (Sr = 0.530, \( P < 0.001 \)). Figure 3 shows the association between levels of nucleosomes and duration of hospitalization.

![Figure 3](image_url)
**DISCUSSION**

In the present study we demonstrate that during painful vaso-occlusive crisis sickle cell patients have significantly higher levels of circulating nucleosomes and neutrophil activation, as shown by increased EA complexes, as compared to sickle cell patients in steady state. Results of the paired analyses support the findings of the between group-analyses. We show that patients developing the severe and potentially life-threatening complication acute chest syndrome were among those with the highest nucleosome and EA levels. Moreover, we found that nucleosome levels correlate with duration of hospitalization. Nucleosome and EA levels correlate significantly with each other during painful crisis. Together, our data provide indirect evidence for NET formation in patients with sickle cell disease suffering from VOC.

Our results are in line with a previous study in sickle cell patients demonstrating significantly increased amounts of circulating cell-free DNA, as determined by quantitative PCR amplification, in sickle cell patients during painful crisis as compared to levels in steady state[43]. Interestingly, in the current study nucleosome levels in sickle cell patients with acute chest syndrome were comparable to levels measured in patients with severe sepsis using the same assay [34, 35]. In these patients with sepsis, circulating cell-free DNA in form of nucleosomes correlated with morbidity and mortality [34, 35].

This study has several limitations that need to be taken into account when interpreting the data collected. Firstly, we have not performed sequential nucleosome and EA analysis during admission for painful crisis in our patients, limiting the findings to a single measurement at presentation with a painful crisis. The observation that nucleosome and EA levels taken at presentation were highest in patients developing an ACS during admission are nevertheless in line with previous findings suggesting neutrophils to be an important player [44, 45] in the pathogenesis of ACS. The observation that neutrophil count does not correlate with EA or nucleosome levels in this study in sickle cell patients is supported by observations from studies in patients with severe sepsis [40]. While EA results reflect general PMN activation, it is likely that during vaso-occlusive crisis neutrophil count is a measure for circulating (“countable”) neutrophils while it does not reflect neutrophils migrated to tissue or adherent to activated endothelial cells, the latter being observed in vaso-occlusive crisis in mice models for sickle cell disease [20, 21]. Secondly, the correlation during painful...
crisis between nucleosome levels with EA levels and PTX3, both being localized in NET[46, 47] (while the latter has previously been demonstrated to be increased during sickle cell painful vaso-occlusive crisis)[48], support the hypothesis of PMN as an important nucleosome source via NET formation, at least during vaso-occlusive complications. This is also in line with the publications reporting circulating nucleosomes with or without markers for neutrophil activation to be a good measure for NET formation in circulation [29, 31]. However, the ELISAs detecting nucleosomes are not specific for nucleosomes released by PMN, and we can not, therefore, exclude the possibility that nucleosomes released into the circulation by other cell types, such as endothelial and parenchymal cells, are detected as well. The statistically significant correlation between nucleosome levels and markers of endothelial activation, vWFag and sVCAM-1, in steady state HbSS/HbSβ0-thal patients might be indirect evidence that damaged endothelial cells contribute to the circulating nucleosomes. Whether this endothelial cell damage is a consequence of local PMN activation, e.g. in form of NET formation being cytotoxic to endothelial cells, remains to be established. Thirdly, results of the analyses performed on the specified genotype groups sometimes diverge from the analyses done when pooling data from and considering the patient population as one group. This may be due to a limited number of patients in the respective subgroups causing a lack of power to show statistical significant findings. Moreover, the influence of the interaction of sickle cell erythrocytes with PMN on NET formation and the role of the genotype in this interaction has to be established yet.

In conclusion, we demonstrate for the first time elevated levels of circulating nucleosomes and neutrophil activation in sickle cell patients with painful crisis suggesting NET formation in these patients. NET, consisting of nucleosomes, proteases and histones, may promote endothelial activation and contribute to longer and more severe sickle cell crisis. The role of NET in the prediction of clinical complications in sickle cell disease painful crisis and as a potential therapeutic target deserves further study.
REFERENCES


