Studies on the patholphysiology, disease severity assessment and management of sickle cell disease

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The studies described in this thesis focused on three main subjects;
- the pathophysiology of sickle cell vaso-occlusion,
- novel markers for assessing sickle cell disease (SCD) severity,
- potential therapeutics for sickle cell patients.

The main findings are summarized in the following paragraph. Related future perspectives for research are presented thereafter.

1.1 Summary

Chapter 2 encompasses a general overview of SCD [1]. Next to its pathophysiological basis, the major complications and its general management are discussed. Also, the complexity of risk assessment for poor outcome is addressed. The frequency of acute vaso-occlusive events is often used as a marker of disease severity in patients with SCD. Based upon our own experience, as well as on findings in the literature, we suggest that for the majority of patients, monitoring therapy efficacy and assessing disease severity by only scoring the frequency of such events may not be accurate [2; 3]. In order to describe the vaso-occlusive process more accurately, we propose three levels of vaso-occlusion in *chapter 3*, namely *silent-*, *non-clinical-*, and *clinical vaso-occlusion* [4]. The fact that even patients who seldomly require medical care acquire significant organ damage throughout life and have a decreased life expectancy, underscores the impact of both non-clinical and silent vaso-occlusion on the morbidity and mortality in SCD. Based upon the increasing understanding of the pathophysiology of sickle cell vaso-occlusion, we discuss several novel potential markers that may prove to reflect the silent vaso-occlusive process in SCD. Also, potential therapeutic strategies based on current insights into the pathogenesis of sickle cell vaso-occlusion (such as anticoagulation and B-vitamin supplementation), are presented [4].

In *chapter 4* a role for interleukin-8 (IL-8), a neutrophil chemotactin, in the pathogenesis of clinical vaso-occlusion in sickle cell patients is demonstrated [5]. Patients presenting with a painful crisis were characterized by strongly elevated IL-8 levels, irrespective of the crisis inducing factor. With amelioration of symptoms IL-8 levels dropped to levels comparable to those in healthy controls. As the Duffy antigen receptor for chemokines on red blood cells binds and inactivates IL-8, we investigated whether a Duffy-positive phenotype is protective for clinical vaso-occlusion [6-8]. No difference in clinical vaso-occlusion could be detected between Duffy-negative and Duffy-positive adult sickle cell patients (*chapter 5*) [9]. The finding of a significantly higher upward deviation of mean IL-8 levels in Duffy-negatives as opposed to Duffy positives suggests that IL-8 blood levels are more tightly regulated in Duffy positive sickle cell patients.

Endothelial activation is an integral part of the sickle cell vaso-occlusive process [10]. We investigated if the degree of endothelial activation, as measured by soluble vascular cell adhesion molecule-1 (sVCAM-1) serum levels, is indicative of the occurrence of clinical vaso-occlusion in SCD. As described in *chapter 6*, this was not the case in HbSS adults [11]. The strikingly elevated sVCAM-1 levels

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A study in pediatric sickle cell pat
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were inversely related to hemoglobin levels in this patient group. Given the role of VCAM-1 interactions with the very late activating antigen-4 on erythroid progenitors and reticulocytes in erythropoiesis, a potential relationship of sVCAM-1 to hematopoietic growth factors was investigated [12, 13]. sVCAM-1 levels did not correlate significantly to interleukin-3, granulocyte-macrophage colony stimulating factor or erythropoietin serum levels, or to serum levels of the soluble transferrin receptor (*chapter 7*) [14]. In *chapter 8* a statistically significant correlation of sVCAM-1 levels with plasma von Willebrand factor (vWF) levels (which to our knowledge is not involved in erythropoiesis) is described [15]. Based upon these findings we conclude that serum sVCAM-1 levels most likely reflect vascular endothelial activation in sickle cell patients.

We investigated whether decreased plasma levels of naturally occurring anticoagulants protein C and S are associated with clinical vaso-occlusion in chapter 8 [15]. The more severe phenotype of SCD (HbSS) was characterized by the more severe derangement of protein C and S levels (as compared to HbSC patients). Apart from slightly lower protein C in HbSC patients who had experienced clinical vaso-occlusion in the years prior to sample collection, no difference was detected between patients that did, or did not experience clinical vaso-occlusion. There was a significant inverse correlation between sVCAM-1 levels and both free and total protein S levels, and there was a significant linear correlation between total protein S levels and hemoglobin levels in HbSS patients.

Increased nitric oxide availability is currently regarded to be of importance in vaso-occlusive complications of SCD [16]. In *chapter 9* we demonstrated that arginine metabolism seems to be shifted to the arginase pathway in HbSS patients, with the extent of this shift being greater in patients with lower hemoglobin levels and higher leukocyte counts [17]. The increased demand for polyamines for red blood cell production (and perhaps also for repair of continuously damaged endothelium) may explain these preliminary results [18].

The results of a pilot study with low adjusted dose acenocoumarol therapy in patients are reported in *chapter 10* [19]. It was demonstrated that thrombin generation can be significantly reduced in these patients with low intensity acenocoumarol therapy (INR 1.6-2.0). However, no effect on clinical endpoints was detectable. Inflammation and coagulation are closely linked, and as *in vitro* data indicate that coumarin derivatives may reduce endothelial activation, we measured available endothelial activation markers in the patients on anticoagulation [20-22]. As described in *chapter 11*, no effect of anticoagulation with acenocoumarol could be detected on endothelial activation [23].

Finally in pediatric sickle cell patients regarding plasma homocysteine levels are described in *chapter 12*. Despite similar folate, vitamin B_6 and B_{12} blood levels as compared to age and race matched controls, homocysteine levels were

higher in our pediatric patients [24]. The lowest achievable homocysteine level was determined by supplementing high doses of folic acid, vitamin B_6 and vitamin B_{12} [24]. Subsequently, the optimal daily dose of folic acid, vitamin B_6 and vitamin B_{12} supplementation in order to achieve this target homocysteine level was determined (*chapter 13*) [25].

1.2 Future perspectives

In this thesis we described the importance of accurately assessing SCD severity for the management of patients with this debilitating disease. Monitoring the effect of (experimental) therapies by solely assessing the effect on clinical vaso-occlusion is inaccurate, as neither the rate of accumulating organ damage, nor mortality, are related to the frequency of clinical vaso-occlusion in most sickle cell patients [4].

We attempted to investigate whether several laboratory markers linked to the pathophysiology of vaso-occlusion could be useful as parameters for assessing SCD severity. The interpretation of the results of our and other comparable studies is limited by several factors. Firstly, disease severity is often arbitrarily defined. Some studies combine hematological characteristics (such as the percentage of fetal hemoglobin [HbF%] and hemoglobin levels) with clinical vaso-occlusion, whereas others only use clinical vaso-occlusion as an indication of disease severity [26-29]. In some studies only clinical vaso-occlusion that requires hospitalization is scored as a vaso-occlusive event, whereas other studies also include emergency room visits [2; 30-35]. Sometimes laboratory data, the rate of clinical vaso-occlusion as well as infectious complications are combined [36]. Secondly, for accurately assessing the value of both established (such as the HbF%) and potential (such as serum sVCAM-1 levels) markers of disease severity, all levels of vaso-occlusion (clinical, non-clinical and silent vaso-occlusion) should be monitored. Thirdly, many studies, including ours, are retrospective. Selection bias occurs when associations of novel potential markers of disease severity are studied in this manner, as blood samples of deceased patients are mostly not available for laboratory testing.

Institution of specialized sickle cell medical centers, such as in the U.S.A., England and Jamaica, has not only improved patient care, but also provided the infrastructure for much needed epidemiological and pathophysiological research [37-39]. In order to minimize the caveats outlined above, we are currently centralizing the out-patient care for sickle cell patients in Curaçao. Both pediatric and adult patients will be seen at a specialized 'out-patient sickle cell center'. In this setting all patients (and/or their parents when applicable) will be asked to give informed consent to participate in a prospective cohort study in order to better define the patients disease course. In order to assess non-clinical vaso-occlusion, patients will be asked to keep pain diaries at home [40]. For analyzing the extent to which silent vaso-occlusion occurs, it seems imperative to determine the extent of ischemic organ damage and dysfunction with objective diagnostics tools (such as imaging studies and specific organ function testing) [41-43]. As acute care for sickle cell patients in Curaçao is almost exclusively confined to one hospital, we feel

that we can accurately measure clinical, non-clinical and silent vaso-occlusion in such a setting. Standard hematological, biochemical, and organ function tests, as well as selected imaging studies will be performed at pre-determined intervals, and blood samples will be stored centrally for subsequent laboratory studies. Hopefully, this comprehensive approach will not only provide us with new insights into SCD pathophysiology and objective additive tools for managing sickle cell patients, but will optimize the care and guidance of patients and their families as well [44; 45].

Which potential laboratory parameters should be studied as markers for assessing the vaso-occlusive disease severity in SCD? Increasing insights into vascular homeostasis are not only unraveling the many functions of endothelial cells, but also provide us with several potential parameters for assessing endothelial activation and damage in SCD. For example, next to monitoring soluble markers such as sVCAM-1, characterization and enumeration of circulating endothelial cells may provide more accurate information on the extent of endothelial damage in SCD [46-49]. Also, novel biomarkers of tissue ischemia are being developed, such as ischemia-modified albumin, which should be studied in SCD [50].

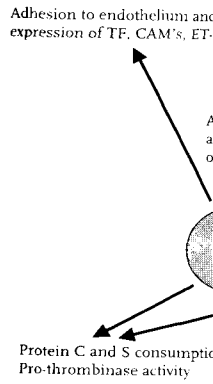
Other laboratory tools that are of interest for predicting the occurrence of SCD-related complications include hemostatic profiles. Indeed, plasma D-dimer levels have been shown to be associated with the occurrence of clinical vaso-occlusion in both a retrospective as well as a prospective setting [51; 52]. The similarity of the SCD multi-organ failure (SCD-MOF) syndrome to thrombotic thrombocytopenic purpura (TTP), together with reports of reversal of SCD-MOF with plasmapheresis, suggests a potential common denominator in these two clinical syndromes [53-57]. Central to the pathogenesis of TTP is a reduced activity of ADAMTS13 (a disintegrin-like and metalloprotease domain with thrombospondin type I motifs), which cleaves the thrombogenic unusually large (UL) vWF multimers upon their release from activated endothelial cells [58]. As has been shown that UL-vWF multimers also mediate the adhesion of sickle red blood cells (SRBC's) to endothelial cells *in vitro*, we are currently studying if reduced activity of ADAMTS13 is associated with the occurrence of SCD related vaso-occlusive complications [59]. Perhaps monitoring of ADAMTS13 in patients with severe painful events may help to predict the SCD-MOF syndrome and allow early institution of aggressive interventions.

Genetic traits that may influence the outcome of sickle cell patients are currently subject of intensive research. For example, specific polymorphisms of the VCAM-1 gene may be associated with a reduced risk of stroke, and patients with nitric oxide synthetase gene polymorphisms may have an increased susceptibility to the acute chest syndrome (ACS) [60-62]. It is generally accepted that monitoring disease severity with blood levels of soluble factors such as cytokines, adhesion molecules and clotting factors is challenging. Such soluble factors can display biological variations with time and within individuals, and their levels may not accurately reflect their concentration or effect at a site of tissue injury [63; 64]. However, SCD is characterized by continuous vaso-occlusion leading to endothelial activation and

damage in the vascular beds of virtually all organs. Therefore, through careful selection of parameters that are widely and continuously expressed on activated endothelium, measuring soluble factors may prove of value in monitoring the vaso-occlusive process in SCD. Pertaining to sVCAM-1, we have provided data indicating its potential as a marker for monitoring endothelial activation in SCD, although we cannot definitively rule out that sVCAM-1 levels are also determined in part by stress erythropoiesis [11; 14; 15]. New studies have shown that sVCAM-1 levels correlate to specific organ dysfunction in sickle cell patients, and a recent study has shown that HbF induction, which is known to reduce (clinical) vaso-occlusion, was associated with a decrease not only in sVCAM-1 levels, but also in vWF levels, inflammatory markers and hypercoagulability during the clinically asymptomatic state [65; 66]. As an induction of HbF also attenuates hemolysis, it remains possible that the sVCAM-1 reduction reflects a reduction of both endothelial activation (and perhaps silent vaso-occlusion) and erythropoiesis [14; 67]. Studies in large and accurately described populations should further address the potential of markers of endothelial activation for monitoring the vaso-occlusive process, taking into account their relation to all levels of vaso-occlusion, the inter- and intra-patient variability, as well as their ability to predict poor outcome. Measuring endothelial activation markers in stored serum and plasma samples of previously described cohort studies will likely be the most appropriate strategy.

The increasing understanding of the pathogenesis of sickle cell vaso-occlusion has shifted its paradigm from simple obstruction of blood vessels by SRBC's to a highly complex mechanism in which most types of blood cells, the coagulation cascade, the endothelium and inflammatory mediators are involved (see figure 1) [16; 68-70]. This has provided us with new potential therapeutic targets for managing SCD. Given the many factors involved in the vaso-occlusive process of SCD, targeting multiple pathophysiological pathways may have a synergistic clinical effect. A case in point is hydroxyurea; whereas its beneficiary effect was initially thought to be solely attributable to HbF induction, it is now clear that reduction of neutrophilic granulocytes via myelosuppression, as well as (directly or indirectly) reduced erythrocyte-endothelial adhesion also are of importance [71-73]. As vaso-occlusion occurs continuously even in clinically asymptomatics, therapeutics aimed at preventing or reducing organ damage should be taken lifelong and should reduce silent and (non) clinical vaso-occlusion [4]. Throughout a great part of their life, many patients may be relatively symptom free, thus requiring such therapeutics to be simple in use and to have minimal side effects. In this light, it should be realized that the greatest burden of SCD occurs in developing countries, with about 120,000 children born with a form of SCD per year in Africa (as opposed to 1,000 newborns with SCD per year in the United States of America) [74]. It is surprising that optimism about current and experimental therapies for SCD is not counterbalanced by the fact that few accepted therapeutics are applicable in financially stricken developing countries [75]. Therefore, we should utilize our 'high-tech' diagnostics to study and develop management strategies that ultimately have the potential to benefit patients globally.

Figure 1



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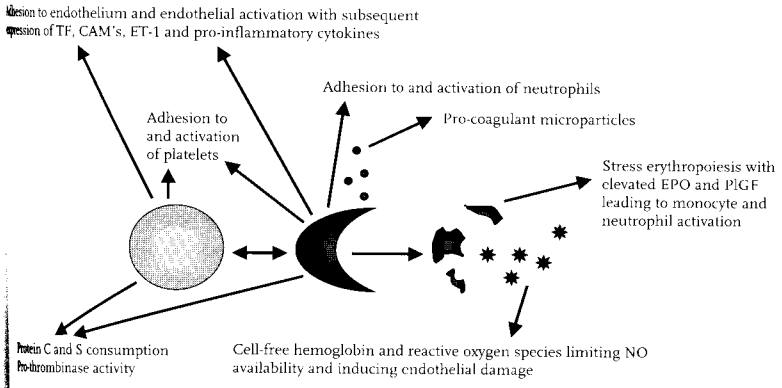

Figure 1: Central role of the sickling cycle and hemolysis in the pathophysiology of sickle cell disease. TF = tissue factor, CAM = cellular adhesion molecule, ET-1 = endothelin-1, PlGF = placenta growth factor, NO = nitric oxide, EPO = erythropoietin.

In the coming months, an intervention study with oral N-acetylcysteine (NAC) will be conducted in Curaçao. NAC is an antioxidant with pleiotropic anti-inflammatory effects, and oral supplementation with NAC replenishes reduced erythrocyte gluthation stores in sickle cell patients (gluthation is the most abundant antioxidant in our body and forms an important defense against free radicals) [76-78]. Reactive oxygen species play an important role in the pathophysiology of SCD related endothelial damage via reperfusion injury, and NAC administration to patients with SCD may be of benefit via multiple mechanisms [76; 78-81]. Examples of promising therapeutics for SCD that are currently being studied by other groups include supplementation of arginine, zinc and n-3 fatty acids, as well as clotrimazole and magnesium salts in order to reduce red cell dehydration [82-86]. Also, inhibition of nuclear factor κB (a transcription factor that regulates several important proteins involved in the vaso-occlusive process of SCD) with sulfasalazine is currently being investigated [4; 87; 88].

So what of anticoagulation as a therapy for SCD? In simple painful crises, the contribution of fibrin formation in the microvasculature may be limited and occurs secondarily to adhesion of SRBC's and leukocytes to endothelial cells [19]. It remains to be seen whether anticoagulation prevents such events (or reduces their duration) [69]. Whereas hydroxyurea prevents the occurrence of both painful crises and ACS's, it may be less effective in preventing ischemic strokes, which are often characterized by large vessel thrombosis [68; 89-94]. Given the burden and risks of chronic transfusion therapy, a randomized controlled trial comparing red cell transfusions to anticoagulation in high-risk pediatric patients for ischemic

stroke seems justified. Also, thrombosis of pulmonary vasculature may contribute to the development and progression of chronic sickle cell lung disease, which is characterized by pulmonary fibrosis and pulmonary hypertension, and has a very poor prognosis [95-101]. It is estimated to occur in at least 30% of adult sickle cell patients [100; 102]. We will conduct a study in which, with modern pulmonary imaging techniques and echocardiography, the presence of pulmonary hypertension and pulmonary vascular occlusion will be assessed. If a decreased pulmonary vascular patency is associated with pulmonary hypertension in these patients, intervention studies with anticoagulation will follow. As in any study regarding anticoagulation, the fear of bleeding complications will necessitate stringent inclusion criteria. However, with the advent of newer and possibly safer anticoagulants, the potential role of anticoagulation, especially for prevention of such major complications as described above, should be investigated [103]. Interestingly, heparin has been shown to reduce SRBC-endothelial cell adhesion, making this an interesting option for therapeutic trials in SCD [104-107].

Given the central role of the sickling process in the pathophysiology of vaso-occlusion, it is clear that for long-term pharmacological management of SCD, the single most effective way to reduce vaso-occlusion would be to limit HbS polymerization. As shown in our work, the extent to which several pathophysiological processes occur is related to the degree of anemia, and based upon large studies, lower hemoglobin levels are associated with many SCD related complications [1; 11; 15; 17; 108]. An exception is the pain-rate and ACS frequency, indicating that relatively high hemoglobin levels without concomitant HbF or HbA_2% increments result in a higher rate of clinical vaso-occlusion [2; 109]. The effect of anemia on silent vaso-occlusion, however, may be the opposite in specific organs as exemplified by the higher risk of ischemic small and large vessel brain injury in absence of overt stroke in patients with relatively lower hemoglobin levels [110; 111]. It is also important to realize that, even though the work in this thesis focused mainly on vaso-occlusion, the hemolytic anemia, with its concomitant hemodynamic changes, is not only a symptom of SCD, but in many ways a major contributor to the pathology observed in these patients. This is supported by the fact that in patients with symptomatic thalassemia the incidence of pulmonary hypertension, strokes and leg ulcers is also high [112; 113]. Furthermore, thalassemia patients (but also patients with other forms of hemolytic anemia) are also characterized by a hypercoagulable state and endothelial activation [113-117].

Currently, HbF inducing agents (such as butyrate and decitabine) are under active investigation as anti-sickling agents [118]. However, it does not seem conceivable that, in the near future, effective red cell sickling prevention will be either widely applicable, or by itself sufficient to effectively prevent organ damage [1]. Bone marrow transplantation, which can cure SCD, remains a high-risk procedure that is unlikely to benefit most sickle cell patients. Therefore, it is likely that a combination of several different drugs targeting multiple pathways involved in the vaso-occlusive process will be needed for effective long-term management of most sickle cell patients. If the different pathophysiological mechanisms involved

n vaso-occlusion are further characterized and their importance in relation to developing organ damage can be established, risk assessment can be optimized, and individualized therapeutic regimens could become daily practice. Perhaps in the future, as a form of 'vascular endothelial supportive care', a widely applicable single 'sickle cell pill' will consist of anti-oxidants, B-vitamins, and arginine. Such a pill could also be used next to an anti-red cell dehydration agent, an HbF inducing agent, an anti-inflammatory agent and/or perhaps an anticoagulant depending on the risk profile of the patient. However, before such therapeutic strategies can be developed, randomized controlled trials should be performed in order to establish their individual and combined efficacies. Importantly, previous studies regarding therapeutic intervention in SCD, such as with hydroxyurea, only included patients with frequent episodes of clinical vaso-occlusion in order to be able to reach clinical endpoints [89]. By doing so, we fail to study the potential of such therapeutics in the majority of patients [119]. If therapeutic interventions are to be studied in the general sickle cell population, future studies should measure clinical, non clinical, and ideally, silent vaso-occlusion [4]. Therefore, the search for accurate markers of silent vaso-occlusion is of cardinal importance. Based on these concepts and through the increasing collaborative work with our colleagues abroad, we hope that in the coming years we will contribute to improving the management of patients with SCD.