Therapeutic targets in sickle cell disease
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Citation for published version (APA):
Sins, J. W. R. (2017). Therapeutic targets in sickle cell disease

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Chapter 1

General introduction and thesis outline

Joep W.R. Sins
Chapter 1

Sickle cell disease; a hemoglobinopathy

SCD is a recessive, genetic disorder of hemoglobin synthesis. It is the result of a single point mutation in the β globin gene, which changes the sixth amino acid from glutamic acid to valine. Yet, this minor change can have devastating effects. SCD was recently ranked 71st in the leading causes of disease burden worldwide, and 18th in central sub-Saharan Africa. The mutation leads to the production of HbS instead of the normal hemoglobin A (HbA). This HbS is insoluble when deoxygenated, forming long polymers. The polymerization of the hemoglobin deforms the highly flexible, donut shaped RBCs into rigid, sickle shaped cells. These cells cause occlusions in the post-capillary venules, preventing essential oxygen from being delivered to the tissues. This initiates a cascade of pathological events, as discussed later under pathophysiology. The clinical hallmarks of SCD are the chronic hemolytic anemia and the episodic microvascular vaso-occlusion, causing severe painful vaso-occlusive crises (VOC) and irreversible organ damage (see section the clinical picture).

Although this hypothesis was initially based on observations in heterozygotes for thalassemia, the first studies confirming Haldane’s hypothesis were performed in populations heterozygous for the hemoglobin S polymorphism (HbS; sickle cell trait). Later cohort studies have confirmed the protective effect of this trait in children in malaria endemic regions. Children with sickle cell trait had a significant survival advantage compared to non-affected individuals. However, if a child receives the mutated allele from both parents, being homozygous for the HbS gene, the protective effect against malaria is overshadowed by a detrimental clinical phenotype with a significantly reduced survival: sickle cell disease (SCD).

Introduction

Haldane’s hypothesis

In 1949, the scientist John B.S. Haldane was first to propose that specific genetic variants can contribute to disease resistance in humans, and that this resistance could potentially be a significant evolutionary force in humans. He noted that mutations expressed in red blood cells (RBCs) were prevalent only in tropical regions where malaria had been endemic and that this genetic variance appeared to protect individuals from acquiring malaria (figure 1). The high mortality and widespread impact of malaria over the past millennia provided a strong selective pressure, resulting in the high incidence of various protective red cell polymorphisms.

Although this hypothesis was initially based on observations in heterozygotes for thalassemia, the first studies confirming Haldane’s hypothesis were performed in populations heterozygous for the hemoglobin S polymorphism (HbS; sickle cell trait). Later cohort studies have confirmed the protective effect of this trait in children in malaria endemic regions. Children with sickle cell trait had a significant survival advantage compared to non-affected individuals. However, if a child receives the mutated allele from both parents, being homozygous for the HbS gene, the protective effect against malaria is overshadowed by a detrimental clinical phenotype with a significantly reduced survival: sickle cell disease (SCD). The term sickle cell disease encompasses all the different genotypes that cause a similar clinical phenotype. Homozygous HbSS disease, usually called sickle cell anemia, accounts for approximately 70% of SCD cases and is generally the most severe type. A further 15 different compound heterozy-
A brief history

The first evidence of the malaria parasite has been traced back to the Paleogene period, approximately 30 million years ago. Yet, the HbS mutation in the genome of mankind appears to be of much more recent origin. It has been estimated to date back 1,350 to 2,100 years ago to a time when severe malaria became a significant selective force in humans.

Gous genotypes can cause sickle cell disease, all including the HbS allele. The most common other genotypes are HbSC diseases and HbSβ thalassemia. The latter has a variable clinical presentation, depending on the type of β-thalassemia mutation that is co-inherited (HbSβ+ or HbSβ0 genotype). Other compound heterozygous genotypes have a mild clinical presentation.

Figure 1. Global distributions of HbS and malaria (figure by Rees, Williams, Gladwin. Lancet 2010; with permission.

(A) This map shows the distribution of the HbS allele. The figures indicate estimates for the combined yearly total number of individuals affected by HbSS, HbSC, and HbS/β-thalassaemia by WHO region.

(B) This map shows the global distribution of malaria (red) before intervention to control malaria.
It was not until 1910 that James B. Herrick ran into ‘an intelligent negro of 20’ from the Caribbean island Grenada and first described the typical sickled red blood cells in this case of severe anemia.\textsuperscript{10} The actual term ‘sickle cell anemia’ was introduced for the first time in 1922 by V.R. Mason.\textsuperscript{11} In 1949, Linus Pauling and colleagues distinguished sickle hemoglobin from normal adult hemoglobin on the basis of its charge, thereby identifying sickle cell anemia as a molecular disease.\textsuperscript{12} In that same year, the autosomal recessive inheritance of SCD was independently reported by Beebe and Neel.\textsuperscript{13,14} Soon after, Hunt and Ingram discovered the specific point mutation on chromosome 11 responsible for the production of HbS.\textsuperscript{15} Since these initial reports, research into SCD has intensified and resulted in an advanced insight into the pathophysiology of this disease, involving multiple pathways and various other cells than merely the RBC.\textsuperscript{7}

**Epidemiology**

SCD is one of the most common monogenic diseases in the world, affecting over 300,000 newborns each year (HbSS only).\textsuperscript{16,17} About 70\% of global cases of sickle-cell disease live in Africa, where the burden is highest in Nigeria and the Democratic Republic of Congo with respectively 91,000 and 39,700 newborns in 2010. India follows closely with approximately 44,000 affected children each year.\textsuperscript{18} Moreover, the global burden of SCD is expected to increase even further over the next decades, due to both a predicted increase in the annual number of newborns with SCD and the introduction of basic health interventions, leading to significant reductions in excess mortality among young children with SCD.\textsuperscript{18}

Increasing globalization and migration streams have increased the number of SCD patients in the Americas and Europe, with annual birth rates of respectively 11,000 and 1,900 of children with homozygous SCD. Also in the Netherlands the incidence of SCD has increased over the years, initially with immigrants coming from former colonies such as Surinam and the Caribbean, and more recently also people from West Africa.\textsuperscript{19} Based on the neonatal screening program, the prevalence of SCD among newborns was estimated at 1 in 4,500 in 2007.\textsuperscript{20} The total number of patients with SCD in the Netherlands is estimated at 2,000 patients.

**Pathophysiology**

All pathological events in SCD can be traced back to the mere presence of HbS and its susceptibility for polymerization, affecting the flexibility and function of the RBC. The rate and extent of this polymerization are determined by the degree of hemoglobin deoxygenation, the concentration of HbS and concurrently, the concentration of fetal hemoglobin in the RBC.\textsuperscript{7} Main predictors of disease severity in SCD are therefore factors that affect this
process. Compound heterozygous forms of SCD, such as HbSC or HbSβ*, have a lower concentration of HbS and tend to be milder in clinical phenotype, although a large variability in clinical presentation is observed. Hereditary persistence of fetal hemoglobin throughout adult age effectively results in a lower intracellular concentration of HbS and was demonstrated to significantly reduce disease severity and mortality.

The polymerization of HbS drives the two major pathophysiological processes in SCD: increased hemolysis and recurrent vaso-occlusive episodes with ischemia and tissue damage. Sickled RBCs are rigid, fragile and prone for lysis. Therefore, with a significantly reduced lifespan of RBCs of 15-20 days, patients suffer from a chronic hemolytic anemia. Due to the hemolysis, increased levels of free hemoglobin are released into the blood stream, generating reactive oxygen species (ROS) that may inflict damage to lipids and proteins, activate cells in the vicinity such as endothelial and immune cells. Importantly, nitric oxide is scavenged by ROS, hampering the crucial vasodilative, anti-thrombotic and anti-inflammatory properties of this molecule and resulting in endothelial dysfunction.

The second pathophysiological phenomenon in SCD is characterized by recurrent episodes of vaso-occlusion. Beyond the mere mechanical obstruction of small blood vessels due to sickled RBCs, the insight in the pathophysiology of vaso-occlusion has significantly increased over the past decades. Adhesive interactions between RBCs, leukocytes and activated endothelium all add to vascular obstruction, triggered by factors such as inflammation, stress, hemolysis or hypoxia. ROS have been demonstrated to be a central mediator in this process. In addition to chronic hemolysis, the recurrent cycles of ischemia and reperfusion are an important source of ROS. This oxidative stress drives a negative feedback loop, adding to further endothelial and immune cell activation, inflammation and vaso-occlusion. In addition, activation of coagulation occurs which further amplifies vascular obstruction. The pathophysiology of VOC is thereby a highly dynamic and complex interplay of many different components.

**The clinical picture**
The first symptoms of SCD may present as soon as the synthesis of fetal hemoglobin is replaced by the production of adult hemoglobin S, few months after birth. In contrast with hemoglobin S, fetal hemoglobin does not contain β globin and therefore does not carry the S mutation. The most common initial symptom in infants is dactylytis, a painful swelling of the hands or feet due to vaso-occlusion. Alternatively, an invasive bacterial infection can also be a presenting feature. Due to recurrent vaso-occlusion and
Chapter 1

The spleen has lost most of its function at an early age (functional asplenia), impairing the immunity against encapsulated bacteria and increasing the risk for fulminant infections. Mortality rates in these infections range up to 15-20%. The main clinical hallmark of SCD is the acute painful VOC, caused by ischemic tissue damage due to vaso-occlusion. Acute pain is the main cause of hospitalization in SCD and a high frequency of painful episodes has even been correlated with early death. However, the majority of pains is coped with at home. Data from a large diary study in the US revealed that patients reported SCD-related pain on 54% of the observed days, whereas healthcare utilization for these pains was only reported on 4% of days. Besides direct effects on physical health, pain also affects other relevant outcomes such as quality of life, school attendance and societal participation of SCD patients.

Other acute and potentially life-threatening complications of SCD include the acute chest syndrome, acute aggravations of anemia and stroke. The acute chest syndrome (ACS) is characterized by a combination of symptoms such as chest pain, fever, cough, dyspnea or tachypnea and a new pulmonary infiltrate on chest radiography. It is a severe complication as the hypoxia may lead to a downward spiral of further sickling, vaso-occlusion and hypoxia. ACS is a frequent complication, occurring up to 12.8 times per 100 patient years in HbSS patients, and is associated with a high mortality. Acute aggravations of anemia can occur due to splenic or hepatic sequestrations, or a parvovirus B19 infection. In the case of sequestrations, a large part of circulating RBCs is trapped in the spleen or liver with subsequent enlargement. It occurs mainly in young children and can be triggered by infection. A parvovirus B19 infection may disturb the production of new RBCs in the bone marrow, causing temporary reticulocytopenia and subsequent acute anemia.

Lastly, stroke is probably the most serious acute complication in SCD, caused by vasculopathy of the large cerebral arteries. Prior to stroke prevention programs, the incidence of stroke was 0.61 per 100 patient years in patients with HbSS disease, with a 11% chance of having experienced a first stroke by the age of 20, with a peak incidence between the age of 6 to 10 years. Beyond overt stroke, also transient ischemic attacks and silent cerebral infarctions may occur. These all add to progressive neurocognitive and behavioral problems throughout life.

Beyond these acute complications, progressive organ damage due to vaso-occlusion and ischemia leads to a wide range of chronic complications in SCD. Almost every organ
system can be affected. Important complications include chronic renal disease, proliferative retinopathy, chronic leg ulcers, pulmonary hypertension, functional asplenia (as discussed previously) and chronic pains. Chronic renal failure has been shown to develop in almost a third of adult patients. In contrast to most forms of organ damage, retinopathy is more common in patients with HbSC disease (more than 50% of patients) but can occur in all genotypes. Leg ulcers occur predominantly in adult patients. They often cause chronic pain, have been related to a high rate of hemolysis and severe anemia and take months to heal. Pulmonary hypertension occurs in approximately 6% of adult patients and has been associated with premature death. Lastly, chronic pain is an important complication of SCD that should be differentiated from acute pain. Chronic pain can either be related to secondary tissue damage, such as avascular bone necrosis or leg ulcers. However, in some cases patients report severe pain in the absence of a clear pathology. These chronic pain syndromes have been related to increased central sensitization to painful stimuli due to the frequent exposure to pain earlier in life.

**Treatment and prevention: from a life threatening childhood illness to a chronic disease of adults**

In western countries, survival of children with SCD has improved dramatically over the past decades. In the early 1970s, over a quarter of all children with SCD would not reach the age of 18 while nowadays over 95% of patients survive into adulthood in high-income countries. This significant increase in life expectancy can be attributed to various interventions that have been implemented over the years.

A major milestone in SCD management was the introduction of a neonatal screening, universally implemented in the United States (US) in 2006 and in the Netherlands in 2007. Screening for SCD at birth allows for an early diagnosis, vital for effective preventative management from a young age onwards. Antibiotic prophylaxis and frequent vaccinations are corner stones in this preventative management, protecting children against severe infections due to functional asplenia and the associated mortality. In addition, parents of SCD children can be educated early so they are able to recognize severe complications timely and seek care when necessary. Another major advancement was the introduction of transcranial Doppler screening for the identification of children at high risk for stroke. Children with abnormal intracranial blood flow are started on chronic transfusion blood therapy, thereby significantly reducing the risk of stroke.

RBC transfusion therapy is one of the few effective interventions in SCD that directly intervene in the pathophysiology of the
disease. By administering blood from healthy donors, the relative proportion of HbS is reduced, resulting in reduced sickling and vaso-occlusion. Chronic transfusion has been demonstrated to be effective in both the primary and secondary prevention of stroke. Acute transfusions (or exchange transfusions) are indicated for VOC related complications such as ACS, acute stroke or multi-organ failure, and pre-operatively to prevent post-operative SCD related complications.6 In prophylactic management, transfusions are usually given every 3 to 6 weeks with the aim to maintain the HbS percentage below 30%.53 Unfortunately, the use of transfusion therapy in SCD is limited by its associated risks. RBC allo-immunization can occur due to antigenic incompatibility between the donor and the patient, restricting the availability of matching donor RBCs for future transfusions and adding to the risk for hemolytic transfusion reactions.54 In addition, frequent transfusion exposure leads to iron overload. Iron chelation therapy is therefore important in chronically transfused patients.6

The range of pharmacotherapeutical options to prevent vaso-occlusive complications in SCD is extremely limited. The only drug with proven efficacy is hydroxyurea (HU). HU is a potent inducer of fetal hemoglobin and has been demonstrated to modulate cell adhesion and nitric oxide generation.55–57 Use of HU in SCD is associated with a reduction in the frequency of VOC, related hospital admissions and even mortality.58–60 There have been concerns about potential oncogenesis or subfertility due to HU.61,62 Yet, long-term follow-up studies do not seem to support these findings and the benefits seem to outweigh the risks.58,60 The application of HU in SCD is thereby expanding to milder patients.53 Unfortunately, HU is not effective in all users and side effects sometimes lead to discontinuation of the drug.59 Currently, the only curative treatment for SCD is hematopoietic stem cell transplantation. Due to the toxicity and risks of the original myeloablative regimens, this was only considered for younger patients with a severe clinical course. Moreover, a matched sibling donor was required.63 Recent studies with new, non-myeloablative allogeneic or gene-edited autologous stem cell protocols have shown promising improvements in these aspects, paving the way for a wider range of patients to be considered for this procedure.64–66

Despite these improvements, even in western countries the life expectancy for patients with SCD is still significantly reduced compared to the general population. The median survival was recently estimated at 58 years for HbSS and HbSβ0 patients and at 66 years for HbSC and HbSβ+ patients.22 Unfortunately, most of the described interventions are not widely available in the countries where the burden of SCD is highest. In sub-Saharan Africa, an estimated 50% to 90% of the children born with SCD die un-
diagnosed before the age of 5, mainly due to malaria and invasive bacterial infections.\textsuperscript{67}

**Scope of research**

As outlined in this introduction, the worldwide burden of SCD is tremendous. Although disease management has significantly improved the life expectancy in developed countries over the past decades, the contrast with low-income countries, where the incidence of SCD is highest, is large. Management options are still poor in these regions and mortality is high.

Yet, even patients with access to proper medical care still experience severe and invalidating symptoms. Already from a young age, patients suffer from a broad range of complications that negatively impact their physical health, cognitive function and psychological well-being. Moreover, actual therapeutic options are still very limited and the main improvements in disease management over the years have mostly been supportive. Therefore, research addressing our understanding of SCD is vital for the advancement of SCD management worldwide. Only when we improve our comprehension of SCD, we will be able to design effective interventions to prevent or at least minimize the devastating complications of this disease.

The aim of this thesis was to gain further insight into the complications, underlying pathophysiological mechanisms and potential therapeutic targets of SCD.

**Outline of this thesis**

Part 1 of this thesis provides further insight into the burden, pathophysiology and current treatment options of SCD. In chapter 2, we evaluate the frequency and characteristics of self-reported pain and VOC in adult patients with SCD living in the Netherlands. Chapter 3 adds to our understanding of the pathophysiology of vaso-occlusion in SCD, focusing on the dynamics of the adhesive glycoprotein Von Willebrand Factor in the course of a VOC. In chapter 4, we provide an overview of the various treatments for SCD that have been evaluated in literature so far. We systematically review the currently available evidence on pharmacotherapeutical strategies beyond HU in the prevention of VOC in SCD.

Part 2 of this thesis discusses the role of oxidative stress as therapeutic target in SCD, and focuses on the effects of the antioxidant N-acetylcysteine (NAC) on clinical, laboratory and societal outcomes of SCD. The primary study for this analysis involved a randomized, placebo-controlled trial that was the result of an international collaboration between 11 sites in the Netherlands, Belgium and the United Kingdom. In this trial, we randomized patients to either placebo or 1200mg of NAC daily for a total duration of 6 months. The clinical results of this trial are discussed in chapter 5, focusing on the effects of NAC on the frequency of SCD related pain, VOC and hospitalizations.
for VOC. **Chapter 6** describes the effects of NAC on the plasma thiol redox balance, as marker of oxidative stress, in participants of this trial. In addition, we explore correlations of various thiol redox markers with both clinical and pathophysiological characteristics of patients with SCD.

In part 3 of this thesis we focus on RBC allo-immunization as an important complication of transfusion therapy in SCD. Allo-immunization against foreign RBC antigens limits the availability of matching blood for patients and may thereby delay the provision of urgent transfusions in medical emergencies. In this part, we discuss the results of a retrospective cohort study in transfused SCD patients in the Netherlands. In **chapter 7**, we evaluate the incidence of allo-immunization in this cohort, and identify various clinical risk factors for allo-immunization. **In chapter 8**, we assess immunogenetic risk factors of allo-immunization. We combined DNA samples from our Dutch cohort with samples from a cohort of French patients. In this joint cohort we evaluated whether polymorphisms in the Fc gamma receptor gene are associated with RBC allo-immunization.

The general discussion of this thesis is presented in **chapter 9**. This section reviews the main results of this thesis and provides a perspective for future research concerning pathophysiological mechanisms, treatment options and prevention of complications in SCD.
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