Sickle cell disease
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Chapter 9

SICKLE CELL DISEASE, 
_pathogenesis and biomarkers_

Summary of results and future perspectives
Chapter 1 is an introduction to this thesis in which we describe the need to establish clinically validated prognostic biomarkers[1, 2] that may help to improve the clinical management of patients with sickle cell disease (SCD). We also give a summary of current knowledge on the complex multifactorial SCD pathophysiology in order to provide the background for our studies on potential biomarkers. Special attention is given to existing data, obtained from mice and human studies, on the role of neutrophils in SCD related acute and chronic complications.

In Chapter 2 we describe the results of a prospective clinical study on plasma levels of both nucleosomes and the neutrophil enzyme elastase in complex with its inhibitor α1-antitrypsine (HNE-α1-AT) in patients with SCD, during vaso-occlusive crises (VOC) and steady state in comparison to levels in healthy controls. In total 74 patients in steady state, 70 patients during VOC and 24 healthy race-matched controls were included in the study. Samples during VOC were collected within 24 hours of admission. Of the patients with VOC, 1 patient was admitted with acute chest syndrome (ACS) and 5 patients developed an ACS during admission. During VOC levels of nucleosomes and HNE-α1-AT, as determined using ELISA, were found to be increased when compared to levels in steady state. There was a significant relation between these markers during VOC (Sr = 0.654, \( P < 0.001 \)). In the 6 sickle cell patients with ACS, nucleosome levels were higher as compared to those in patients during VOC without ACS. In addition, nucleosome levels in sickle cell patients during VOC correlated significantly with duration of hospital stay (Sr = 0.441, \( P < 0.001 \)).

As both neutrophil elastase and nucleosomes are constituents of neutrophil extracellular traps (NETs) and a correlation between circulating levels of both markers has been described to be a reflection of the presence of NETs (“soluble NET markers”), [3-5] the results of this study indicate the presence of NETs in patients with SCD in VOC. In a subsequent prospective clinical trial, we aimed to study the relation between these “soluble NETs markers” (nucleosomes and HNE-α1-AT) and established markers of inflammation (C-reactive protein (CRP), pentraxin3 (PTX3)) and neutrophil
(calprotectin) and endothelial activation (Von Willebrand Factor antigen (VWFAg) and Von Willebrand Factor propeptide (VWFpp)). The results of this study are described in chapter 3. To gain more insight into the kinetics of events during VOC levels of all markers were determined on subsequent days of admission for VOC. In this clinical study, 24 patients with SCD admitted for VOC were included, accounting for 32 admissions. Five patients developed an ACS during admission. Four weeks after discharge steady state blood samples were collected. The results of this study suggest that in patients with SCD, VOC is an acute inflammatory process with prompt neutrophil activation, on top of chronic endothelial activation. We confirm the presence of elevated levels of nucleosomes in patients with SCD during VOC as described previously,[6] although levels of HNE-a1-AT were not significantly elevated in this study, which is probably due to the smaller number of patients in this cohort. Interestingly, the highest nucleosome level in the second study (chapter 3) was more than a ten-fold higher than the highest nucleosome level we measured in our first clinical study (chapter 2), namely 27550 vs 2065 U/ml, although median levels during SCD VOC were in the same range, namely 20.0 vs 26.4 U/mL. The very high nucleosome levels in SCD patients with VOC complicated by ACS in the latter study were in the same range as non-survivors in patients with severe sepsis or septic shock[7] or non-surviving children with meningococcal C sepsis.[8]

In both cohorts we showed that levels of circulating nucleosomes significantly correlate with levels of HNE-α1-AT in patients with SCD during VOC,[6] suggesting an increased presence of NETs during VOC.[3, 4] In Berkeley sickle mice, NETs were identified as important players in the pathogenesis of VOC including related lung complications.[5] Increased levels of nucleosomes and HNE-α1-AT,[6] and also PTX3[9] during VOC and their relation with clinical severity indicate that NETs may be involved in the pathogenesis of VOC in sickle cell patients.[6] Data of both cohort studies support these findings, since the highest levels of nucleosomes, HNE-α1-AT and calprotectin were observed in patients with ACS. However, the number of patients included in both studies was limited. In the cohort described in chapter 3, 15% of patients admitted with VOC developed an ACS, in chapter 2 this percentage was 8.5%. Whether plasma levels of nucleosomes and HNE-α1-AT can be used as biomarkers to access the risk of ACS, should be verified in a prospective clinical study in a large cohort, preferably in a multi-center setting.
Several other aspects need to be addressed. Caution must be taken to attribute elevated levels and correlations between these markers solely to the presence of NETs, because circulating markers might not necessarily reflect processes at cellular level. Nucleosomes might indeed originate from NETs, but it is also possible that VOC-ischemia induced tissue damage, like endothelial or parenchymal cell damage, may have induced the release of nucleosomes as nucleosomes are the basic unit of DNA-organization.

To further investigate the role of NETs in patients with SCD, experimental in vitro studies are warranted to study the contribution of these NETs to SCD pathophysiology, also because NETs constituents are known to induce cell damage through direct cytotoxic effects to both parenchymal as well as endothelial cells and are able to induce coagulation.

In chapter 3 we reported on stable levels of and VWFpp and stable elevated levels of VWFAg during VOC and steady state, indicative of chronic endothelial activation in patients with SCD, which is in line with previous publications.[10, 11] In Chapter 4, we describe a follow-up study designed as an in depth study on the role of VWF in SCD VOC pathogenesis. Despite the relatively stable levels of VWFAg and VWFpp during VOC, elevated VWF reactivity is found during VOC, as we observed significantly higher levels of aVWF, VWF:GPIbM and HMW multimers during the first days of VOC compared to steady state. This higher VWF reactivity might partly be explained by concomitantly lower plasma levels of ADAMTS13:ag and ADAMTS13:act observed during VOC. Although no pronounced ADAMTS13 deficiency was observed. We hypothesize that reactive oxygen species, released by activated neutrophils, may inhibit VWF cleavage by ADAMTS13, as previously described by oxidation of its cleavage site[12]. This was supported by the strong correlations between VWF reactivity and markers of neutrophil activation like calprotectin and HNE-α1-AT. As the hyper-adhesive VWF is potent in scavenging platelets and sickle erythrocytes in the circulation, thereby promoting (thrombotic) microvascular occlusion, it is of interest to further elucidate in future studies if the higher VWF reactivity during VOC is merely a marker of severity of disease or actually contributes to VOC pathogenesis.

As we show in previous chapters and in concordance with observations by others, neutrophil activation is present in SCD VOC. Neutrophils isolated from patients with SCD have an higher expression of adhesion molecules,[13-15] including Mac-1, when
compared to neutrophils from healthy controls.[13] Interestingly, an important role for Mac-1 in regulation of NETs formation was previously suggested, [16] as in vitro pretreatment of neutrophils with a inhibitory monoclonal antibody to the Mac-1 integrin adhesion receptor drastically reduced NETs formation. Therefore, it would be interesting to investigate if neutrophils of patients with SCD are more prone to make NETs when compared to neutrophils from healthy donors, which may lead to complications in affected patients. In addition, one wonders if there are factors in the circulation of patients with SCD that might induce NETs formation by neutrophils, like heme, as recently suggested by the group of Chen et al.[5] This, as in their experimental sickle mice model in which they induced VOC by the administration of TNF-α plus hemin, NET formation could be reduced by infusion of hemopexin (Hpx), the natural heme-scavenger.

As we were interested to investigate the potential therapeutic use of Hpx in patients with SCD, we aimed to determine, as described in chapter 5, whether ex vivo addition of Hpx to sera obtained from patients with SCD would prevent NET formation by healthy donor (HD) neutrophils. In this experimental study, the sera of patients with SCD admitted for VOC and in steady state were used. We used confocal fluorescence microscopy and staining for extracellular DNA with the cell impermeable DNA-binding dye Sytox Green to investigate NET formation by human neutrophils. NET formation was quantified as the surface area of confocal images covered by positive fluorescence staining of NETs. In our study, sera of patients with SCD were found to be able to induce NETs in neutrophils obtained from healthy donors. NET formation was significantly enhanced with VOC sera, $P=0.008$, when compared to steady state sera, despite the fact that cell-free heme levels in sickle cell patients in VOC were comparable to those in patients in steady state. Exogenous hemin (ferriprotoporphyrin IX) was also able to induce NETs in neutrophils obtained from HD, while protoporphyrin IX (lacking the iron-moiety) did not trigger NET formation in neutrophils. This indicates that the iron-moiety is essential in hemin induced NET formation. The formation of NETs induced by exogenous hemin was abrogated by adding plasma-derived Hpx. Intriguingly, adding plasma-derived Hpx to SCD sera failed to prevent NET formation in all sera from patients with SCD tested. However, when the iron chelator deferoxamine or the iron-binding protein apotransferrin was added to sera, NET formation was prevented in a subset (half) of sera of patients with VOC. Upon ranking the patients according to the level of labile plasma iron, it became apparent that DFO addition affected NETs release in those sera with high labile iron.
levels. Therefore, we suggest a role for free extracellular iron in the induction of NETs by serum in a subset of patients with SCD. Further studies should reveal if iron binding compounds may form an interesting therapeutic in the treatment of VOC in patients with SCD. Interestingly, in half of sera of patients with SCD tested NET formation could not be abrogated using iron-binding agents. Future studies should reveal what (other) compounds are present in SCD sera that may induce NET formation in neutrophils. Possible candidates are pro-inflammatory cytokines (like IL-8 and TNF-α) as well as complement factors.[17, 18]

In chapter 6 we investigated possible mechanisms responsible for an increased urinary zinc loss in patients with SCD which may lead to the commonly observed zinc deficiency in these patients. We studied relations between urinary zinc levels, adjusted for urinary creatinine, and urinary markers of bone degradation (pyridinoline (PYD) and deoxypyridinoline (DPD)), urinary markers of renal damage (Kidney Injury Molecule-1 or Heart-type Fatty Acid Binding Protein) and established plasma markers of hemolysis (LDH, bilirubin, reticulocyte count) in two cohorts of patients with SCD and one cohort with healthy controls. In the primary cohort, 38 patients with SCD in steady state, 27 patients in VOC and 25 healthy race-matched volunteers were included. Urinary zinc levels were higher in patients with SCD in steady state when compared to levels in healthy controls($P <0.001$), but lower when compared to levels in patients with SCD in VOC ($P = 0.004$). Both fractional excretion of zinc (FEZ) and sodium (FENa) were higher in patients during VOC when compared to steady state. Urinary levels of zinc did not correlate with reticulocyte counts nor with levels of LDH and bilirubin. In patients during VOC a significant relation was observed between urinary zinc and urinary PYD levels ($r_{0.417}, P = 0.030$) but not with DPD levels. In the second cohort 24 patients with SCD accounting for 32 VOC were included. Samples were obtained on succeeding days of admission for VOC and an increase in urinary zinc excretion was observed on subsequent days of admission for VOC. No significant correlations between urinary zinc levels and urinary levels of KIM-1 and HFABP were observed in patients in VOC. During VOC, in the first sample taken after admission, urinary levels of zinc correlated significantly to urinary levels of DPD ($S_{0.439}, P = 0.012$) and PYD ($S_{0.388}, P = 0.028$). In an additional cohort including twelve healthy male controls, urinary FENa increased over time after intravenously sodium infusion, while urinary FEZ decreased, indicating a zinc-sparing effect of exogenous sodium load in these subject. This is opposed to an increased urinary FEZ in SCD patients in VOC, indicating that the increased FEZ is specific for SCD and not the result of
iatrogenic sodium load. We conclude that there is increased urinary zinc excretion in patients with SCD, especially during VOC. In VOC urinary zinc levels correlated with markers for bone resorption, indicating that locally ongoing microvascular ischemia of the bones and subsequent bone degradation are the major source of increased renal zinc excretion. Relations between urinary zinc levels and the development of ACS were not assessed in this study, but we observed that urinary zinc levels on the first morning after admission for VOC correlated significantly with the duration of admission (\( R = 0.437, P = 0.02 \)). Whether urinary zinc is an ideal biomarker in the prediction of SCD related complications requires more clinical studies. In favor of urinary zinc determination as a potential useful biomarker in patients with SCD, is the easy accessible collection of a urinary marker.

In the introduction, we described that both chronic hemolysis and recurrent and generalized (micro)vaso-occlusion in patients with SCD ultimately lead to a wide range of organ complications. The occurrence of organ damage in patients with SCD is a crucial determinant for both medical treatment and prognosis. To be able to intensify treatment of patients with SCD at risk for developing organ complications or at risk of early death, for example by commencing a transfusion program, it is of utmost importance to have clinical or biochemical parameters that allow identification of these patients.

Previously, in US cohorts, both elevated N-terminal prohormone brain natriuretic peptide (NT-proBNP) plasma levels (>160 pg/mL) and tricuspid regurgitant flow velocity (TRV ≥ 2.5 m/s) have been related to the presence of pulmonary hypertension (PH) and risk of early death in patients with SCD. In chapter 7, we describe the results of our cohort study that included 85 consecutive ambulatory SCD patients who were followed for 6 years, during which 12 deaths occurred. We confirm that a plasma NT-proBNP level ≥ 160 pg/mL is a strong predictor for mortality in these patients (Hazard ratio (HR) 10.0 [CI 2.9-34.4], \( P < 0.001 \)). Interestingly, this was found to be independent of TRV: HR for mortality was 11.0 [CI 3.1-38.4], \( P < 0.001 \) after adjustment for elevated TRV. HR for mortality for patients with only TRV levels ≥ 2.5 m/s at baseline was not significantly elevated: 1.6 [CI 0.5-5.2], \( P = 0.4 \). This study, as well as other observations, suggests that plasma NT-proBNP values in patients with SCD are probably influenced by several other factors besides the presence of PH. This is important to realize as recent guidelines advise determination of levels of NT-proBNP as a screening surrogate for PH in patients with SCD when transthoracic
Doppler measurement of TRV is not available.[19] Nonetheless, a plasma level of NT-proBNP ≥ 160 pg/ml is a strong prognostic marker for mortality in patients with SCD, also in our Dutch cohort. It is intriguing that in non-SCD patients with heart failure a much higher NT-proBNP cut-off value (generally ≥1000 pg/ml) is used to identify patients at risk for complications like mortality.[20-22] NT-proBNP is released upon stretching of the atrial and failing ventricular myocardium. Probably in patients with SCD additional mechanisms leading to increased natriuretic peptides play a role. Results of the (limited, due to power problems) multi-variate analyses in our study demonstrate that none of the included variables (such as age, renal function, TRV or hemoglobin levels) that may affect NT-proBNP plasma levels can solely explain the increased HR for death for patients with NT-proBNP plasma levels of ≥ 160 pg/ml. Therefore, additional variables may play a role. Possible candidates are hypoxia inducible factors (HIFs). HIFs are a family of transcription factors and the transcription of natriuretic peptides is probably directly influenced by these HIFs.[23, 24] Several factors, other than natriuretic peptides, that use HIF for transcription of genes are also increased in SCD patients, including angiogenic growth factors erythropoietin, angiopoietin-2 and stromal-derived growth factor-1α [25], but also other vasoactive molecules like endothelin, [26], VEGF [27, 28] and heme oxygenase-1 (HO-1)[27, 29]. An upregulation of HIFs in SCD patients can result from an increased exposure to hypoxia [Abstract 2229, Cabanas-Pedro, ASH Conference 2013/ Sun, Curr Opinion Hemat 2013], due to recurrent transient episodes of tissue ischemia during VOC, as well as from the chronic inflammatory state in patients with SCD under normoxic conditions. [27] It might therefore be that increased levels of NT-proBNP in SCD are a reflection of the systemic oxygen condition of the patient with SCD and that it is actually this factor that determines an increased risk of mortality in these patients. It might be of interest to conduct a study to determine levels of HIFs in patients with SCD, both in the steady state as well as during VOC.

Apart from predictors of early death, we were interested to study the course and incidence of the various forms of organ damage and SCD related complications in patients with SCD in our Dutch tertiary teaching hospital. The results of this study are described in Chapter 8. Previously, in a cohort of 104 consecutive ambulatory SCD patients the prevalence of SCD related organ damage and complications was systematically analysed.[30] This cohort was subsequently followed for 7 years and patients were prospectively screened biannually for sickle cell related complications. The following forms of sickle cell-related organ damage were screened according to
international guidelines: microalbuminuria, renal failure, elevated TRV ≥2.5 m/sec, retinopathy, iron overload, avascular osteonecrosis and cholelithiasis. Sickle cell related complications like: leg ulcers, acute chest syndrome (ACS), frequent VOC (≥3 episodes/year), stroke and priapism were scored. Of the 104 patients included in the original study, 9 were lost to follow-up. Overall, 59 patients (62%) developed a new form of organ damage or new complication since baseline. As expected, patients with the HbSS/HbSβ0-thalassemia genotype were at increased risk of development of any form of new organ damage compared to patients with the HbSC/HbSβ+-thalassemia genotype (HR 2.4 [CI 1.3-4.5], P < 0.007). Interestingly, the risk of developing any new form of organ damage did not differ between patients in whom organ damage was not manifest at baseline and patients who already had some form of organ damage. In addition, the use of hydroxycarbamide (scored at baseline) did not protect patients with SCD from the development of organ damage or SCD related complications (HR 1.1 [CI 0.6-2.2], P = 0.64). Furthermore, there was no relation between the frequency of VOC experienced during follow-up and the risk of developing any new form of organ damage or complication (HR 0.93 [CI 0.8-1.1], P = 0.5). With this study we again confirm the need for continued screening for occult organ damage in all patients with SCD, but especially in those with the HbSS/HbSβ0-thalassemia genotype.

**In conclusion**, patients with SCD suffer from a wide range of complications and so far biomarkers that identify patients at risk for development of these SCD related complications, and enabling improvement in the clinical management of these patients, are lacking. In this thesis, we studied several potentially useful biomarkers in prospective clinical studies.

Several of these studies focused on biomarkers identifying patients with SCD VOC (to support the clinical diagnosis) and/or at risk for development of VOC related complications. The potential of the urinary biomarker zinc as an indicator for the presence of VOC is an interesting focus for a prospective trial. We think the most promising biomarker for identifying patients at risk for development of the VOC related complication ACS is the plasma level of nucleosomes, because in two prospective studies we have found the highest levels of nucleosomes in patients with ACS. The observed relation between levels of nucleosomes and neutrophil activation markers, found in both studies, might be an indication for the presence of NETs in patients with VOC and underline the role of neutrophils in the complex pathophysiology of VOC. If higher VWF reactivity during VOC, which correlates with markers of inflammation and
neutrophil activation, is a result of VOC or actually contributes to its pathogenesis remains to be studied.

Sickle cell sera of patients with SCD, especially in VOC, are able to induce NET formation in neutrophils obtained from healthy donors. In a subset of patients this is partly caused by the presence of extracellular iron. This makes iron-binding agents like apotransferrin or oral iron chelators interesting possible therapeutics for SCD VOC.

In two chapters we describe the results of studies with a longer follow-up to identify biomarkers related to the development of SCD related long-term organ complications and mortality. NT-proBNP is a promising independent biomarker for identifying patients with SCD at risk for early mortality. The origin and pathophysiological relevance of increased levels of NT-proBNP in patients with SCD remain to be elucidated.

It might seem a bit futile, to develop screening methods to identify patients with SCD at risk for development of SCD related complications as long as therapeutic options for patients with SCD remain limited. Nonetheless, we believe that results of the studies presented in this thesis are useful for further improvement of the clinical management of patients with SCD and helpful to stratify patients at risk for complications. Moreover, these studies provide new and important insights into the pathophysiology of SCD that might form the basis for the development of novel therapeutic modalities.
REFERENCES


