Sickle cell disease
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Chapter 1

General introduction and scope of the thesis
INTRODUCTION TO THE PROBLEM

Sickle cell disease (SCD) is caused by a mutation in the hemoglobin (Hb) gene causing a valine for glutamic acid replacement at the sixth position of the β-globin chain of the Hb molecule leading to a structural variant of normal adult hemoglobin (HbA), namely sickle hemoglobin (HbS).[1] The word ‘sickle’ was introduced by James B. Herrick who was in 1910 the first to describe the morphological changes that occur to the affected erythrocyte, when sickle hemoglobin, which is packed by millions inside erythrocytes, polymerizes if subjected to hypoxia.[2]

After the discovery of its genetic basis, for long SCD was considered a simple molecular disorder, and it was thought that the solution for this (mono) genetic problem would not take long to find. Unfortunately, now, more than 50 years after the discovery of the genetic base of SCD, still only one FDA approved medicine is on the market, hydroxyurea. Despite its proven efficacy in reducing the incidence of vaso-occlusive crises (VOC), hydroxyurea can cause serious side effects, and not all patients experience benefit from the treatment.[3] Hematopoietic stem cell transplantation is currently the only existing cure for the disease but is limited available, certainly in areas where SCD is most prevalent. For patients experiencing painful VOC, current available therapies mainly deal with the symptoms patients experience rather than targeting the underlying pathogenesis of these crises.

In summary; proven and accessible therapeutic options are very limited for patients with SCD.[4] In the meantime, patients with SCD are suffering from recurrent painful VOC and cumulating secondary organ damage resulting in a strongly reduced quality of life in both children and adults with SCD.[4-10] In addition, the median life expectancy of patients with sickle cell anemia is still strongly limited[11-15] despite an improved survival in childhood in countries with comprehensive care programs.

An important cause of the limited available therapeutic options is the complex pathophysiology of the complications of this hemoglobinopathy which is still incompletely understood. A better understanding of all factors involved in the development of VOC may result in the identification of potential new therapeutic targets. In addition, the identification of patients at risk of development of SCD related complications is difficult, as up to now more than 100 of biomarkers have been studied in patients with
SCD, which has not resulted in a validated prognostic biomarker that could improve the clinical management of patients with SCD. [16, 17]

In this thesis, we aim to investigate the use of several different biomarkers in identifying patients with SCD at risk of development of SCD related complications. In addition, we aim to unravel the complex pathophysiological processes involved in SCD in both experimental and clinical studies hopefully resulting in identification of new potential targets of therapy.

DETAILED DESCRIPTION OF THE PROBLEM

BIOMARKERS in sickle cell disease
Previous studies have tried to identify factors and biomarkers that predict which patients with sickle cell disease (SCD) are at increased risk of developing organ complications and/or have an increased risk of death both during vaso-occlusive crisis (VOC) and in steady state. Ideally, identification of high risk patients makes it possible to monitor these patients more closely, or to start specific therapeutics in a timely manner. Currently, numerous factors and biomarkers have been identified in patients with SCD, additionally revealing the complex multi-factorial pathophysiological processes involved in SCD. Most of these biomarkers however, have been shown of limited clinical value and there is still a need for clinically validated prognostic biomarkers.[16, 17] According to the Biomarkers Definitions Working Group a biomarker is defined as a “characteristic that is objectively measured and evaluated as an indicator of normal biological or pathogenic processes, or pharmacological responses to a therapeutic intervention”. [18] We like to add to this that an ideal clinical biomarker should be easy to obtain with limited burden for the patient. The biomarker should not be present when the patient with SCD is not at risk for the development of complications. However, it should be present (or elevated) in patients at risk for complications.

EPIDEMIOLOGY of sickle cell disease
The global frequency of the HbS gene is highest in tropical areas, as a result of relative carrier protection against malaria.[19] It is expected that the annual number of newborns with SCA, estimated 305,800 in 2010, will increase in years to come with the majority of affected babies born in Sub-Sahara Africa with high contributions of Nigeria, Democratic Republic of Congo and India.[20] The 2013 Global Burden
of Diseases, Injuries, and Risk Factors Study (GBD) concludes that whereas the worldwide burden of communicable diseases is diminishing, the global burden of inherited hemoglobinopathies is on the rise. Slave trade and natural migration has led to the spread of the HbS gene causing increasing numbers of patients with SCD in the Americas and Northern Europe. [21] The incidence of sickle cell disease in The Netherlands was estimated to be 2.1 per 10,000 live births in the period 2003-2009, which is probably an underestimation due to under-reporting[22]

**CLINICAL PICTURE of sickle cell disease**

Homozygosity for the HbS allele leads to the most common type of sickle cell disease; sickle cell anemia (SCA). Other common variants of the at least 14 other genotypes, are the heterozygous hemoglobinopathies HbSC disease and HbS/β-thalassemia.[23] Genotype is the major determinant of clinical severity in patients with SCD whereby patients with sickle cell anemia (HbSS) and phenotypical comparable HbSβ0-thalassemia are considered worst affected, followed by HbSC and HbSβ+-thalassemia patients.[15, 24] Intriguingly, even within the different genotypes a wide range of clinical phenotypic expression exist between patients, of which the underlying mechanisms are incompletely understood.[25, 26] Besides genotype, also fetal hemoglobin concentrations and co-inheritance of α-thalassemia are important mediators of disease severity. [27]

The clinical hallmarks of sickle cell disease are chronic hemolytic anemia, an increased susceptibility to infections, recurrent painful VOC and a reduced life expectancy.[23, 28] A painful VOC is the main reason for acute care utilization of patients with SCD[29, 30] and is defined as: “the occurrence of pain in the extremities, back, abdomen, chest, or head, lasting at least two hours, leading to a clinical visit that cannot be explained otherwise.” [31] VOC may precede the development of acute chest syndrome (ACS), which is defined as: “an acute illness characterized by fever and/or respiratory symptoms, accompanied by a new pulmonary infiltrate on chest X-ray.”[32] ACS is a severe complication for which up to 10% of patients may require mechanical ventilation and bears a high risk of death,[27, 33, 34] especially for adult patients.[35] A patient not experiencing an acute SCD related complication like a painful VOC, is referred to as being in “steady state”. [36]

Patients with SCD suffer from a wide range of organ complications, including stroke, retinopathy, nephropathy, leg ulcers, osteonecrosis, cardiomyopathy, and pulmo-
nary hypertension (PH).[37-49] Organ complications are thought to derive from both (micro)vaso-occlusion as well as chronic hemolysis.[50] A classic division was made between SCD patients with a more vaso-occlusive phenotype (characterized by a relatively higher hemoglobin level) resulting in more frequent VOC, ACS and avascular osteonecrosis in contrast to patients with a more hemolytic phenotype (characterized by low hemoglobin level and high LDH levels) that more frequently suffer from complications like: nephropathy, stroke, pulmonary hypertension and leg ulcers.[50, 51] However, in clinical practice these phenotypes represent more likely the extremes of a wide spectrum of complications that can be found in patients with SCD. Interestingly, even though frequency of VOC is considered a marker for disease severity, as patients experiencing an average of ≥ 3 VOC per year are at increased risk for mortality,[31] (cumulative) organ damage occurs irrespective of VOC rate. [52] Cohort studies with a long follow-up period are needed to further examine and confirm these findings, and are needed to find factors and biomarkers that better identify patients at risk of developing organ complications.

**PATHOPHYSIOLOGY of sickle cell disease**

Central in the pathophysiology of SCD is the HbS induced polymerization upon hypoxia resulting in chronic hemolysis and (micro) vaso-occlusion. These processes are characterized by a generalized vasculopathy with endothelial activation, enhanced cellular adhesion, coagulation activation, oxidative stress and a generalized systemic inflammatory response. The interaction between these processes is extremely complicated and not completely clarified yet.

The rate of hemolysis is influenced by sickle red blood cell (sRBC) dehydration, enhanced auto-oxidation of sickle-cell oxyhemoglobin,[53, 54] and altered intracellular iron/heme decompartmentalization,[53, 55] making the affected erythrocytes prone to hemolysis, with a mean estimated erythrocyte life span of 10-20 days.[56, 57] Ongoing hemolysis results in severe anemia and the release of excessive amounts of free intravascular hemoglobin (Hb) which has multiple toxic effects including the consumption of nitric oxide (NO),[58, 59] leading to decreased vasodilation,[59] enhanced endothelial activation,[60] leukocyte adhesion [61] and a pro-thrombotic state,[62] finally culminating in generalized vasculopathy with microvascular vaso-occlusion,[63] Together with these cascade of events cell-free Hb also reacts with peroxide resulting in the formation of ferric Hb (Hb-Fe$^{3+}$), ferrylHb (Hb-Fe$^{4+}$), and associated reactive oxygen species.[64] These oxidation induced unstable heme-adducted
Hb species[65] ultimately lead to the release of heme, making heme available as ligand for molecular signaling.[66-68] Heme can directly activate the endothelium as it induces degranulation of Weibel-Palade bodies leading to P-selectin and von Willebrand Factor expression on endothelial cells[68, 69] and can also directly activate neutrophils.[70-72] Infusion of heme in mice (wild type and NY1DD or Townes sickle mice models) had detrimental effects; it induced coagulation (shown by induced TAT levels),[73] increased vascular permeability in heart, lung and kidneys,[73] activated endothelial cells (shown by upregulation of P-selectin, VWF, and VCAM),[68, 74] and caused a lethal hemolytic crisis in sickle mice.[75] Importantly, these were all experiments with exogenous administered heme, and although heme can dissociate from ferrihemoglobin and be transferred to other globins and albumin,[76] and was shown to be present in erythrocyte microparticles (MPs),[77-79] it remains uncertain if this highly hydrophobic molecule truly exists in a free form in plasma to fulfill all its assigned detrimental effects.[80]

In sickle mice models, generated to study vaso-occlusion in detail, leukocytes, and especially neutrophils, were identified as important players in the pathogenesis of VOC. In Berkeley sickle mice, only expressing human SS β-globin, induction of VOC (by cremasteric preparation and intravenous administration of TNF-α) led to increased numbers of rolling leukocytes and heterotypic RBC/WBC interactions when compared to wild type mice.[81] This could be reversed by intravenous administration of human immunoglobulin (IVIG) prior to VOC induction, leading to especially reduced neutrophil recruitment, and an improved blood flow and survival of IVIG treated mice.[82, 83] For neutrophil rolling on endothelium, P- and E-selectin interactions with integrins were shown to be crucial, [81, 82, 84-88] and IVIG inhibits leukocyte adhesion to endothelium and the heterotypic RBC/WBC interactions as well as Fc RIII mediated β-2 integrin Macrophage-1 Antigen (Mac-1) activation.[89] In the human setting, observational clinical studies with SCD patients show that leucocytosis and neutrophilia are related to morbidity like frequency of VOC and risk of ACS[90-92] as well as to an increased risk of mortality. [27] Moreover, neutrophils, the most abundant leukocytes present in blood, circulate in a primed state in patients with SCD, evidenced by higher expression of adhesion molecules[93-95] like an upregulation of Mac-1[93], and a lower expression of CD 62L (L-selectin). [95] Also in SCD patients Mac-1 was shown to be essential for the increased neutrophil rolling and arrest on endothelium seen in sickle cell blood when compared to controls.[88, 96]
Neutrophils are the primary cellular responders to acute inflammation and have several effector functions, like phagocytosis, granular release and the formation of neutrophil extracellular traps (NETs).[97, 98] NETs consist of extracellular DNA fibres associated with neutrophil proteins[99] and are released from activated neutrophils in a highly organized manner. NETs (components) have been ascribed an anti-pathogenic action.[97, 100, 101] However, NETs (components) also have a dark side, as they are able to induce coagulation,[102-104] auto-immunity[105, 106] and cell damage.[107-109] In clinical studies, circulating nucleosomes, the basic units of DNA organization,[110, 111] and markers for neutrophil activation were described as suitable markers for the presence of NETs.[112, 113] As NETs (components) can induce downstream problems, like cell damage or induction of coagulation, we hypothesize that they may contribute to the complex pathophysiology of SCD when present in (the circulation) of patients with SCD. Other important immunological cellular players identified in SCD VOC are mononuclear cells,[114, 115] platelets as mediators between inflammation and coagulation,[90, 116-119] and Natural killer (iNK)-cells[120, 121] and cytotoxic T cells seem to play a role in SCD related lung injury. [122]

TREATMENT of sickle cell disease

An extensive discussion of this topic is beyond the scope of the introduction of this thesis and others have written excellent general reviews on the management of patients with SCD.[4, 123]

The current only curative therapy for patients with SCD is hematopoietic stem cell transplantation (HSCT).[124] The clinical utility of autologous hematopoietic stem cell gene therapy still needs to be defined.[125, 126]

There are two disease-modifying therapies that are currently used in the treatment of patients with SCD; Hydroxycarbamide and chronic blood transfusions. Hydroxycarbamide is the only FDA approved drug for the treatment of patients with SCD. [127] Its clinical effect, the reduction of frequency of VOC and risk of ACS[127, 128] but also reducing mortality[129, 130] has been related to an increase in intracellular HbF and concomitant decrease of intracellular HbS concentration, making the erythrocyte less prone for both polymerization and hemolysis.[128] Additional working mechanisms include the associated decreased leukocyte count, decreased adhesive properties of neutrophils[131, 132] and an exogenous nitric oxide supply directly from hydroxycarbamide. [133] With transfusion therapy a distinction is made
between the use of acute (exchange) transfusions and the application of chronic transfusion schemes. Acute transfusions are mostly applied in the treatment of acute anemia, ACS or life threatening conditions like multi-organ-failure, sepsis and acute stroke or preoperatively to prevent perioperative complications. Chronic transfusion is mostly applied for the primary and secondary prevention of cerebral infarcts. [134]

New therapies under investigation of their clinical use in treatment of patients with SCD include anti-inflammatory drugs and anti-adhesive drugs,[87, 135, 136] (like the P-selectin inhibitor Crizanlizumab and the pan-selectin inhibitor Rivipansel, both showing promising results in patients experiencing VOC in recently completed Phase 2 trials);[137, 138] anti-oxidants;[139, 140] and anti-sickling agents and anti-platelet activation drugs; [141-143] besides numerous compounds that have a HbF inducing capability.[144, 145]

**PROGNOSIS of sickle cell disease**

Comprehensive care programs, including newborn screening programs, screening on cerebral vascular stenosis by transcranial Doppler, the prophylactic administration of antibiotics (leading to a huge drop in infectious-disease related SCD death[146]) and an extensive immunization program, has attributed enormously to the increased life expectancy of children with SCD.[12, 13] Unfortunately, in most “high SCD burden-low income” areas, these comprehensive care programs are not available resulting in remaining high rates of mortality among children with SCD in those areas.[11, 147] In countries with comprehensive care programs more than 95% of the children with SCD survive into adulthood.[12, 13] However, despite this improved survival in childhood the last decade, the median life expectancy of patients with sickle cell anemia is still strongly reduced.[14, 15]
OUTLINE OF THE THESIS

As described above, patients with SCD suffer from a wide range of complications and biomarkers that identify patients at risk of development of these SCD related complications, to improve the clinical management of these patients, are lacking. In this thesis, we studied several potentially useful biomarkers in prospective clinical studies. As neutrophils play a pivotal role in SCD pathogenesis (see Pathophysiology) we were especially interested to study the role of (biomarkers reflecting) neutrophil activation in SCD related complications. Some studies focused at biomarkers identifying patients with SCD in vaso-occlusive crisis (VOC) (to support the clinical diagnosis) and/or development of VOC related complications, and 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. In addition, by examining changes in these chosen biomarkers we hope furthermore to unravel the complex multifactorial pathophysiological processes involved in SCD in both experimental and clinical studies. Hopefully this may result in the identification of new potential targets of therapy.

As neutrophils play a pivotal role in SCD (pathogenesis), we aimed to investigate the presence of markers for NETs formation during VOC in a clinical study. In chapter 2, we describe elevated levels of nucleosomes and neutrophil elastase in complex with its inhibitor α1-antitrypsin (HNE-α1-AT) in patients with SCD with VOC when compared to levels in steady state. These markers correlated strong and highly significant with each other during VOC, and appeared to reach the highest plasma levels in patients developing ACS. In this chapter the role of NET formation and neutrophil activation in the pathogenesis of VOC is further discussed.

Chapter 3 is a sequel of the study described in chapter 2. Apart from neutrophil activation, VOC is characterized by inflammation, endothelial activation and cell death. In this clinical study, we aimed to gain increased insight into the kinetics of these events during VOC. Next to levels of nucleosomes and HNE-α1-AT, levels of C-reactive protein (CRP), pentraxin3 (PTX3) (as markers of inflammation) and calprotectin (as an additional marker of neutrophil activation) and von Willebrand Factor antigen (VWFAg) and von Willebrand Factor propeptide (VWFpp) (as markers of endothelial activation) were determined on subsequent days of admission for VOC.
The dynamics of these markers during VOC, their relation with VOC severity, as well as mutual relations between these markers are described in chapter 3.

In **Chapter 4** we aimed to further focus on the role of VWF in SCD VOC pathogenesis. For this purpose we determined, next to levels of VWFAg and VWFpp, levels of active VWF, VWF activity, high-molecular weight multimers of VWF, platelet count and levels of ADAMTS13 and ADAMTS13 activity. Clear differences were observed in changes of levels of the different markers on subsequent days of admission for VOC and in steady state which we describe and discuss in chapter 4.

Following the observations in clinical studies, in which we found markers suggestive of the presence of NET formation in plasma of patients with SCD, we developed experimental models to investigate NET formation in the SCD context *ex vivo*. We aimed to determine whether (a component in) serum of patients with SCD in VOC is able to activate neutrophils and induce NET formation. In **chapter 5** we describe that serum of patients with SCD, especially from patients in VOC, is indeed able to induce NET formation in healthy donor neutrophils. Others, very recently, ascribed this effect to heme. [148] In chapter 5, we investigate this hypothesis and suggest another factor in SCD serum that is able to induce the release of NETs in donor neutrophils.

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.[149-152] The hypothesized responsible mechanisms include chronic hemolysis, releasing intracellular zinc from erythrocytes and renal zinc loss due to tubular injury as a result of chronic oxidative stress and inflammation, especially during VOC.[153] Alternatively, increased bone degradation due to recurrent bone ischemia, especially during VOC,[154] may also contribute to the increased urinary zinc loss in patients with SCD. The results of this clinical studies are discussed in chapter 6.

Both elevated N-terminal prohormone brain natriuretic peptide (NT-proBNP) levels (>160pg/mL) and an increased 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. [44, 155-157] However, the exact relation between these markers and their relation to PH and mortality in patients with SCD remains to be defined. In **chapter 7**, we describe the 6 year follow-up of a Dutch cohort of 85 con-
secutive ambulatory SCD patients, in which elevated NT-proBNP levels appeared to be the strongest risk factor for mortality, independent of TRV. In chapter 7, we discuss the relation of these elevated NT proBNP levels with renal function, cardiac function, age and tricuspid regurgitation flow velocity.

In chapter 8, we describe the follow-up of a second prospective cohort study that included 104 ambulatory patients with SCD. These patients were followed for seven years to gain insight in the course and incidence of the various forms of secondary organ damage and SCD related complications in patients in a tertiary academic hospital.

In Chapter 9, we summarize all findings and discuss future perspectives deriving from this thesis.
REFERENCES


General introduction and scope of the thesis


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