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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 9

Summary, General Discussion and Conclusion

Joep W.R. Sins
Summary and General Discussion

Therapeutic targets in sickle cell disease

The aim of this thesis is to gain further insight into the underlying pathophysiological mechanisms, associated complications and potential therapeutic targets of SCD. The presented studies are divided in three main topics. In the first part, we address the physical burden and the pathophysiological mechanisms of SCD, and provide an overview of potential treatments for SCD that have been evaluated so far. Part 2 focuses on oxidative stress as a novel therapeutic target, and discusses the results of an intervention study with the antioxidant N-acetylcysteine (NAC). In part 3, we concentrate on allo-immunization as a major complication of RBC transfusion therapy in SCD. We identify various potential risk factors of allo-immunization that may help in the prevention of this complication, ultimately adding to the safe use of vital RBC transfusions in SCD. This final chapter will provide a summary and general discussion of the results of our studies, addressing limitations, implications for clinical practice and directions for future research.

Part I – Burden, pathophysiology and current treatment options

In chapter 2 of this thesis, we present the first European study on the frequency and characteristics of pain in a cohort of adult SCD patients. In this observational pain diary study, we demonstrated that patients report-ed SCD related pain on almost a fifth of the observed days (17%). These pains were defined as painful, vaso-occlusive crisis (VOC) on 3% of days and hospitalization for these pains occurred on 1% of days. Use of analgesics was reported in approximately half of the days with pain, and was mainly limited to acetaminophen and non-steroidal anti-inflammatory drugs. Importantly, the reported frequency of pain reflects a highly heterogeneous population, as 20% of patients did not report any pain at all and 11% had pain on more than half of the observed days.

This study demonstrates that pain in daily life in adult SCD patients in the Netherlands is significantly less frequent as compared to previous observations in the United States (US), where patients reported having pain on over half of the observed days.\textsuperscript{1–5} Besides differences in patient characteristics and study design, we hypothesize that disparities in the organization and accessibility of healthcare between the US and the Netherlands may be an important explanation of this discrepancy. Nevertheless, the observed frequency of daily life pain in SCD patients in the Netherlands is still too high. These pains have a vast impact and affect the societal participation and quality of life of patients.\textsuperscript{6–11} The large majority of these pains are invisible to healthcare providers as patients do not seek professional care and manage their pains at
home. Interestingly, only a small proportion of these pains are reported as typical vaso-occlusive crises (VOC). In part, these non-crisis pains are likely to be milder episodes or early prodromes of acute VOC. However, beyond these acute pains, a wider spectrum of pains can be distinguished in SCD. Chronic, long-lasting pains appear to be a main contributor to the high frequency of pain as observed, especially in patients reporting pain on more than half of the observed days. These chronic pains may be the result of secondary tissue damage such as avascular osteonecrosis. In others, no clear physical cause can be identified and may be the result of altered perception of pain due to central sensitization, for example due to frequent exposure to acute pains in the past. As little is still known on the pathogenesis of these pains, this should be subject of future studies. Understanding of these mechanisms is essential to discern interventions targeting this invalidating complication of SCD.

Our findings emphasize the significant burden of pain in SCD, comprising predominantly of daily non-crisis pains which are suffered and treated at home and are associated with chronic, long-lasting pains. In addition, patients suffer from acute, episodic VOC that are associated with hospitalizations. Thereby, the current management of these distinct pains will require different approaches, where clinicians should be attentive of the largely invisible burden of pain that is suffered at home. However, ultimately these distinct pains in SCD share the same initial trigger, which is vaso-occlusion. Therefore, the ultimate goal on the long term would be to prevent these episodes of vaso-occlusion. This would reduce both the number of acute VOC and the secondary development of chronic pain syndromes. The prevention of vaso-occlusion can in part be achieved by a more widespread, optimal use of hydroxyurea, starting at young age. However, not all patients are responsive to hydroxyurea and it does not relieve the symptoms completely. Therefore, new effective treatments to prevent vaso-occlusion in SCD are urgently needed. The advancement of such new interventions is discussed in chapter 4.

Pain remains a difficult outcome to study, especially in SCD. Currently used outcomes such as pain days, VOC days or visual analogue scale pain scores, as marked in a pain diary, are highly subjective and appear to have great interpatient variability. Other measures, such as VOC with healthcare contacts or actual hospital admissions, may appear more objective but still involve the subjective decision of the patient to ask for professional help. Moreover, these outcomes do not fully reflect the significant proportion of disease-related pains that
patient suffer at home. While a solution for this issue remains difficult, it would at least be helpful to have an international consensus on the definition of pain-related outcomes for future research in SCD.

**Chapter 3** investigates the dynamics of the adhesive, hemostatic protein Von Willebrand Factor in the pathogenesis of vaso-occlusion in SCD. In this observational study in adult patients with SCD, we assess the quantity and reactivity of VWF and its regulating protease ADAMTS13 during the course of admission for a VOC and in steady state. This study provides first evidence that VOC in SCD are characterized by an increased reactivity of VWF as compared to steady state, while the total quantity of VWF (VWF antigen) remained relatively stable. Platelet counts and plasma levels of ADAMTS13 antigen and activity were concomitantly lower during VOC. Yet, no pronounced ADAMTS13 deficiency was observed during VOC. We hypothesized that this hyper-reactivity of VWF can be explained by decreased susceptibility of VWF to proteolysis. It has been demonstrated that reactive oxygen species, released by activated neutrophils, can inhibit VWF cleavage by ADAMTS13 through oxidation of its cleavage site, thereby increasing its resistance to proteolysis.\(^{15,16}\) Moreover, the pro-oxidative free hemoglobin has been shown to competitively bind to the ADAMTS-13 cleavage site, also blocking proteolysis and promoting microvascular thrombosis.\(^{17,18}\) In support of this, markers of hemolysis, inflammation and neutrophil activation correlated strongly with markers of VWF reactivity in our study. The observational study design did not allow us to elucidate whether high VWF reactivity during VOC is merely a marker or an actual causal agent in the pathophysiology of VOC in SCD. In-vitro studies have suggested that thick bundles of VWF can obstruct blood flow, binding platelets, leukocytes and erythrocytes.\(^{19}\) In addition, increased VWF reactivity has also been observed in other disease models with microvascular thrombosis.\(^{20–22}\)

Interventions targeting hemolysis, inflammation or oxidative stress may therefore be effective inhibitors of VWF activation, and thereby potentially also of vaso-occlusion-related complications in SCD. Interestingly, the antioxidant NAC has been demonstrated to reduce disulfide bonds within VWF multimers in in-vitro and in-vivo models, thereby reducing the size and activity of VWF multimers.\(^{23}\) In addition, the therapeutic value of recombinant ADAMTS13 in SCD also deserves further exploration,\(^{24}\) as this could restore ADAMTS13 to normal values, optimizing its proteolytic activity. Future studies will have to elucidate if hyper-adhesive VWF is merely a marker or an actual contributor to the pathophysiology of VOC in SCD, and could thereby represent a new therapeutic target of clinical relevance.
Chapter 4 describes the results of a systematic review, providing an overview of the various treatment modalities beyond hydroxyurea that have been evaluated over the years in the prevention of VOC in SCD. A total of 36 controlled studies were identified, covering 26 different prophylactic interventions. Most promising interventions, reducing the frequency of VOC or hospitalizations, are the antioxidants L-glutamine and omega-3 fatty acids, and the anti-adhesive P-selectin inhibitor Crizanlizumab. Studies on the anti-platelet agent ticlopidine, the dietary supplement zinc and anti-malarial prophylaxis for patients in malaria endemic regions provided encouraging results. The other studies either did not show any beneficial effect of the intervention under investigation or were methodologically inadequate to draw strong conclusions. This study highlights the discrepancy between the significant burden of this disease worldwide, the low number of well-designed and adequately powered trials performed so far and the overall lack of effective treatments. In future trials, we must draw better lessons from prior studies and focus major trial efforts on interventions with greatest potential and a solid preclinical evidence base. In addition, as described earlier, we must strive for international consensus on a standardized set of outcomes. This would significantly improve the quality and applicability of future studies.

There remains a significant unmet need for new, accessible therapies for SCD. It is hope giving that the number of clinical trials in SCD in the past decade seems to be increasing and various new treatments are in advanced stages of clinical evaluation, close to implementation. Moreover, beyond the scope of this review, there are many new interventions in a pre-clinical phase of development, targeting a wide range of pathophysiological targets, that may give rise to effective treatments in the future.26–28

Part II – N-acetylcysteine and oxidative stress

In chapter 5 we report the results of a randomized clinical trial, evaluating the effect of the oral antioxidant N-acetylcysteine (NAC) on the frequency of SCD-related pain in SCD. In this study, patients were randomly assigned to either 1200mg of NAC daily or placebo for 6 months. The primary outcome was the rate of SCD-related pain days. In total, 96 patients were randomized, of which 27 patients in the NAC arm and 40 in the placebo arm met the minimum required observation time of 110 days. Unfortunately, in this modified intention-to-treat population we did not observe any differences between the treatment arms for the primary and secondary clinical outcomes of this study. No -tably, only half of the patients could be considered adherent, based on tablet counts. Per protocol analysis limited to a small group of adherent patients did suggest a significant reduction in the rate of days with VOC in the NAC group compared to placebo.

Subsequently, chapter 6 describes the effects
of oral NAC administration on the plasma thiol redox balance in patients of this randomized clinical trial, and in patients from a previous uncontrolled pilot study with NAC.\textsuperscript{29} In the extracellular milieu, cysteine (Cys) is the main thiol reservoir. Upon reacting with oxidants, free Cys will form disulfide bonds with other thiols (thiol-disulfide exchange reactions). Thereby, both total, free (reduced) and disulfide (oxidized) forms of Cys are important indicators of the plasma redox state and markers of oxidative stress.\textsuperscript{30} Clinically, these thiol markers have been associated with aging, smoking, a high cholesterol, vascular disease, and even mortality in coronary artery disease.\textsuperscript{31–39} One of the potential mechanisms of action of the antioxidant NAC is that it can act as a reducing agent by breaking these disulfide bonds, due to its free thiol group.\textsuperscript{23,40} In both the uncontrolled pilot as well as the randomized controlled trial, oral NAC significantly increased levels of free (reduced) and total NAC, and free Cys. Moreover, treatment with NAC decreased levels of protein bound (oxidized) Cys. Thereby, NAC significantly improved the thiol plasma redox balance in patients with SCD. Unfortunately, we were unable to demonstrate any direct effects of oral NAC on both pathophysiological as well as clinical outcomes in the randomized clinical trial (chapter 5), most likely due to poor adherence and a too low dosage of NAC.

However, at baseline the thiol redox markers were independently associated with the number of hospital admissions, age, SCD genotype, endothelial and coagulation activation, and advanced glycation end products. This provides indirect evidence that improvement of the thiol redox balance may affect other pathophysiological processes of the disease, and could potentially even reduce the number of hospital admissions. Therefore, the use of NAC and other thiol antioxidants in SCD deserves further investigation.

Various lessons can be learned for future studies. Adherence was not optimal in the randomized clinical trial. By including patients with only 1 VOC per year, a subgroup with relatively mild disease may have been less inclined to maintain good adherence. In addition, significantly more patients in the NAC arm reported gastro-intestinal adverse events, possibly adding to a higher drop-out rate here. Lastly, the dosage of NAC in this study appears to have been too low to elicit any clinical effects. As higher oral doses may result in the occurrence of more adverse events, the potential of oral use of NAC may be limited. Therefore, the value of other routes of administration and of other oral thiol antioxidants should also be explored further. Encouragingly, the therapeutic potential of intravenous, high-dose administration of NAC during VOC is currently being pursued (NCT01800526).
**Part III – Allo-immunization**

Part 3 of this thesis addresses both clinical as well as genetic risk factors for RBC allo-immunization, as a complication of transfusion therapy. **Chapter 7** is a retrospective multicenter cohort study, in which we aimed to elucidate the association between the cumulative transfusion exposure, first occurrence of allo-immunization and independent risk factors. A total of 254 patients were included, of which 43 patients (18%) developed allo-antibodies. The cumulative risk of first allo-immunization increased with higher transfusion exposures. Yet, half of the allo-immunized patients formed their first allo-antibody already before the 8th transfusion. In a unique comparison with a general transfused population, adjusting for the cumulative transfusion exposure, the risk of first allo-immunization was significantly higher in our SCD cohort. In SCD patients, the risk of allo-immunization increases twofold for patients that receive the first transfusion in life after the age of 5, receive incidental episodic transfusions as compared to chronic scheme transfusions, and receive non-extended matched units as compared to extended matched units for at least Rhesus phenotype and Kell. While the role of age at first transfusion remains unclear due to inconsistent findings among different studies\(^41–43\), the other two risk factors appear to have a more solid evidence base. The higher allo-immunization risk associated with episodic transfusions has recently also been observed in a different cohort\(^41\), and adds to the notion that RBC units given during acute, inflammatory events are associated with an increased risk for allo-immunization.\(^44–46\) The efficacy of extended matching was already demonstrated in earlier studies that have led to the clinical implementation of this practice in the Dutch national transfusion guidelines.\(^47–52\) Our study now confirms the efficacy of this policy in clinical practice in the Netherlands (at least for Rhesus phenotype and Kell).

This study indicates that allo-immunization remains a frequent problem in SCD and that the majority of first alloantibodies are formed after a small number of transfusions. A cautious, evidence based transfusion policy in patients with SCD is therefore imperative. Early identification of patients at high risk of allo-immunization will enable a more tailored and cost effective approach to prevent allo-immunization. In these patients extended matching should be pursued whenever possible.

In **chapter 8**, we used DNA samples from our Dutch cohort (chapter 9) and combined these with samples of a similar cohort of transfused SCD patients from France. In this case-control study, we aimed to evaluate the association between genetic variation in Fc gamma receptors (FcγRs) and RBC allo-immunization in SCD. These FcγRs are expressed on immune cells, and are import-
ultimately add to the development of preventive strategies for allo-immunization in SCD.

Importantly, further identification and confirmation of both clinical as well as genetic risk factors of allo-immunization will require more prospective studies. So far, most observations are of retrospective nature, and therefore have their limitations. An important research focus for future studies lies in the further exploration of the pathophysiological mechanism of allo-immunization, and the role of the immune system. This will help us to identify potential therapeutic targets for prevention of allo-immunization. In addition to our own observations, various studies have suggested that pro-inflammatory events of SCD (or episodic transfusions associated with these events), appear to add to the risk of allo-immunization. In line with this, it could be interesting to assess the potential of immunomodulatory therapy during transfusion at high risk events to prevent this complication.

To further ascertain the clinical relevance of allo-immunization, it would also be helpful to include more patient-centered outcomes of allo-immunization. Most studies now focus on the absence or presence of serological allo-antibodies. Yet, no information is provided on the extent of morbidity or mortality associated with allo-immunization in sickle cell disease.
Conclusion and perspectives

SCD is often described as the first known molecular disease.\textsuperscript{53} Yet, advances in disease management and treatment have lagged behind compared to later discovered molecular diseases that affect far fewer people, such as cystic fibrosis. Only recently, this tide seems to be changing. With increasing global migration streams, the number of SCD patients in western countries has significantly increased over the past years. This has promoted interest for SCD research in these countries, thereby gaining access to more funding and greater expertise. Exemplary for this change was the launch of a global initiative in 2016, the SCD coalition, uniting both public and private stakeholders in the goal to advance SCD care and promote awareness around the globe.

Despite this late progress and the fact that therapeutic options still remain scarce, the future of children with SCD in high-income countries has changed profoundly over the past decades. A broad range of supportive measures, including neonatal screening and antibiotic prophylaxis, has significantly improved the life expectancy of SCD patients.\textsuperscript{54} With a growing group of patients that survives into adulthood, one of the main challenges of research in SCD now lies in the prevention of chronic complications of SCD, affecting quality of life and leading to significant morbidity and mortality later in life. Many of these complications, including recurring vaso-occlusive crises and progressive neurological, cardiovascular and pulmonary disease, are still not well understood and management is mostly symptomatic. It is encouraging that many new therapies are currently under investigation, targeting these complications.\textsuperscript{26–28} Moreover, significant advances are being made with various methods of gene therapy in SCD with the potential of actually curing patients from their genetic defect.\textsuperscript{55,56} There are still many hurdles yet to overcome, but the first results are promising.

This thesis has increased our insight into the burden and pathogenesis of complications in sickle cell disease. We have evaluated several potential therapeutic targets, associated with vaso-occlusion and red blood cell allo-immunization, thereby advancing the clinical implementation of urgently needed, new treatment strategies. Major challenges remain, including the majority of patients living in low-income countries where the life expectancy is still low. Yet, the prospect for patients with SCD anno 2017 seems brighter than ever before. A new moon rises.
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